ISSC

Interstate Shellfish Sanitation Conference

Task Force I Proposals for Consideration At the 2027 Biennial Meeting

Newport, Rhode Island
January March 24-29, 2027

ISSC Task Force I
2027 Proposal Inventory

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Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Alison Sirois and Jackie Knue
Affiliation	Department of marine Resources and Alaska State Environmental Health Laboratory
Email	Alison.Sirois@maine.gov and Jacqueline.Knue@alaska.gov
Proposal Subject	PSP HPLC-PCOX Species Expansion
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	4. Approved Limited Use Methods for Marine Biotoxin Testing PCOX This submission presents data to support the use of PCOX method for Quahogs (M. mercenaria and A. icelandica), Surf Clams (S. solidissima), Geoducks (P. generosa), Butter Clams (S. giganteus), Little Neck Clams (P. stamineais), and Razor Clams (S. patula) for regulatory paralytic shellfish toxin (PST) testing. Results of the 2009 Interstate Shellfish Sanitation Conference (ISSC) proposal 09- 104 concluded the PCOX method approved for official use as a Type IV method; subsequently after single laboratory validation (SLV) and collaborative studies, ISSC proposal 13-309 accepted PCOX method as an AOAC official method of analysis (OMA) in 2013. Currently PCOX is an "Approved for Limited Use" method for mussel, clam, oyster and scallop. SLV work will be presented for quahogs, surf clams, geoducks, butter clams, little neck clams, and razor clams that demonstrates comparable performance characteristics for these species as with mussels, clams, oysters, and scallops using the PCOX method. The cost and challenges associated with maintaining both the MBA and PCOX methods for these species are high; differing laboratory skill sets are required and state laboratories have limited budgets and staff resources. Additionally, the recent shortage of the NIST saxitoxin standard used for MBA proficiencies is of concern if laboratories are expected to maintain MBA for verification purposes for these species. The requested action is being made and data presented for the purpose of inclusion of quahogs, surf clams, geoducks, butter clams, little neck clams, and razor clams as approved species (by addition to the footnote that includes mussels, clams, oysters, and scallops or as the ISSC deems appropriate) within the NSSP Guide Section IV Guidance Documents Chapter II. Growing Areas .11 Laboratory Tests Methods Table, Methods for Marine Biotoxin Testing with Biotoxin Type: Paralytic Shellfish Poisoning (PSP), Application: Growing Area Survey & Classification Sample Type: Shellfish And Application: Controlled Relaying Sample Type: Shellfish.
Public Health Significance	The PCOX method was developed to provide a rapid, high throughput chemical assay that would eliminate the need to sacrifice animals, AOAC mouse bioassay (MBA), for toxin detection. There is a worldwide move to replace assays that use live animals as test subjects. Laboratories currently using PCOX for regulatory

	PST testing have found that the lower detection limits of the PCOX method allow for better early warning therefore better management of PST closures and significantly improved public health decision-making. The addition of the proposed species will allow regulatory laboratories to move away from the costliness of maintaining MBA and eliminate the need to sacrifice animals as well as improve management of species specific closure decision–making.
Cost Information	Total consumable costs for the analysis is estimated at $10/sample. A chemistry laboratory will usually be equipped with an LC system and a post column reactor to carry out the analysis. Total capital costs for the instrumentation required for the analysis is approximately $120,000. Although the upfront investment for instrumentation is high, the removal of care, maintenance, and cost of mice quickly offsets this expenditure.
Action by 2015 Laboratory Method Review Committee	Recommended referral of Proposal 15-109 to an appropriate committee as determined by the Conference Chair for evaluation of data and until additional data are received.
Action by 2015 Task Force I	Recommended adoption of Laboratory Method Review Committee recommendation on Proposal 15-109.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-109.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-109.
Action by 2017 Laboratory Committee	Recommended referral of Proposal 15-109 to an appropriate committee as determined by the Conference Chair.
Action by 2017 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 15- 109.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 15-109.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 15-109.
Action by 2019 Laboratory Committee	Recommended referral of Proposal 15-109 to an appropriate committee as determined by the Conference Chair.
Action by 2019 Task Force I	Recommended the adoption of Laboratory Committee recommendation on Proposal 15-109.
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 15-109.

Action by FDA February 21, 2020	Concurred with Conference action on Proposal 15-109.
Action by 2023 Laboratory Committee	Recommended referral of Proposal 15-109 to an appropriate committee as determined by the Conference Chairperson.
Action by 2023 Task Force	Recommended adoption of the Laboratory Committee recommendation for Proposal 15-109.
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 15-109.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 15-109.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.
Specific NSSP Guide Reference	Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14 (Approved Laboratory Tests), Table 2 (Approved Methods for Biotoxin Testing) and Table 4 (Approved Limited Use Methods for Marine Biotoxin Testing)
Text of Proposal/ Requested Action	The intention is for this method to be an Approved Method for Marine Biotoxin Testing for clams and that it should appear in Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14 (Approved Laboratory Tests), Table 2 (Approved Methods for Marine Biotoxin Testing) under the new heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP), and the applications should be (1) Growing Area Survey and Classification and (2) Controlled Relaying with the sample type of Shellfish for both. In addition, the method should also be included in Table 4 (Approved Limited Use Methods for Biotoxin Testing) for mussels and oysters. Additional validation will be submitted later in order to move mussels and oysters also to Table 2.
Public Health Significance	Method will be used to control hazard from Diarrhetic Shellfish Poisoning (DSP) in shellfish. No methods for DSP are currently listed in the NSSP yet shellfish harvesting closures have occurred due to these toxins in Texas since 2008, in the Pacific Northwest since 2011, and in the New England region since 2015. Regulatory laboratories in these regions are currently using best available science of LC-MS/MS according to the EU reference SOP for LC-MS/MS determination of lipophilic shellfish toxins
Cost Information	Capital equipment purchases: $500,000. Consumable cost per sample: $10.00

Research Needs Information

a. Proposed specific research need/ problem to be addressed	No methods are currently approved for use to control DSP hazard under the NSSP. The EU has adopted LC-MS/MS as the reference method for all of the lipophilic shellfish toxins, including DSP. This method is a modified version of the EU LC-MS/MS method optimized specifically for DSP.
b. Explain the relationship between proposed research need and program change recommended in the proposal	The proposal will provide full SLV data for the detection of DSP toxins in clams. Therefore it would be considered an Approved Method for clams (Table 2). Based on the immediate need for this method, it was felt that the submission should be made with the available data for clam with the intention of subsequent validation for mussels and oysters, for which only preliminary data is provided here. Therefore, the method should be considered for Approved Limited Use at this time for mussel and oyster and be included in Table 4 for these matrices.
c. Estimated cost	$10,000
d. Proposed sources of funding	FDA Internal Funding
e. Time frame anticipated	Submission of all materials I order to

	be reviewed prior to the 2017 bi-annual ISSC meeting
Action by 2017 Laboratory Committee	Recommended the following: 1) Adoption of Proposal 17-103 as an Approved Method for clams 2) Referral of Proposal 17-103 to an appropriate committee as determined by the Conference Chair to determine the appropriateness of the method for mussels and oysters
Action by 2017 Task Force I	Recommended adoption of Laboratory Committee recommendations on Proposal 17-103
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-103.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 17-103.
Action by 2019 Laboratory Committee	Recommended referral of Proposal 17-103 to an appropriate committee as determined by the Conference Chair
Action by 2019 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 17-103.
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 17-103
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 17-103
Action by the 2023 Laboratory Committee	Recommended referral of Proposal 17-103 to an appropriate committee as determined by the Conference Chairperson.
Action by 2023 Task Force I	Recommended adoption of the Laboratory Committee recommendation for Proposal 17-103
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-103
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 17-103

Single Laboratory Validation (SLV) Protocol for Submission to the Interstate Shellfish Sanitation Conference (ISSC) For Method Approval

Name of the New Method:

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Name of Method Developer:

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A. Need for the New Method

1. Need for Which the Method has been Developed:

Shellfish contaminated with natural toxins can cause consumer illnesses. The Food and Drug Administration has established guidance levels for five groups of natural toxins in shellfish responsible for the following illnesses: amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). Shellfish hazards for domestic products are managed under the National Shellfish Sanitation Program (NSSP), but at present approved and approved limited use methods are only available for ASP, NSP, and PSP. Shellfish harvesting closures have been required due to DSP toxins in excess of the established regulatory guidance level of 16 µg OA eq./100 g shellfish on the Texas Gulf Coast since 2008, in the Puget Sound region since 2011, and in the New England region since 2015. This report describes the validation of an LC-MS/MS method for DSP toxins for use in the NSSP for the control of this hazard in clams. Once found to be fit for purpose for clams, the method will be fully validated for the additional matrices of mussel and oyster. Due to the immediate need for approved methods for this toxin group it was felt that submitting this proposal now, with the available full SLV data for clam, was important. Preliminary data is available for mussel and oyster such that the method can be used for these matrices in an approved limited use capacity.

2. Purpose and Intended Use of the Method: The intention is for this method to be an Approved Method for Biotoxin testing for DSP toxins under the NSSP (for clams) and that it should appear in Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14 (Approved Laboratory Tests), Table 2 (Approved Methods for Marine Biotoxin Testing) under the new heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP), and the applications should be (1) Growing Area Survey and Classification and (2) Controlled Relaying with the sample type of Shellfish for both. Preliminary data is also provided for the additional matrices of mussel and oyster such that the method should be included in Table 4 (Approved Limited Use Methods for Biotoxin Testing) for these matrices while additional SLV data is generated.

3. Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods:

The regulatory guidance level in the U.S. for diarrhetic shellfish poisoning toxins (DSTs) is 16 µg total (free plus esterified) toxins/100 g shellfish. The European Union (EU) recently adopted LC-MS/MS as the reference method for lipophilic shellfish toxins, which include DSTs.[1] However, because the EU harmonized protocol also measures a number of additional lipophilic toxins, including pectenotoxins, yessotoxins, and azapriracids, the protocol contains multiple method modifications and variations to account for this, depending on the needs of individual laboratories. This work optimized the EU lipophilic toxin reference method specifically for the quantitation of DSTs in clams for use in the NSSP. Some labs in the U.S. are already using best available science based on the EU LC-MS/MS reference method; thus, an NSSP-validated method for use in the U.S. is urgently needed. This LC-MS/MS method would be complimentary to other available testing methods such as the *in-vitro* protein phosphatase inhibition assay (PPIA), which has also been submitted for approval to the ISSC under a previous proposal, and comparative data is provided between these two methods in the comparability section.

4. Type of Method (Chemical, Molecular, or Culture):

Chemical Confirmatory Method: Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) specifically measures okadaic acid, dinophysistoxin 1, and dinophysistoxin 2.

B. Method Documentation

Method Title:

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Method Scope:

This method is fully validated for the determination of diarrhetic shellfish poisoning toxins in clams. Preliminary and comparative data only is provided for mussels and oysters.

References:

1. European Union Reference Laboratory for Marine Biotoxins. Harmonized Standard Operating Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS. Version 5, January 2015.
2. Gerssen, A.; McElhinney, M.A.; Mulder, P.P.J.; Bire, R.; de Boer, J. Liquid chromatograph-tandem mass spectrometry method for the detection of marine lyophilic toxins under alkaline conditions. Journal of Chromatography A, 1216 (2009) 1421-1430.
3. ICH Q2B, Validation of Analytical Procedures: Methodology, Fed. Regist. 1997, 62 (96), 27463-27467.
4. McNabb, P.; Selwood, A. I.; Holland, P. T. Multi-residue method for determination of algal toxins in shellfish: single-laboratory validation and inter-laboratory study. Journal of AOAC International, 88 (2005) 761.
5. AOAC International "Appendix K: Guidelines for Dietary Supplements and Botanicals, Part 1 AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals", 2013.
6. Smienk et al. 2012 Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins. Toxins, 5, 339-352.
7. Smienk et al. 2013. Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal of AOAC International Vol. 96, No. 1.

Principle:

Liquid chromatography tandem mass spectrometry is an analytical technique that provides both physical separation (liquid chromatography) and mass analysis (mass spectrometry) of sample components. In this work, a Waters ACQUITY® Ultra Performance Liquid Chromatography system was coupled with an AB Sciex 5500 QTrap® triple quadrupole mass spectrometer by electrospray ionization. Pre-validation studies were performed to assess the impacts of acidic versus basic chromatography and the use of neat versus matrix matched standard curves on overall method performance (data included). For targeted quantitation of DSTs, structurally informative transitions were specified for multiple reaction monitoring (MRM). In MRM mode, an ion of interest (precursor ion) is preselected in the first quadrupole and fragmented in the second quadruple. Multiple product ions resulting from fragmentation of the precursor ion are then mass analyzed in the third quadrupole. Two analyte specific transitions, one for quantitation and one for confirmation, are specified for each analyte and monitored in both the calibration standard solutions and in the extracted shellfish matrices. A linear fit is applied to the peak area data for the quantitation ion collected for the calibration standards, and the equation for this line is utilized to calculate the concentration of each analyte in the spiked matrix samples. The enhanced resolving power afforded by LC and the selectivity gained by tandem mass spectrometry permitted the accurate detection and quantitation of DSTs in complex shellfish matrices. Individual toxin values are converted to a single integrated okadaic acid equivalents value through the use of established

toxicity equivalency factors (OA: 1, DTX1: 1, DTX2: 0.6). Only OA and DTX1 have been found to date in the U.S.

Analytes of Interest:

Diarrhetic shellfish poisoning toxins: Okadaic Acid (OA), Dinophysistoxin-1 (DTX1), and Dinophysistoxin-2 (DTX2). Shellfish metabolites (fatty acid acyl-esters for all 3 toxins, collectively referred to as DTX3) are included through the use of a required alkaline hydrolysis step.

Proprietary Aspects: None

Cost of the Method:

Capital equipment purchases:

1. Ultra Performance Liquid chromatograph (UPLC) or High Performance Liquid Chromatograph (HPLC), capable of running in gradient mode [example: Waters Corporation Acquity UPLC system (Manchester, UK) (approx. $60,000, new with government (GSA) discount)]

2. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities [example: AB Sciex QTrap 5500 equipped with a Turbo V ionization source (Framingham, MA) (approx. $320,000, new with government (GSA) discount)]

Cost per sample (approx. for consumables): $10/sample

Sample Turn Around Time: Analysis of 10 samples, including extraction, hydrolysis, sample analysis, and quantitation can be accomplished in approx. 6 -7 hours. Analysis time increases by 2.5 hours for every 10 additional samples.

Equipment Required:

1. Instrumentation for sample preparation: knives for shucking, stainless steel laboratory spatulas, sieve for draining
2. 4 oz. plastic screw top specimen cups (if a sample of unextracted homogenate is to be saved) (e.g., Covidien #17099)
3. Balance, accuracy to the nearest 0.01 g
4. Blender or food processor
5. Laboratory homogenizer (e.g., Polytron, Ultraturax, etc.) equipped with a generator appropriate for shellfish (e.g. 20 mm saw toothed)
6. Vortex mixer (either single or multi-tube)
7. Centrifuge, capable of 2000 x *g*, with adaptors for 50 mL and 15 mL centrifuge tubes
8. Heat block or water bath capable of maintaining 76 ± 2 °C
9. Volumetric flasks or graduated cylinders capable of accurately measuring 20, 100, 500, and 1000 mL

10. Disposable 50 mL centrifuge tubes
11. Disposable glass Pasteur pipettes with bulbs
12. 20 mL glass scintillation vials with coned polyethylene lined caps (if a sample of non-hydrolyzed methanolic extract is to be saved) (e.g., Wheaton #986560)
13. Disposable 16 × 100 mm glass screw cap tubes with phenolic PTFE lined caps (e.g., Fisher #14-959 35AA (tubes), Corning #9998-15 (caps))
14. 0.01, 0.05, 0.1, 0.5, and 1 mL positive displacement microdispensers or syringes (e.g., Drummond #3-000-510, 3-000-575, 3-000-590 or Hamilton #80530, 80630, 81330, 81365)
15. 1 mL disposable syringes (e.g., Becton Dickinson #309602)
16. PTFE syringe tip membrane filters, 13 mm, pore size 0.2 µm (e.g., Pall #PN4542)
17. LC-MS autosampler vials with pre-slit caps (e.g., Agilent #5182-0715)
18. Reversed Phase HPLC Column (e.g., Acquity UPLC BEH C18 1.7 µm particle size, 1.0 × 150 mm)
19. Ultra Performance Liquid chromatograph (UPLC) or High Performance Liquid Chromatograph (HPLC), capable of running in gradient mode
20. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities

Reagents Required:

1. Certified Reference Standards

Okadaic acid: e.g. (NRC-CRM-OAc) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-1: e.g. (NRC-CRM-DTX1) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-2: e.g. (NRC-CRM-DTX2) NRC-CNRC Institute for Marine Biosciences, Canada

2. Chemicals

Acetonitrile, HPLC Optima Grade
Methanol, HPLC Optima Grade
Water, HPLC Optima Grade
Ammonium formate (≥98% purity)
Formic Acid (≥98% purity)
Hydrochloric acid (37%)
Sodium hydroxide (≥98% purity)
Hexanes (Certified ACS, ≥98.5%)

Solution Preparation

1. Extraction solvent: 100% methanol

2. Hydrochloric Acid 2.5 M: Add 60 mL of water to a 100 mL volumetric flask or graduated cylinder. To the water, add 20 mL of hydrochloric acid and then dilute with water to 100 mL. Place in an appropriate glass jar for storage marked with the date of creation. This solution may be stored at room temperature and can be used for up to three months.

3. Sodium hydroxide 2.5 M: Dissolve 10 grams of sodium hydroxide in 75 mL of water in a glass beaker and add to a 100 mL volumetric flask or graduated cylinder. Make up to 100 mL with water and transfer to an appropriate storage vessel marked with the date of creation. This solution may be stored at room temperature and used for up to 3 months.

Matrix or Matrices of Interest:

Clam, mussel, and oyster. Full SLV validation data is provided here for clam but pre-validation and method comparability studies (data provided) indicate that the method will also be applicable to additional shellfish matrices such as mussel and oyster. Data for additional matrices will be provided upon approval of the method for clam.

Sample Collection, Preservation, and Storage requirements:

CRM-DTX1, CRM-DTX2, CRM-OA stock solutions (in MeOH in sealed ampules) were purchased from the National Research Council Canada and stored at −20 °C according to manufacturer's recommendations.

For each bivalve type, animals were collected from four different growing areas (Table 1). Shellfish were shucked, rinsed and drained to remove salt water. Ten to twelve animals were combined and homogenized using a blender/food processor and/or a Polytron homogenizer. Homogenized tissues were stored in plastic screw top specimen containers at −20 °C until used.

Table 1. Sources of blank shellfish matrices for spiking experiments

Source	Clam (*Mercenaria mercenaria*)	Mussel (*Mytilus edulis*)	Oyster (*Crassostrea virginica*)
A	Provided by Maryland Department of Natural Resources	Provided by MD Department of Natural Resources (used for pre-validation studies)	Provided by Maryland Department of Natural Resources (used for pre-validation studies)
B	Purchased live directly from harvester in Virginia	Washington Department of Health (used for method comparability data)	Provided by Texas Department of State Health Services (used for method comparability data)
C	Purchased live from retail. Harvest location Northport, Maine	Not performed yet	Not performed yet
D	Provided by Florida Wildlife Research Institute. Collection location Cedar Key, Florida.	Not performed yet	Not performed yet

Safety Requirements:

Proper precautions should be taken to avoid inhalation of harmful reagents or contact with skin or eyes. Analyst should wear a lab coat, gloves and safety glasses when working with chemicals. Chemical reagents that are flammable and/or toxic should be used within a chemical fume hood to protect laboratory workers.

Other Information:

Technical Skills Required: General laboratory skills (i.e., ability to accurately pipette small volumes, etc.). Experience with operation and general maintenance of liquid chromatography mass spectrometry equipment is required.

Abbreviation and acronyms:

DTX1: Dinophysistoxin-1
DTX2: Dinophysistoxin-2
OA: Okadaic acid
LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry

Step by Step Test Procedure:

Shellfish Preparation:

1. Clean outside of the shellfish with fresh water.
2. Cut the adductor muscles to open and rinse the inside with fresh water to remove any debris.
3. Remove the meat from the shell and drain tissue using a sieve to remove salt water.
4. Combine 10–12 animals and homogenize using a blender/food processor or a laboratory homogenizer.

Extraction Procedure:

1. Accurately weigh 2.00 g ± 0.05 g of tissue homogenate into a 50 mL disposable centrifuge tube.
2. Add 9.0 mL of methanol and mix thoroughly using a vortex mixer for 3 min.
3. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
4. Transfer the supernatant to a clean 20 mL graduated cylinder, volumetric flask, or glass scintillation vial.
5. Re-extract the residual tissue pellet with an additional 9.0 mL of methanol and homogenize using a laboratory stick homogenizer (e.g. Polytron, Ultraturax).
6. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
7. Transfer the supernatant to a 20 mL volumetric flask or graduated cylinder and combine with the first extract. Adjust total extract volume to 20 mL with 100% methanol.
8. Transfer the 20 mL of total extract back into the 20 mL scintillation vial for storage (if desired).

Hydrolysis:

Note: *For this procedure, total DSP toxins (i.e. free toxin plus toxin fatty acid esters) must be quantified. To do this, all samples must go through an alkaline hydrolysis step prior to analysis.*

Accurately transfer a 2 mL aliquot of the 20 mL methanolic extract to a 16 × 100 mm glass tube with a phenolic PTFE lined screw cap using a positive displacement microdispenser or syringe. Add 250 µL of 2.5 M NaOH to the 2 mL extract aliquot. Homogenize with a vortex mixer for 30 seconds and record the total weight of the tube. Make sure caps are securely fastened and heat the mixture at 76 °C for 40 minutes. [Note: 76 °C is above the boiling point for methanol, therefore sample loss will occur if tube caps do not fit well and are not securely fastened.] Dry the water from the outside of the tubes, allow the tubes to cool to room temperature (approx. 5-10 minutes), and then re-weigh each tube. If the weight has dropped by more than 0.1 g, replace lost weight using 100% MeOH. Finally, neutralize samples with 250 µL of 2.5 M HCL, mix by vortexing for 30 seconds.

Sample Clean-up:

Partitioning of the methanolic shellfish extracts with hexane is performed to remove nonpolar lipids such as triglycerides, which could contaminate the source region of the instrument and/or suppress the responses of the DSTs.[4] Add 5 mL of hexanes directly to the 2.5 mL hydrolyzed methanolic extract. Mix by vortexing for 1 minute. Partition by centrifuging at 2,000 *g* for 10 min. Using a disposable glass pasture pipette, collect the hexanes (top layer) and discard to an appropriate waste container. Transfer approximately 1 mL of the methanolic extract (bottom layer) into a 1 mL disposable syringe equipped with a 0.2 µm PTFE syringe tip filter. Filter directly into an LC-MS certified glass vial and cap.

Quality Control Steps:

1. If available, a divert valve should be used to divert LC flow at the beginning and end of each chromatographic run. If a divert valve is not used, the ion source region and curtain plate will need to be cleaned between each batch (approximately every 24 hours) to maintain adequate sensitivity.
2. Use a new calibration curve each day of analyses. The calibration curve should be analyzed before and after each set of samples, and the data from both curves should be fitted with a line using least squares linear regression. Each calibration curve should be derived from at least six calibration points and the linear regression should yield a correlation coefficient (R^2) greater than or equal to 0.98. Analysts should also visually inspect the plot of the calibration to confirm linearity. If a calibration curve yields a correlation coefficient less than 0.98 or if non-linearity is visually observed, a new calibration curve should be prepared and samples should be reanalyzed.
3. The variation in the calibration curve slopes between the first and second set of calibration standards should not exceed 25%.
4. Reagent Blanks (methanol solvent) should be analyzed after the high calibration standard and periodically after fortified samples to insure that analyte carryover is not occurring; toxins

should not be detected above 10% of the lowest calibration point or should be below LOD. If carryover is observed, the LC gradient should be extended to allow for a longer wash at high organic (99% B).

5. Procedural Blanks (methanol carried through sample preparation process at the same time as the samples) should be analyzed before and after extracted samples.
6. One mid-scale calibration standard (e.g., 10 ng/mL) should be analyzed bracketing at least every 10 samples to assure that no retention time shifts (possibly due to column failure) or loss in signal intensity (due to fouling of the column or mass spectrometer) has occurred.
7. The retention time of analytes in all matrix solution should be within 3% that of the neat toxin standards.
8. Each chromatographic peak must be defined by at least 10 data points.
9. To confirm the presence of each DST, two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio(S/N) is used for quantitation, and the transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation must be ≥ 3.
10. Ion ratios between the quantitative and qualitative ion transitions should be within ±20% that of the relative ion abundance of the neat toxin standards.
11. Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (R_s) of OA/DTX2 should be calculated using the equation below and must be ≥ 1 for correct identification).

$$R_s = \frac{2x(RT_2 - RT_1)}{W_1 - W_2}$$

LC-MS/MS Method

Instrumentation Used for Validation: AB Sciex QTrap 5500 equipped with a Turbo V ionization source (Framingham, MA) and a Waters Corporation Acquity UPLC system (Manchester, UK).

LC Parameters:

UPLC column used for validation: Waters BEH C18 (1.7 µm, 1.0 mm × 150 mm)
Column Temperature: 40 °C
Autosampler Temperature: 10 °C
Injection Volume: 5µL

LC Gradient:

Mobile phase A: 2mM ammonium formate and 50 mM formic acid in 100% water.

Preparation of 1000 mL: dissolve 128 ± 10 mg ammonium formate in water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with water and add 1.9 mL of formic acid. Fill to mark with water.

Mobile phase B: 2mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water.

Preparation of 1000 mL: dissolve 128 ± 10 mg ammonium formate in 48.1 mL water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with acetonitrile and add 1.9 mL of formic acid. Fill to mark with acetonitrile.

Weak needle* and strong needle* wash solvent composition matched that of mobile phase A and B, respectively (*specific to Waters Acquity UPLC).

For the gradient in Table 2, LC flow should be diverted to waste from time 0.0–3.5 min and from 9.0–15.0 min.

Table 2. LC Gradient

Time (min)	Flow Rate (mL/min)	%A	%B
0.0	0.120	50	50
2.0	0.120	50	50
6.0	0.120	30	70
8.0	0.120	1.0	99
10.0	0.120	1.0	99
10.5	0.120	50	50
15.0	0.120	50	50

Analyte retention times for this gradient and column can be seen in Figure 1.

MS Ion Source Parameters:

Turbo V ion source parameters were optimized in negative ionization mode for all analytes under the acidic chromatographic conditions listed above. These parameters will vary between different instrument platforms or ionization sources.

Table 3. Turbo V ion source parameters

Source Temperature	550 °C
Ion Spray Voltage	−4500 V
Curtain Gas	25 au
Gas 1	40 au
Gas 2	40 au

MRM Parameters:

Manual or automatic compound optimization must be performed by every laboratory to determine the optimal settings for the MRM parameters as these may vary between different instrument platforms. Ruggedness studies (detailed later in the document) found that compound re-optimization was required even for detector replacement with the same make and model. Instrument must be correctly calibrated for negative ionization mode. OA, DTX1, DTX2 should be monitored in negative ionization mode. The precursor and product ion mass-to-charge (*m/z*) should be confirmed by acquiring full scan MS1 and MS/MS spectra for each toxin. At least two product ions must be monitored for each toxin, one for quantitation and one for confirmation. Dwell times for transitions should be set such that at least 10 data points are acquired across each peak. Declustering Potential (DP), Entrance Potential (EP), Collision gas (CAD), Collison Energy (CE), and Collision Cell Exit Potential (CXP) should be optimized for each MRM transition monitored. Table 4 lists the compound-dependent parameters optimized for two different AB Sciex QTRAP 5500 detectors. For method validation, two confirmatory ions were monitored; however, for routine analysis monitoring the product ion at *m/z* 151 is not required.

Table 4. MRM Parameters used for Method Validation on an AB Sciex QTrap 5500 Mass Spectrometer

Compound	Polarity	Q1 (*m/z*)	Q3 (*m/z*)	Dwell Time (ms)	DP† (V)	EP (V)	CAD Gas	CE (V)	CXP (V)
OA	Negative	803.5	255.2	100	−110	−10	High	−70	−15
OA	Negative	803.5	113.1	100	−110	−10	High	−100	−19
OA*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	255.2	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	113.1	100	−110	−10	High	−100	−19
DTX2*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	255.2	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	113.1	100	−110	−10	High	−100	−19
DTX1*	Negative	817.5	151.1	100	−110	−10	High	−70	−15

*One additional confirmatory ion transition was monitored for method validation purposes.

† Compound re-optimization for ruggedness testing using a second AB Sciex QTRAP 5500 detector found that the declustering potential had to be changed to -5 V for optimum method performance.

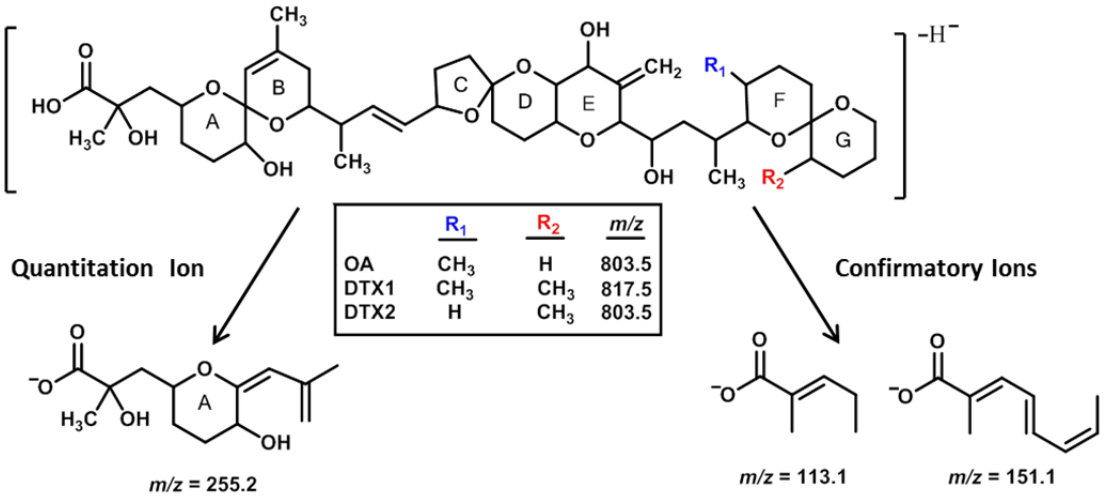


Figure 1. Structure of precursor ions and proposed product ion structures for DSTs.

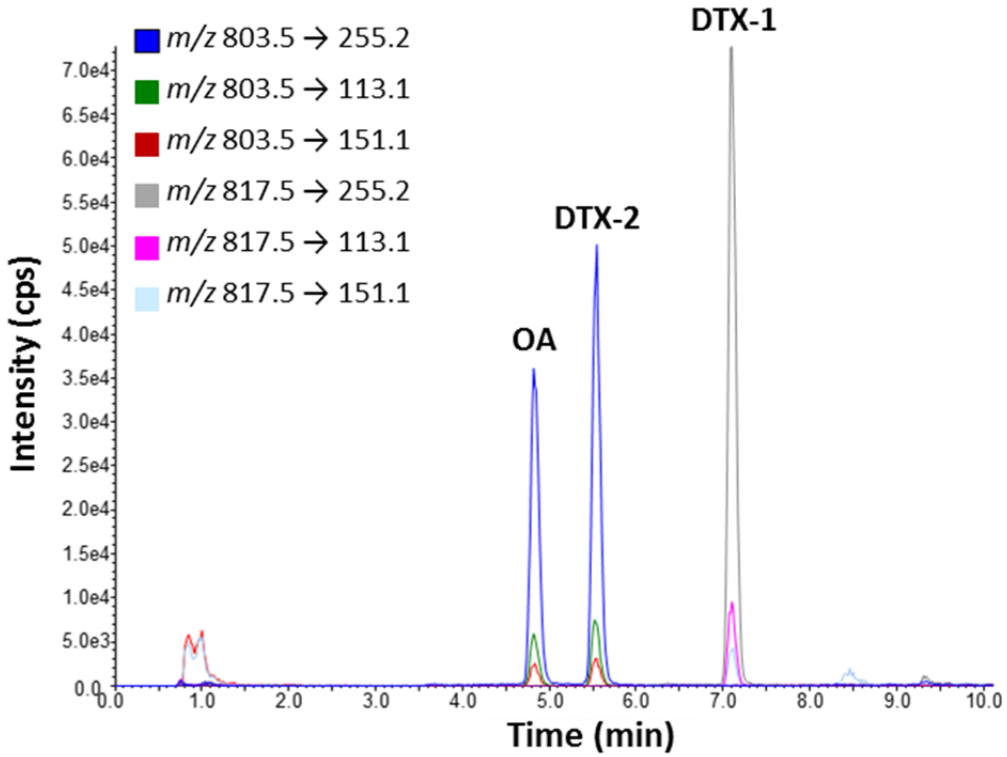


Figure 2. MRM chromatogram for the 12.5 ng/mL (regulatory level = 12.8 ng/mL) spike of OA, DTX1, and DTX2 into blank clam matrix.

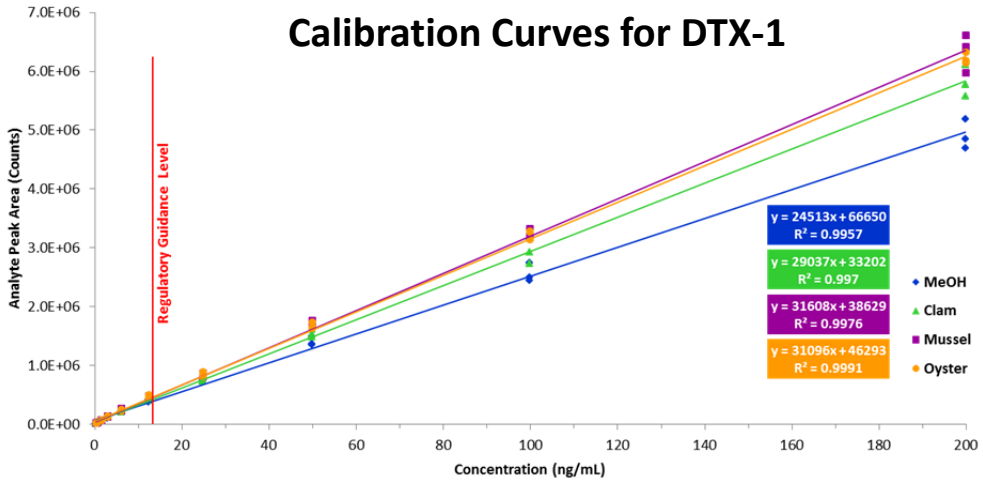
Pre-Validation Study Results (Testing for potential shellfish matrix effects for clam, mussel, and oyster, and ruggedness testing for acidic versus basic chromatography)

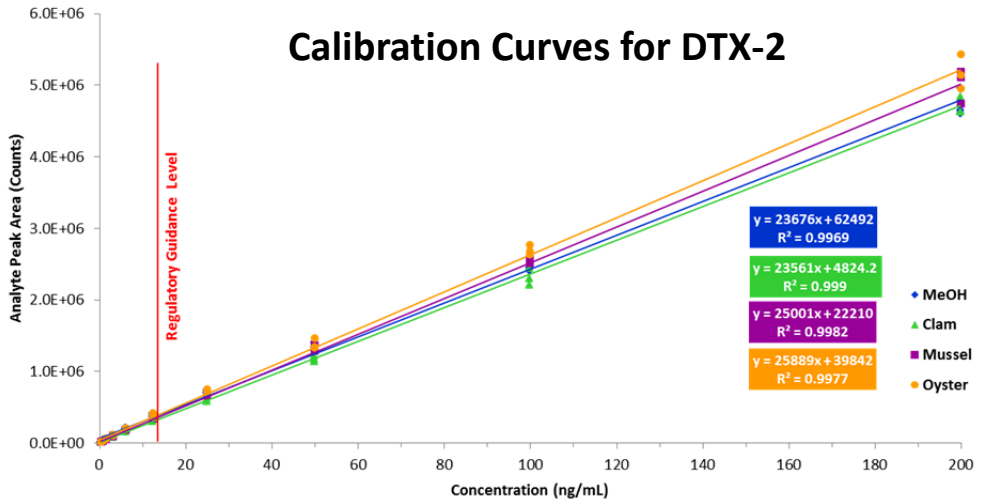
Initial pre-validation testing investigated the impact of mobile phase pH on method performance, and potential matrix effects (i.e. MS signal suppression or enhancement) for clam (*Mercinaria mercinaria*), mussel (*Mytilus edulus*), and oyster (*Crassostrea virginica*) in neat versus matrix matched spiked standard curves. Various laboratories in the United States and abroad are using different chromatographic mobile phase conditions, based on individual lab preference and need. Specifically, some labs are utilizing acidic chromatography (pH 2.4) while others are using basic chromatography (pH 11). Acidic chromatography is routinely used for quantifying total DSP toxin following hydrolysis. However, for methods that require polarity switching to investigate many classes of lipophilic toxins, basic chromatography is advantageous in that analytes that are preferentially ionized in positive mode are sufficiently separated from those that are preferentially ionized in negative mode.[2] Basic chromatography has also been reported to enhance sensitivity for certain lipophilic toxins, but due to matrix effects often requires the use of matrix matched standard curves, which adds additional cost and time to the method.

Figures 3 and 4 illustrate calibration curves from neat standard solutions in methanol compared to spiked methanolic extracts of clam, mussel, and oyster analyzed under acidic and basic chromatographic conditions, respectively. For the preparation of blank shellfish extracts, homogenates from 5 previously tested composite shellfish samples (10-12 animals each) for each species, found to have no detectable DSTs, were re-extracted, hydrolyzed, and hexane washed following the procedure described above. These 5 extracts were then pooled per species and used for the preparation of matrix-matched standard curves. Ten fortification concentrations: 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 ng/mL (equivalent to 0.49-250 µg/100g) of each toxin (from 2 µg/mL stocks) were used to construct each calibration curve. Fortification with DSTs was done post extraction to evaluate potential matrix interferences without having to account for analyte recovery. Each curve was analyzed in triplicate. All calibrations curves were shown to be linear ($R^2 \geq 0.99$) within the range of 0.391–200 ng/mL using both acidic and basic chromatography.

Our results indicate that under acidic chromatographic conditions over an extended working range, the slope of the calibration curves for OA and DTX2 in the presence of matrix is within 10% that of the neat toxin standards in methanol for all three matrices. DTX1 response, on the other hand, showed signal enhancement in matrix compared to neat standards, especially at high toxin concentrations (>50 ng/mL in the hydrolyzed shellfish extract). The percent difference in the slope of the solvent-only calibration curve and the matrix-matched calibration curve for DTX1 was 19% in clam, 29% in mussel, and 27% in oyster. However, at the regulatory guidance level (16 µg/100g shellfish, 12.8 ng/mL on column, in the hydrolyzed shellfish extract), the difference in peak areas for DTX1 in matrix versus solvent is <20% for all three matrices. In comparison, under basic chromatographic conditions OA and DTX2 signals were suppressed in the presence of matrix, resulting in significantly lower calibration curve slopes (up to 19% lower) for matrix-matched calibration curves. At the regulatory guidance level, matrix suppression resulted in response differences as high as 40% for OA and 37% for DTX2. LODs and LOQs were comparable for both acidic and basic chromatography, and both were well below the level of concern for these toxins (additional information below). Based on this information, acidic chromatographic conditions were chosen for the SLV study because they would allow the use of neat standard curves in methanol, saving both time and expense. In addition, the greatest risk from the use of acidic chromatography with non-matrix-matched standard curves is potentially overestimating DTX1 concentrations (increased chance of false positive result of exceeding guidance level), while the greatest

risk from basic chromatography with non-matrix matched standard curves is underestimating OA or DTX2 concentrations (increased chance of false negative result of exceeding guidance level). From a public health perspective, overestimation is more protective than underestimation and it was felt that this slight risk was acceptable when weighted against the additional burden in both time and expense in requiring the use of matrix-matched standard curves. Furthermore, additional validation including the use of matrix matched standard curves from multiple matrix sources would likely be required for accurate quantitation of DSTs when using mobile phases at high (basic) pH.





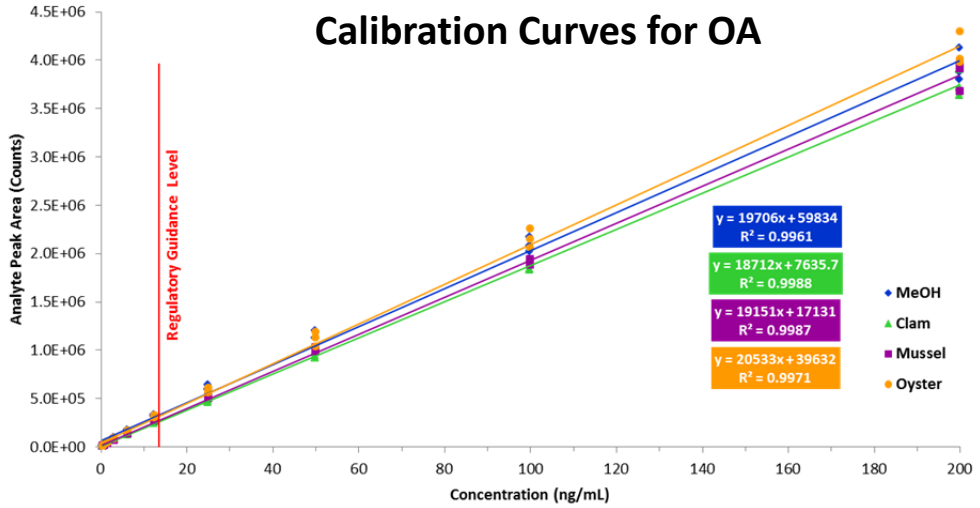
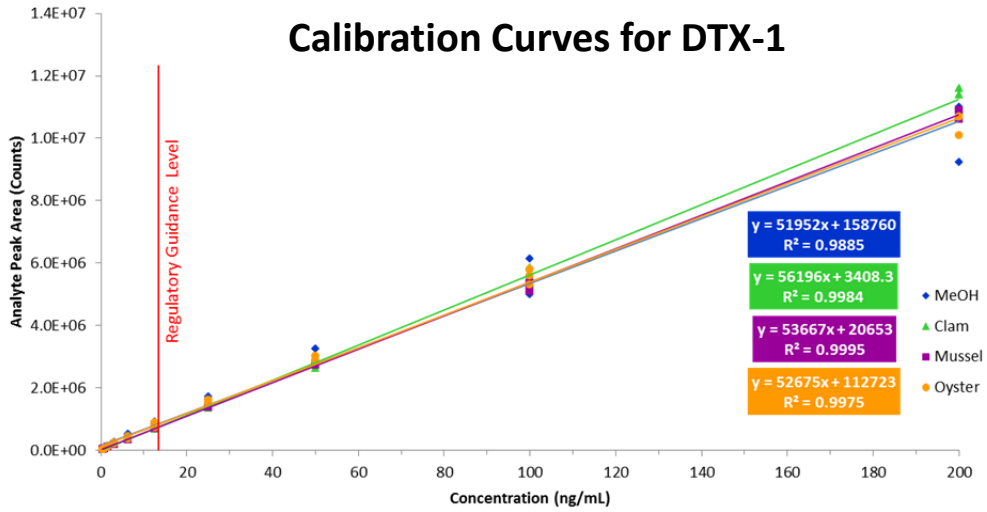


Figure 3. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under acidic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.



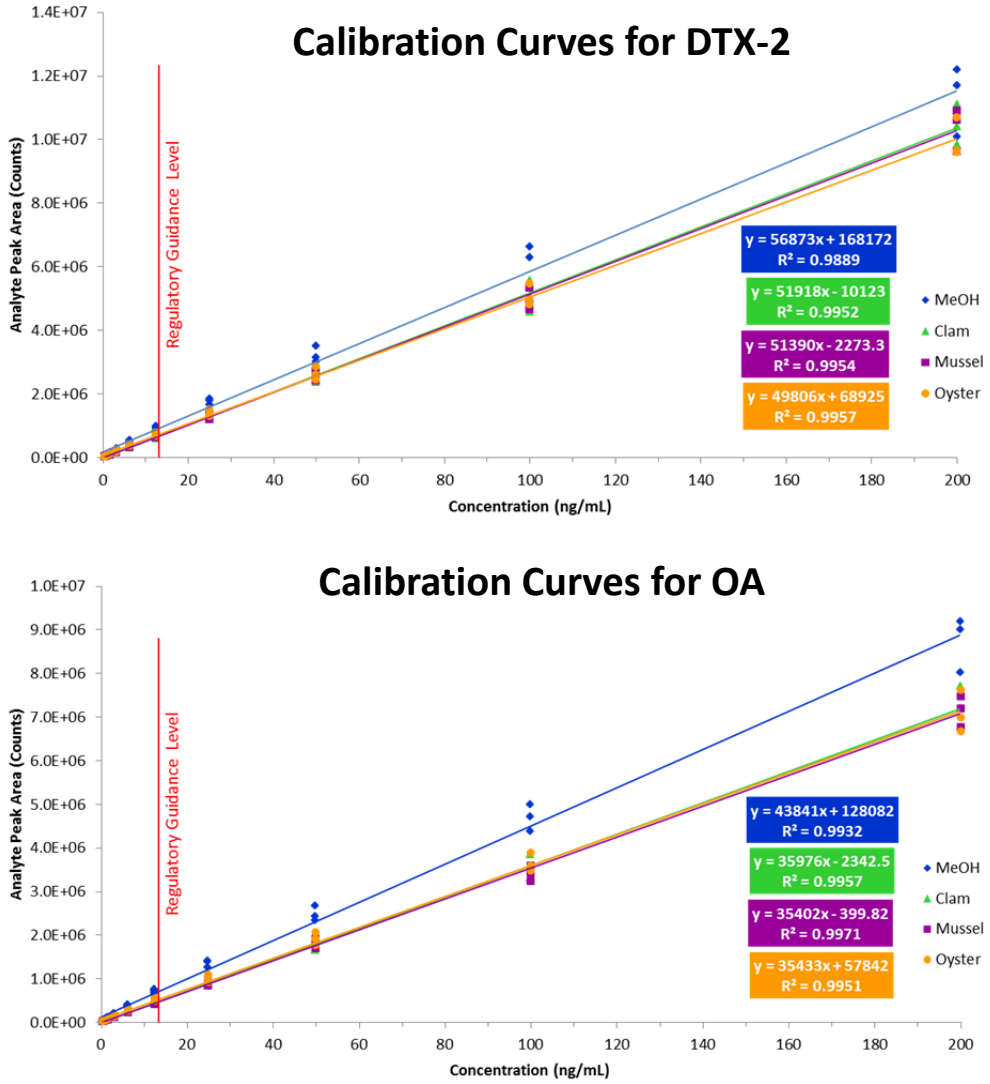


Figure 4. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under basic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.

Pre-validation Limits of Detection (LODs) and Quantitation (LOQs):

LODs and LOQs are based on the standard deviation of the response and the slope.[3] The equations for each are expressed as:

$$LOD = \frac{3.3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

Where σ = the standard deviation of the response from five blank matrix samples
S = the slope of the calibration curve

	LOD (ng/mL)			**LOQ (ng/mL)**		
	OA	DTX-2	DTX-1	OA	DTX-2	DTX-1
	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic	Acidic / Basic
Clam	0.055 / **0.040**	0.010 / **0.003**	**0.032** / 0.043	0.166 / **0.120**	0.031 / **0.009**	**0.096** / 0.129
Mussel	0.019 / **0.007**	0.007 / **0.006**	0.018 / **0.008**	0.057 / **0.020**	0.022 / **0.018**	0.053 / **0.023**
Oyster	0.017 / **0.015**	**0.008** / 0.011	0.018 / **0.016**	0.050 / **0.046**	**0.025** / 0.034	0.054 / **0.049**

Method Validation

Preparation of Stock Solutions for Validation:

Certified reference materials from the National Research Council Canada are supplied in sealed glass ampoules containing at least 0.5 mL of MeOH containing each toxin at a certified concentration that differs between toxins and lots. Thus, different volumes of each toxin standard, in 0.5 mL aliquots transferred using a 0.5 mL positive displacement Hamilton syringe, were transferred to a 20 mL glass scintillation vial and diluted with methanol to achieve stock standard solutions for each toxin at a concentration of 2 µg/mL. Stock solutions were stored at −20 °C.

Table 4. Toxin Stock Solution Preparation for Clam Validation Study

Certified Reference Material	Certified Concentration (µg/mL)	Lot #	Volume (mL)	Solvent (ml)	Total Volume	Final Concentration (µg/mL)
CRM-DTX1	15.1 ± 1.1	20071024	2	13.10	15.10	2
CRM-DTX2	7.8± 0.4	20071121	4	11.60	15.60	
CRM-OA-c	13.7 ± 0.6	20070328	2.5	14.625	17.125	

Validation Criteria:

Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).

Accuracy/Trueness

Each shellfish sample used for this study was screened for DSTs using the described LC-MS/MS method to assure that each matrix did not contain any naturally accumulated DSP toxins. Once these samples were established to be free of contamination, twenty sample homogenates (five each from matrix sources A through D from Table 1) were spiked at five concentrations ranging from 8 µg/100 g to 32 µ/100 g. Concentrations for spiking were selected to cover one half to two times the regulatory limit for DSTs in shellfish which is 16 µg of toxin per 100 g of shellfish tissue. Detailed procedures are described below.

1. For each sample, ten-twelve animals were rinsed, shucked, drained, and homogenized in a commercial food processor.
2. 2.0 ± 0.05 g of each homogenate was weighted into a 50 mL disposable centrifuge tube using a stainless steel laboratory spatula.
3. 9.0 mL of 100% methanol was added to the centrifuge tube

4. Individual DSP toxin standards were added into the tube, using positive displacement pipettes, so that the final concentration of each toxin was 8, 12, 16, 24, or 32 µg/100 g
5. Each sample was mixed for 3 min using a vortex mixer
6. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
7. The supernatant was transferred to a 20 mL glass scintillation vial.
8. The residual tissue pellet was re-extracted with 9.0 mL of methanol and homogenized using a Polytron homogenizer, followed by vortexing for 3 min.
9. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
10. The supernatant was transferred to a clean 25 mL graduated cylinder, combined with the first extract, and the total extract volume was adjusted to 20 mL by adding 100% methanol. The adjusted extract was then transferred back into the original 20 mL glass scintillation vial.
11. A 2 mL aliquot of the 20 mL extract was transferred to a 16 × 100 mm glass tube using a 1 mL positive displacement Hamilton syringe and 250 µL of 2.5 M NaOH was added. Each tube was sealed with a phenolic PTFE lined screw cap and vortexed to mix for 30 seconds. Tube weights were recorded, then placed in a 76 ± 2 °C water bath for 40 minutes.
12. Sample tubes were dried, allowed to cool to room temperature for 5–10 minutes, and re-weighted to assess any evaporative sample loss. No samples required volume adjustment during these experiments. Samples were then neutralized with 2.5 M HCL, followed by vortex mixing for 30 seconds.
13. Approx. 5 mL of hexane was added to each 2.5 mL hydrolyzed methanolic extract and vortexed for 30 seconds to mix. Samples were partitioned by centrifuging at 2,000 *g* for 10 min at 20 °C. Using a disposable glass Pasteur pipette, the upper hexane layer was removed and discarded into an appropriate waste container, and approximately 1 mL of the remaining methanolic extract (bottom layer) was transferred into a 1 mL disposable syringe equipped with a 13 mm, 0.2 µm syringe tip filter using a clean disposable glass Pasteur pipette. Each filtrate was collected directly into an LC-MS certified glass vial for analysis.

Data for OA, DTX1, and DTX2 in clam are reported in tables 4, 5, and 6 respectively. These data were collected on four different days over the course of nine days.

Precision and Recovery

Matrix fortification and extraction were conducted as described above for Accuracy/Trueness. However, each of the ten samples (five samples each from sources A and B from Table 1) was fortified at concentrations of 8, 16, and 32 µg/100 g. Even number samples, comprised of clams from two source locations, were prepared on the same day and ran within 24 h. Odd number samples also consisting of clams from the two sources were prepared and analyzed nine days later than the even number samples. Data for OA, DTX1, and DTX2 are reported in tables 7, 8, and 9, respectively. Precision is reported as percent relative standard deviation (%RSD).

Specificity

All three DSTs were analyzed in the presence of the potentially co-occurring lipophilic toxins azaspiracids (NRC-CRM-AZA 1, NRC-CRM-AZA 2, NRC-CRM-AZA 3), pectenotoxin (NRC-CRM-PTX2) and yessotoxin (NRC-CRM -YTX). For each sample three aliquots of blank tissue matrix were used. One aliquot served as a control blank, one sample contained a ½ action level spike (8 µg/100 g) of each DSTs, and one

contained the same concentration of DSTs and also a moderate to high concentration of each potential interfering compound. Five technical replicates of each aliquot, excluding the negative control blank, were analyzed. The specificity index is reported for each DST.

Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity

Matrix fortification and extraction were conducted as described above for Accuracy/Trueness. However, each of ten replicate clam samples (five each from source locations A and B from Table 1) were fortified at five concentrations spanning 50-150% of the range of interest (4, 8, 16, and 32, 48 µg/100 g). Two replicate injections of each fortified sample were analyzed. The linear range for OA, DTX1, and DTX2 extracted from fortified clam is shown in figures 5, 6, and 7. These data were collected on four different days over the course of ten days.

Ruggedness

In addition to pre-validation ruggedness testing of acidic versus basic chromatography and neat verses matrix matched calibration curves (data presented earlier in this report), two additional factors were assessed: 1. Effect of the hexane washing step on accuracy/trueness, and 2. Effect of using a different mass spectrometer of the same make and model on method performance.

To assess the effect of the hexane washing step on accuracy/trueness, two sub-samples from each of 10 extracts from previously spiked samples, representing two different matrix sources (5 samples each from matrix sources A and B), each spiked at 5 different concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were hydrolyzed following the procedure above. For each sample, one hydrolyzed extract was put through the above described hexane washing step, while the other was filtered straight into an LC vial for analysis. This entire procedure was repeated on different days so that in total 20 samples were tested both with and without the hexane washing step. The data handling procedures outlined in the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness were used for reporting these results.

To assess the effect of using a different mass spectrometer of the same make and model on method performance (i.e. method transferability), ten samples (spiked at either 8, 16, or 32 µg/100 g) were analyzed using two different AB Sciex QTrap 5500 detectors equipped with a Turbo V ionization source (same make and model but purchased several years apart). The data handling procedures outlined in the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness were used for reporting these results.

Matrix Effects

See Pre-Validation Study Results above.

Comparability

Method comparability**,** typically accomplished through comparison to a reference method, was not directly possible due to the fact that no reference method has been established under the NSSP. LC-MS/MS is the established reference method for the measurement of lipophilic shellfish toxins in the EU. The method described here is a version of the EU reference method optimized specifically for DSP toxins. One of the biggest differences between the method described here and the EU reference method is that the EU reference method is designed to detect multiple groups of lipophilic toxins in addition to DSP such as pectenotoxins, azaspiracids, and yessotoxins. But the analysis of these additional toxin groups in a single chromatographic run requires switching between positive and negative ion modes and initial analysis without sample hydrolysis as hydrolysis destroys several of these toxin groups. Analysis of total DSP toxins (free plus esterified) requires a second sample injection after alkaline hydrolysis of a sub-sample of the shellfish extract. Pectenotoxins and yessotoxins are not required to be analyzed for under the NSSP as they have not been proven to cause human illness, and while azaspiracids are required to be monitored, an optimized LC-MS/MS method for azaspiracids would be performed in positive ion mode and without sample hydrolysis. Therefore we are treating the LC-MS/MS method for azaspiracids as a separate method, even though the same extract, mobile phase, and equipment can be used for the analysis of both toxins. Different labs in the EU do run different versions of the "reference method" including some analyzing with acidic chromatographic conditions and some using basic, but pre-validation studies performed here found that analysis under basic chromatographic conditions would require the use of matrix matched standards. Furthermore, the two regulatory laboratories in the U.S. currently running a version of the EU reference LC-MS/MS method for lipophilic shellfish toxins as best available science are both using acidic chromatographic conditions. Therefore, running a different version of the EU reference LC-MS/MS method, such as basic chromatography or analysis without hydrolysis did not seem relevant for the method comparability requirement.

Analysis of certified reference materials would be another way to assess method performance as these naturally contaminated materials are certified to contain a known amount of all three of the target compounds. Until recently the only source of CRMs for DSP toxins, NRC Canada, only certified these materials for free toxins (i.e. toxins present pre-hydrolysis not taking into account the potential presence of fatty acid acyl ester shellfish metabolites (DTX3), which are known to be present in naturally contaminated shellfish samples. But new materials produced by NRC, both a frozen shellfish homogenate and a lyophilized material, are now provided with informational concentrations for total toxins (free plus esterified). Five aliquots of the frozen CRM-DSP-Mus-c (at $185.00 each) and one aliquot of the lyophilized NRC-FDMT1 (at $1,175 each) were purchased from the NRC Canada Certified References Materials Program. The frozen CRM contains 4 g ± 0.5 g of homogenized material therefore can only be reliably tested once using the required 2 g extraction method. The lyophilized material reportedly contains enough material for approximately 8 extractions. This material was extracted and tested 5 times, for a total of 10 CRM replicate tests (5 frozen and 5 lyophilized). Each extract was hydrolyzed and tested twice on separate days (for a total of 20 analyses) to assess the methods performance.

Although LC-MS/MS is the only reference method currently accepted in the EU for the analysis of lipophilic shellfish toxins, EU regulations do allow for the use of supplementary methods if they are shown to be equally protective (Commission Regulations (EC) No.853/2004 and No.15/2011). One such supplementary method that has been both single[6] and multi-laboratory[7] validated and is recognized as equally protective for DSP toxins in the EU is the OkaTest, produced by ZEU Inmunotec in Spain. The colorimetric protein phosphatase inhibition assay (PPIA), OkaTest, complies with the criteria stipulated by the European Reference Laboratory on Marine Toxins and Commission Regulation 15/2012 for determination of OA-group toxins in molluscs, according to the European Commission (DG-SANCO). But since this method specifically detects DSP toxins while the EU reference LC-MS/MS method detects a number of different lipophilic shellfish toxins, the OkaTest is considered a supplementary method for the detection of DSP and not a full alternative to LC-MS/MS for the lipophilic shellfish toxins by the EU. The OkaTest is now sold in the United States by Abraxis LLC as the Protein Phosphatase Inhibition Assay (PPIA) for DSP toxins. Since the PPIA OkaTest provides a composite toxicity score for all DSP toxins present, it was not possible to test all of the spiked samples generated during this validation directly by both methods as all samples in this study were spiked with all three of the target compounds. Furthermore, the linear range of the OkaTest is 6.3 – 35.4 µg OA eq./100 g. Only two of the spiking levels used in this study were within the linear range of the OkaTest, the 4 and 8 µg/100 g spiking levels (n=10 each; 20 samples total) from the liner range determination, which equated to 12 and 24 µg/100 g in total DSP toxins, closely bracketing the regulatory level of 16 µg OA eq./100 g. In addition, all shellfish homogenates (n = 10 each; 30 samples total) from the three species of shellfish used in the pre-validation matrix effect studies (clams, *Mercinaria mercinaria*; mussels, *Mytilus edulus*; and oysters, *Crassostrea virginica*), that had been previously tested and found to be <LOD for DSP toxins by LC-MS/MS were also tested using the OkaTest to show that they were also negative by this alternative method. Lastly, naturally contaminated shellfish from a variety of species and geographic locations including softshell clams (*Mya arenaria*) from New York (n=9) and containing both OA and DTX1 (range <LOD – 37.3 µg OA eq. /100 g), blue mussels (*Mytilus edulus*) from Washington (n=12) containing DTX1 only (<LOD – 52.5 µg/100 g), and eastern oysters (*Crassostrea virginica*) from Texas (n=11) containing OA only (1.6 – 56.3 µg/100g) were tested by both methods to assess comparability of the LC-MS/MS method with PPIA.

Results

To be provided to the LMRC with sufficient time to be reviewed prior to the 2017 ISSC meeting.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.
Name of the Method Developer	Jonathan Deeds
Developer Contact Information	Jonathan.deeds@fda.hhs.gov; 240-402-1474 US FDA, 5001 Campus Drive, College Park, MD 20740

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	
2. What is the intended purpose of the method?	Y	
3. Is there an acknowledged need for this method in the NSSP?	Y	
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	
B. Method Documentation		
1. Method documentation includes the following information:	Y	
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any Proprietary Aspects	Y	
Equipment Required	Y	
Reagents Required	Y	
Sample Collection, Preservation and Storage Requirements	Y	
Safety Requirements	Y	
Clear and Easy to Follow Step-by-Step Procedure	Y	
Quality Control Steps Specific for this Method	Y	
C. Validation Criteria		
1. Accuracy / Trueness	Y	
2. Measurement Uncertainty	Y	
3. Precision Characteristics (repeatability and reproducibility)	Y	
4. Recovery	Y	
5. Specificity	Y	
6. Working and Linear Ranges	Y	
7. Limit of Detection	Y	

8. Limit of Quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix Effects	Y	

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	
D. Other Information		
1. Cost of the Method	Y	
2. Special Technical Skills Required to Perform the Method	Y	
3. Special Equipment Required and Associated Cost	Y	
4. Abbreviations and Acronyms Defined	Y	
5. Details of Turn Around Times (time involved to complete the method)	Y	
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	
Submitters Signature Jonathan R. Deeds -S Digitally signed by Jonathan R. Deeds -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, 0.9.2342.19200300.100.1.1=1300218767, cn=Jonathan R. Deeds -S Date: 2017.05.31 12:46:30 -04'00'	Date: 5/31/2017	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - the ability of a method to measure only what it is intended to measure.[1]
18. Working Range - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Pacific Rim Shellfish Sanitation Association
Affiliation	Sitka Tribe of Alaska
Email	michael,jamros@sitkatribe-nsn.gov
Proposal Subject	Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity **Determination to Allow Use with Geoduck**
Specific NSSP Guide Reference	Section IV, Chapter II.14 -- NSSP Approved Laboratory Tests (p. 261 Table 2. Approved Methods for Marine Biotoxin Testing -- footnote 2, and/or p. 263 Table 4. Limited Use Methods for Marine Biotoxin Testing -- footnote 5)
Text of Proposal/ Requested Action	This submission presents the 'Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck' for consideration as an NSSP Approved Method for Marine Biotoxin Testing for PSP in Geoduck. The RBA is a competition-based assay that employs radiolabeled saxitoxin (3H-STX) to compete with PSP toxins present in standards/samples for binding sites on natural receptors in the assay. Following incubation with the receptors, unbound 3H-STX is removed and the remaining labeled toxin is measured with a scintillation counter. The amount of remaining 3H-STX is inversely proportional to standard/sample toxicity. The RBA offers a high-throughput, sensitive, and quantitative alternative to the mouse bioassay (MBA), which has been the long-standing reference method for PSP toxicity. Further, the RBA eliminates the use of live animals for detection of these toxins. While the RBA still uses receptors prepared from animals, the number of animals required for analysis is significantly reduced. Using native receptors as the analytical recognition elements for the assay allows for a composite measure of overall toxicity, as opposed to toxin concentrations measured by liquid chromatographic methods that require conversion factors of equivalent toxicity to calculate the overall toxicity. The RBA has undergone AOAC single and multi-laboratory validation and is designated through AOAC as an Official Method of Analysis (OMA 2011.27). The RBA is currently an NSSP Approved Method for Marine Biotoxin Testing for PSP in mussels as well as a NSSP approved for Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP (ISSC 2015 Summary of Actions Proposal 13-114). Here we provided results from a single laboratory validation study for use of RBA with the matrix geoduck (*Panopea*) viscera for submission for the RBA to be considered for approval as an NSSP Approved Method for Marine Biotoxin Testing for PSP.

Public Health Significance	Paralytic shellfish poisoning intoxications result from the consumption of seafood (primarily bivalve molluscs) contaminated with neurotoxins known as paralytic shellfish toxins (PSTs). This suite of toxins binds to voltage-gated sodium channels and may result in paralysis if enough toxin is consumed. In extreme cases when respiratory support is not available to the patient, the intoxication may prove fatal. Since the toxins cannot be destroyed during cooking and there is no way to remove the toxins from seafood, the best control strategy is to ensure that contaminated product never reaches the market. To protect public health, harvesting closures are implemented when toxicity exceeds the guidance level of 80 micrograms saxitoxin equivalents per 100 grams of shellfish tissue. As such, accurate analytical methods are needed to monitor shellfish toxicity for making decisions regarding opening and closing shellfish growing areas accordingly. Acceptance of the RBA as an NSSP Approved Method for Marine Biotoxin Testing for PSP toxicity determination in geoduck (*Panopea*) would provide monitoring and management programs with an additional tool that can be used for monitoring toxin levels and making regulatory decisions. Not only does the RBA eliminate the need for live animals for PSP testing, it is also more sensitive than the MBA, thereby providing an early warning system for monitoring programs as toxin levels begin to rise.
Cost Information	For the assay: The estimated cost per 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample[ranging from 3.5-600 µg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced. (Van Dolah 2013) For proposal: The cost of RBA work for geoduck matrix expansion is covered by and existing grant awarded to the Sitka Tribe of Alaska. Naturally contaminated samples from Washington and Alaska are pulled from regular samples tested by the respective state agencies that are part of routine shellfish testing. Therefore, there is no additional cost or funding necessary for the proposal.
Research Needs Information a. Proposed specific research need/ problem to be addressed	Paralytic shellfish poisoning (PSP) is a foodborne illness caused by ingestion of contaminated shellfish. The paralytic shellfish toxin, saxitoxin (STX), and its analogs are potent neurotoxins responsible for PSP. Marine dinoflagellates and freshwater cyanobacteria produce STX. The STX can accumulate in filter-feeding bivalve mollusks to levels that are toxic to humans. Symptoms of PSP include: tingling and numbness of the perioral area and extremities, drowsiness, incoherence, loss of motor control, and following high dose consumption, respiratory paralysis. In 1965 the mouse bioassay (MBA) was adopted as an official AOAC method for STX determination. The MBA has been the only method available for PSP testing for the last five decades. Both North American and European regulatory agencies have expressed the desire to transition to a more humane PSP testing method that does not require the use of live animals and is not subject to the matrix effects documented for the MBA

	(Turner 2012). Recently, the NSSP approved a post- column oxidation liquid chromatographic (PCOX) method and a receptor binding assay (RBA) as alternatives to the MBA. The PCOX method is approved for full use; whereas, the RBA is approved for limited use (the RBA is only approved for shellfish matrices evaluated in the single lab and multi-lab validation studies). Both the PCOX and RBA are sensitive quantitative assays for STX detection, and they do not require the use of live animals. The RBA is approved for regulatory testing of mussels as an alternative to the MBA and is approved for limited use as a screening tool for clams and scallops, but is not yet approved for use with geoduck (*Panopea*) due to a lack of data. Geoduck are a major commercial product, with large dive fisheries in Southeast Alaska and the Puget Sound that require STX testing. This proposal requests consideration for the NSSP RBA approval to be expanded to include geoduck. The proposal provides data from a single laboratory validation (SLV) of the RBA for geoduck testing as support for this request.
b. Explain the relationship between proposed research need and program change recommended in the proposal	This method is intended for use as an NSSP Approved Limited Use Method for screening for PSP toxicity in shellfish. The RBA serves as an alternative to the MBA in these applications, offering a measure of composite toxicity with high throughput and the elimination of live animal testing. (Van Dolah 2013) This application is for the addition of geoduck to the list of matrices approved for use with the RBA. There is an acknowledged need for this method in NSSP. A significant portion of the Washington and Alaska state shellfish industries are comprised of the harvest of geoduck. Approval of the RBA for use with geoduck would provide an alternative to (1) the MBA, which uses live animals, and (2) the PCOX HPLC method, which requires costly equipment and skilled personnel and offers low throughput. Acceptance of the RBA as an NSSP Approved Method for Marine Biotoxin Testing for PSP toxicity determination in geoduck would provide monitoring and management programs with an additional tool that can be used for monitoring toxin levels and making regulatory decisions. Not only does the RBA eliminate the need for live animals for PSP testing, it is also more sensitive than the MBA. References: Van Dolah 2013. ISSC application: Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP)Toxicity Determination. Van Dolah et al. 2012. Determination of paralytic shellfish toxins in shellfish by receptor binding assay: collaborative study. J AOAC Int. May-Jun;95(3):795-812. Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. J AOAC Int. Nov- Dec;92(6):1705-13.

	Ruberu et al. 2012. Evaluation of variability and quality control procedures for a receptor-binding assay for paralytic shellfish poisoning toxins. Food Addit Contam Part A Chem Anal Control Expo Risk Assess.29(11):1770-9. Turner et al. 2012. Investigations into matrix components affecting the performance of the official bioassay reference method for quantitation of paralytic shellfish poisoning toxins in oysters. Toxicon : official journal of the International Society on Toxicology 59, 215-230. OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org.
c. Estimated cost	
d. Proposed sources of funding	Thid research was performed by the Sitka Tribe of Alaska using funds from an ANA ERE grant.
e. Time frame anticipated	
Action By 2017 Laboratory Committee	Recommended referral to an appropriate committee as determined by the Conference Chair.
Action By 2017 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 17-106
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-106
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 17-106
Action By 2019 Laboratory Committee	Recommended referral to an appropriate committee as determined by the Conference Chair.
Action By 2019 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 17-106
Action by 2019 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-106
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 17-106
Action By 2023 Laboratory Committee	Recommended referral to an appropriate committee as determined by the Conference Chair.
Action By 2023 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 17-106
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-106
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 17-106

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method
Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck

Name of the Method Developer
Michael Jamros, Chris Whitehead

Developer Contact Information
Sitka Tribe of Alaska, 456 Katlian St, Sitka, AK 99835
907-747-7356 phone
907-747-4915 fax
michael.jamros@sitkatribe-nsn.gov
chris.whitehead@sitkatribe-nsn.gov

Checklist		

A. Need for the New Method

1. Clearly define the need for which the method has been developed.
Paralytic shellfish poisoning (PSP) is a food born illness caused by ingestion of contaminated shellfish. The paralytic shellfish toxin, saxitoxin (STX), and its analogs are potent neurotoxins responsible for PSP. Marine dinoflagellates and freshwater cyanobacteria produce STX. The STX can accumulate in filter-feeding bivalve mollusks to levels that are toxic to humans. Symptoms of PSP include: tingling and numbness of the perioral area and extremities, drowsiness, incoherence, loss of motor control, and following high dose consumption, respiratory paralysis.

In 1965 the mouse bioassay (MBA) was adopted as an official AOAC method for STX determination. The MBA has served as the primary method available for PSP testing for the last five decades. Both North American and European regulatory agencies have expressed the desire to transition to a more humane PSP testing method that does not require the use of live animals and is not subject to the matrix effects documented for the MBA (Turner 2012). Recently, the NSSP approved a post-column oxidation liquid chromatographic (PCOX HPLC) method and a receptor binding assay (RBA) as alternatives to the MBA. The PCOX HPLC method is approved for full use; whereas, the RBA is approved for limited use (the RBA is only approved for shellfish matrices evaluated in the single lab and multi-lab validation studies, which does not include geoduck (*Panopea*). Both the PCOX and RBA are sensitive quantitative assays for STX detection, and they do not require the use of live animals. The PCOX HPLC requires skilled personnel and offers low throughput in comparison to the RBA.

2. What is the intended purpose of the method?
The RBA is approved for regulatory testing of mussels as an alternative to the MBA and is approved for limited use as a screening tool for clams and scallops, but is not yet approved for use with geoduck (*Panopea*) due to a lack of data. Geoduck are a major commercial product that requires PSP testing. This proposal requests consideration for the NSSP RBA approval to be expanded to include geoduck. The proposal provides data from a single laboratory validation (SLV) of the RBA for geoduck testing as support for this request.

This method is intended for use as an NSSP Approved Limited Use Method for screening for PSP toxicity in shellfish, specifically geoducks. The RBA serves as an alternative to the MBA in these applications, offering a measure of integrated toxicity with high throughput and the elimination of live animal testing (Van Dolah 2013). This application is for the addition of geoduck to the list of matrices approved for use with the RBA.

3. Is there an acknowledged need for this method in the NSSP?

There is an acknowledged need for this method extension in the NSSP. A significant portion of the Washington and Alaska state shellfish industries are comprised of the harvest of geoduck. Approval of the RBA for use with geoduck would provide an alternative to (1) the MBA, which uses live animals, and (2) the PCOX HPLC method, which requires costly equipment and skilled personnel and offers low throughput.

Acceptance of the RBA as an NSSP Approved Method for Marine Biotoxin Testing for PSP toxicity determination in geoduck would provide monitoring and management programs with an additional tool that can be used for monitoring toxin levels and making regulatory decisions. Not only does the RBA eliminate the need for live animals for PSP testing, it is also more sensitive than the MBA.

4. What type of method? i.e. chemical, molecular, culture, etc.

Molecular. The RBA is a functional assay, whereby toxins present in the standard/sample bind to sodium channel preparations in the assay. Radiolabeled toxins (3H-STX) compete with toxins present in the standard or sample for sodium channel binding sites in a microplate format. Thus a decrease in signal from radiolabeled toxins represents an increase in standard/sample toxicity. This competitive RBA allows for quantitation that directly relates to the composite toxicity of the sample.

B. Method Documentation

1. Method documentation includes the following information:

Method Title

Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck

Method Scope

This submission presents the 'Matrix Expansion for the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination to Allow Use with Geoduck' for consideration as an NSSP Approved Method for Marine Biotoxin Testing for PSP in Geoduck.

The RBA offers a high-throughput, sensitive, and quantitative alternative to the mouse bioassay (MBA), which has been the long-standing reference method for PSP toxicity. Further, the RBA eliminates the use of live animals for detection of these toxins. While the RBA still uses receptors prepared from animals, the number of animals required for analysis is significantly reduced. Using native receptors as the analytical recognition elements for the assay allows for a composite measure of overall toxicity, as opposed to toxin concentrations measured by liquid chromatographic methods that require conversion factors of equivalent toxicity to calculate the overall toxicity.

The RBA has undergone AOAC single- and multi-laboratory validation and is designated through AOAC as an Official Method of Analysis (OMA 2011.27). The RBA is currently an NSSP Approved Method for Marine Biotoxin Testing for PSP in mussels as well as a NSSP approved for Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP (ISSC 2015 Summary of Actions Proposal 13-114). Here we provided results from a single laboratory validation study for use of RBA with the matrix geoduck viscera for submission for the RBA to be considered for approval as an NSSP Approved Method for Marine Biotoxin Testing for PSP.

References

Van Dolah 2013. ISSC application: Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP)Toxicity Determination.

Van Dolah et al. 2012. Determination of paralytic shellfish toxins in shellfish by receptor binding assay: collaborative study. J AOAC Int. May-Jun;95(3):795-812.

Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. J AOAC Int. Nov-Dec;92(6):1705-13.

Ruberu et al. 2012. Evaluation of variability and quality control procedures for a receptor-binding assay for paralytic shellfish poisoning toxins. Food AdditContam Part A Chem Anal Control Expo Risk Assess.29(11):1770-9.

Turner et al. 2012. Investigations into matrix components affecting the performance of the official bioassay reference method for quantitation of paralytic shellfish poisoning toxins in oysters. Toxicon: official journal of the International Society on Toxicology 59, 215-230.

OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org.

Principle

The RBA is a competition-based assay that employs radiolabeled Saxitoxin (3H-STX) to compete with PSP toxins present in standards/samples for binding sites on natural receptors in the assay. Following incubation with the receptors, unbound 3H-STX is removed and the remaining labeled toxin is measured with a scintillation counter. The amount of remaining 3H-STX is inversely proportional to standard/sample toxicity.

Any Proprietary Aspects

None

Equipment Required

The following list identifies the equipment and supplies needed for conducting the RBA.
For the assay:
(a) Scintillation counter (traditional or microplate)
(b) An 8-channel pipettor (5-200 ul variable volume and disposable tips)
(c) Micropipettors (1-1000 ul variable volumes and disposable tips)
(d) 96-well microtitre filter plate (1 µm pore size type GF/B glass fiber filter/0.65 um pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50)
(e) MultiScreen vacuum manifold (Millipore; Cat. No. NSVMHTS00)
(f) Vacuum pump
(g) Centrifuge tubes (15 and 50 ml, conical, plastic)
(h) Mini dilution tubes in 96-tube array
(i) Reagent reservoirs
(j) Ice bucket and ice
(k) Vortex mixer
(l) Sealing tape (Millipore; Cat. No. MATA HCL00)
(m) Volumetric flask or graduated beaker (1 L)
(n) -80 °C freezer
(o) Refrigerator

For sample extraction:
(p) Blender or homogenizer for sample homogenization
(q) Pipets
(r) Centrifuge tubes (15 ml, conical, plastic)
(s) pH meter or pH paper
(t) Hot plate or water bath
(u) Graduated centrifuge tubes (15 ml)
(v) Centrifuge and rotor for 15 ml tubes

Reagents Required

For the assay:
a) STX diHCl standards (NIST RM 8642; available through the National Institute of Standards and Technology; www.nist.gov) [This is the same standard used for the MBA] or (CRM-STX; National Research Council of Canada;

www.nrc-cnrc.gc.ca/eng/solutions/advisory/crm/list_product.html#B-PSP)
(b) 3H-STX (0.1 mCi per ml, ≥10 Ci per mmol; available through American Radiolabeled Chemicals, St. Louis, MO [or equivalent])
(c) 3-Morpholinopropanesulfonic acid (MOPS; Sigma; St. Louis, MO; Cat. No. M3183-500G [or equivalent])
(d) Choline chloride (Sigma; Cat. No. C7527-500G [or equivalent])
(e) Ultima Gold liquid scintillation cocktail (PerkinElmer Inc.; Waltham, MA; Cat. No. 6013321 [or equivalent])

For the sample extraction:
(f) Hydrochloric acid (HCl; 1.0 and 0.1 M)
(g) Sodium hydroxide (0.1 M)
(h) Water (distilled or deionized [18 μΩ])

Sample Collection, Preservation and Storage Requirements
Samples should be kept cool until meat is removed from shell, meat should be removed from shell within 48 hours of collection and either frozen or extracted.

Safety Requirements
General safety requirements (e.g., personal protective equipment including gloves, safety glasses, and laboratory coat) for working with toxins, biological reagents, and radioactive material must be followed. Users must be trained in and follow all in-house safety procedures for working with toxins and radiolabeled materials. Even though low levels of radiation are used for this assay, users must follow all local, state and federal laws and procedures regarding the receipt, use, and disposal of isotopes.

Clear and Easy to Follow Step-by-Step Procedure
Please see the detailed protocol AOAC OMA 2011.27 (Appendix 1)

Quality Control Steps Specific for this Method
Only data falling within the linear part of the curve (0.2-0.7 B/B0) is used for quantitation. Binding curve data shown here is from 14 RBA plates run on separate days. All analysis was performed using GraphPad Prism version 7.02.

The following parameters are required for quality control and acceptance of RBA results and were met by all assays included in this study:

(a) Slope must be between -0.8 and -1.2 (theoretical slope is -1). In this study, the average slope was -0.98 +/- 0.08.
(b) IC50 (inhibitory concentration at which CPM is 50% maximum) is in the acceptable range (2.0 nM ± 30%), between 1.4 and 2.6 nM. In this study, the average IC50 was 1.7 nM +/- 0.1 nM.
(c) A QC sample (1.8 x 10 M-8 STX concentration, 3 nM STX in-well concentration) should be within 30% (2.1 nM to 3.9 nM in-well concentration). In this study, the measured QC had an average value of 3.1 nM +/- 0.4 nM.
(d) The RSDs of triplicate counts per minute must be less than 30%. All standards, QC samples, and geoduck samples in this study met these criteria.

C. Validation Criteria

1. Accuracy / Trueness
Accuracy was evaluated based on recovery of known amounts of saxitoxin added as a QC check sample. A QC check sample is included in every receptor binding assay. Recovery of the QC check sample (3nM in-well solution) was 105% +/- 13% (Table 1).

Table 1: Calibration curve and QC check parameters in receptor binding assays

RBA ID	Slope	R^2	IC_{50} (nM)	IC_{70} (nM)	LOQ (ug STX eq/ 100g tissue)	QC (nM)
17-001	-0.86	0.99	1.6	0.59	2.6	2.9
17-002	-0.88	0.99	1.8	0.68	3.0	2.8
17-003	-0.94	0.96	1.6	0.65	2.9	2.5
17-004	-0.99	0.96	1.7	0.71	3.2	2.6
17-005	-0.92	0.98	1.5	0.60	2.7	3.1
17-006	-0.98	0.98	1.8	0.78	3.5	3.1
17-009	-0.95	0.94	1.5	0.62	2.8	3.6
17-010	-1.00	0.96	1.5	0.66	2.9	3.0
17-011	-1.15	0.96	1.9	0.92	4.1	3.7
17-012	-1.08	0.97	1.7	0.77	3.4	3.3
17-013	-1.04	0.97	1.8	0.81	3.6	3.1
17-014	-0.99	0.95	1.7	0.70	3.1	3.1
17-015	-0.95	0.99	1.5	0.62	2.8	3.7
17-016	-1.04	0.96	1.8	0.77	3.4	3.4
Average	-0.98	0.97	1.7	0.71	3.2	3.1
+/-	0.08	0.02	0.1	0.09	0.4	0.4

2. Measurement Uncertainty

3. Precision Characteristics (repeatability and reproducibility)

Repeatability was determined by analyzing each sample in three assays performed on independent days. The average RSD was 14.6%, with a range of 5.4% to 25.6% (Table 2). These results are consistent with the mean RSD of 17.7% (Van Dolah 2009), used to demonstrate repeatability in ISSC 2015 Proposal 13-114.

Table 2: Receptor binding assay results, summary statistics, and comparison to MBA results

Sample ID	RBA (ug/100g)			RBA mean (ug/100g)	MBA (ug/100g)	% MBA value	SD	RSD (%)
1823	29	32	38	33	42	79	5	13.9
2095	22	37	34	31	45	69	8	25.6
1594	45	74	58	59	58	102	15	24.6
2094	51	56	48	52	59	88	4	7.8
1607	60	43	47	52	67	78	12	23.3
1865	88	111	86	95	75	127	14	14.6
1933	88	85	74	82	88	93	7	9
1830	121	108	83	104	116	90	19	18.6
2315	93	97	82	91	128	71	8	8.6
2420	103	98	111	104	129	81	7	6.3
2071	129	141	163	144	140	103	17	11.9
2072	169	152	158	160	142	113	9	5.4
2138	406	344	332	361	447	81	40	11
1595	25	31	19	25	<38	-	6	24
1674	3	9	6	6	NTD	-	3	50*
Average						90	12	14.6

*RSD value omitted due to value below LOQ

4. Recovery

The average recovery of the QC check sample (3 nM in-well solution) was 105% +/- 13%.

5. Specificity

The RBA is specific to toxins that bind to site 1 of voltage-gated sodium channels. This includes all PSP congeners, whereby binding affinity is proportional to potency. Tetrodotoxin also binds to site 1 of the sodium channels, yet the typical combinations of sources, vectors, and geographical regions of tetrodotoxin and the saxitoxins differ.

6. Working and Linear Ranges

The dynamic range of the RBA is 1.2-10.0 nM in-well concentration (Van Dolah 2012). When necessary, samples must be diluted prior to analysis so that they are within the dynamic range of the RBA. Sigmoidal dose response with variable slope analysis is used to generate a binding curve from standard STX concentrations evaluated on each plate.

7. Limit of Detection

See Table 3 in the next section for a description of the limit of detection (LOD) for this method

8. Limit of Quantitation / Sensitivity

The limit of quantitation (LOQ) was determined from the average IC70 of all assays ran in the study, which was 0.71 nM +/- 0.09 nM.Using an adaptation of Eurachem Guide definitions for limit of detection (LOD) and LOQ by Van Dolah et. al. (2012), where B/B0 = 0.7 (average IC70 value) is used as the cutoff for quantitation, we obtain the below values for LOD and LOQ (Table 1). The numbers are for a sample diluted 1/10 (the established minimum dilution to avoid matrix effects) and extraction according to the AOAC protocol.

Table 3: LOD and LOQ for RBA matrix expansion of geoduck SLV

	Equation	SLV Results
LOD	IC_{70} + 3 x SD	4.4 ug STX eq/100 g
LOQ	IC_{70} +10 x SD	7.2 ug STX eq/100 g

9. Ruggedness

Previous work has been done to identify critical steps to ensure accuracy and ruggedness (Ruberu et al. 2012, Van Dolah et al. 2012, VanDolah et al. 2009). It was deemed important to clarify the shellfish extracts by centrifugation prior to performing the assay, particularly if the sample was refrigerated or frozen. The rat brain preparations should be vortexed frequently to ensure the synaptosomes are in suspension, and the buffer should be ice cold to ensure that toxins are not released from the receptor. Assay plate filtration should be at a rate of 2-5 seconds for well clearance. Lastly, a minimum of 30 minutes should be allowed before reading the plates after scintillation liquid is added such that scintillant can penetrate the filters (Van Dolah 2013).

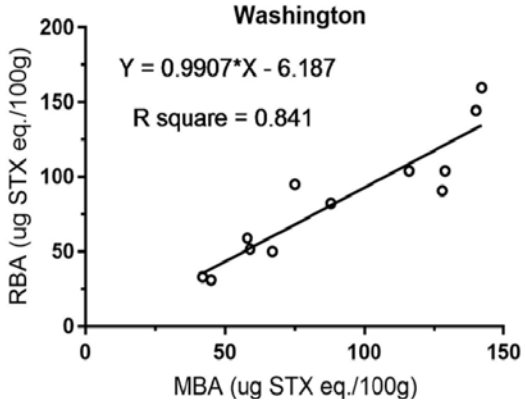
10. Matrix Effects

No matrix effects were reported. Minimum dilutions of shellfish extracts were 10-fold and were found to be sufficient to eliminate matrix effects. (Van Dolah 2013)

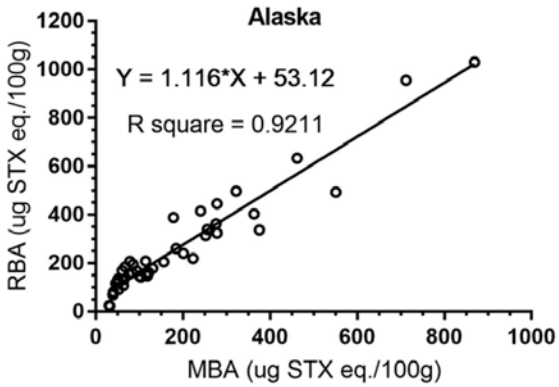
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)

Comparability to MBA

A comparison of STX concentration assayed in naturally contaminated samples by the MBA and the RBA was performed using linear regression analysis (GraphPad Prism, version 7.02). MBA results for samples from Washington were analyzed by the Washington Department of Health Shellfish Biotoxins & Water Bacteriology Laboratories and samples from Alaska were analyzed by the Alaska Department of Environmental Conservation Environmental Health Lab. All RBA results are from analysis by the Sitka Tribe of Alaska Environmental Research Laboratory. 57 total samples were compared, with the RBA yielding no false negatives relative to the regulatory limit of 80 ug/100g. Overall there were 12 false positives relative to the MBA.



	RBA +	RBA -
MBA +	7	0
MBA -	1	7



	RBA +	RBA -
MBA +	28	0
MBA -	11	3

Comparability to Previous RBA Validation Work

Previous work by (Van Dolah et al. 2012, Van Dolah et al. 2009) was submitted to the ISSC as ISSC 2015 Proposal 13-114, resulting in approval of the RBA as a NSSP Approved Method for PSP in mussels and as a NSSP Approved Limited Use Method for clams and scallops for the purpose of screening and precautionary closure for PSP. The results from this SLV for matrix expansion of RBA for geoduck matrix is consistent with the data from the previous validation studies.

A comparison of this SLV to previous validation work for the RBA demonstrates the ability of the RBA to withstand minor changes in analytical technique, reagents, and environmental factors (Table 4).

Table 4: Comparison of SLV results to previous RBA validation studies

	Accuracy (recovery of QC)	Repeatability (Average RSD)	Linear Range (slope, R^2)	IC_{50} (nM)	LOQ (mean IC_{70} - nM)	Comparison to MBA (R^2 from linear regression analysis)
STA Geoduck	104.5%	14.6%	-0.98, 0.97	1.7 +/- 0.1	0.7	0.84, 0.92
Van Dolah et. al. 2009 - SLV	99.3%	17.1%	-0.98, 0.97	2.3 +/- 0.3	1.1	0.98, 0.88
Van Dolah et. al. 2012 - MLV	106.9%	17.1%	-1.03, ND*	1.9 +/- 0.5	0.8	0.84

*No data available

D. Other Information

1. Cost of the Method

The estimated cost per 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample[ranging from 3.5-600 µg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced.
(Van Dolah 2013)

2. Special Technical Skills Required to Perform the Method

General laboratory training is necessary (this would include being able to prepare reagent solutions, pipetting, centrifugation, and simple calculations). Additional training for working with low levels of radioactive material is required.

3. Special Equipment Required and Associated Cost

A microplate scintillation counter is needed and the cost is ~$50-120K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$15K.

4. Abbreviations and Acronyms Defined

3H-STX Tritiated saxitoxin
AOAC, Association of Analytical Communities
ARC, American Radiolabeled Chemicals
B, Bound CPM
Bo, Maximum bound CPM
CFSAN, Center for Food Safety & Applied Nutrition
CPM, Counts per minute
diHCl,Dihydrochloride
Eq, Equivalents
HCl, Hydrochloric acid
IC50, Inhibitory concentration at which CPMs are at 50% of maximum
LC-FD, Liquid chromatography with fluorescence detection
LOD, Limit of detection
LOQ, Limit of quantitation
MBA, Mouse bioassay
MOPS, 3-Morpholinopropanesulfonic acid
NaOH, Sodium hydroxide
NIST, National Institute of Standards and Technology
NSSP, National Shellfish Sanitation Program
OMA, Official method of analysis
PCOX, Post-column oxidation liquid chromatography with fluorescence detection
Pre-COX, Pre-column oxidation liquid chromatography with fluorescence detection
PSP, Paralytic shellfish poisoning
PSTs, Paralytic shellfish toxins
QC, Quality control

QS, Quality System
RBA, Receptor binding assay
RSD, Relative standard deviation
SLV, Single laboratory validation
STX, Saxitoxin

5. Details of Turn Around Times (time involved to complete the method)

Microplate scintillation counting provides the ability to test multiple samples simultaneously with a turn around time for data in approximately 3 hours. Up to six plates per analyst are possible in one day, yielding a throughput of 42 samples per day. If the assay is run in screening mode where only a single dilution (1/10) is run, then through-puts of >120 samples per day can be achieved.

6. Provide Brief Overview of the Quality Systems Used in the Lab

The Center for Food Safety and Applied Nutrition (CFSAN) Quality System (QS) provides guidance to (1) design and develop processes, products, and services related to CFSAN's mission, the FDA's regulatory mission, and critical management and administrative support services, and (2) continually improve and strengthen product and service quality. The Laboratory Quality Assurance program serves as CFSAN's logical application of QS to Center laboratories and lab-based activities. The third edition (October 2009) of the Laboratory Quality Manual was followed. Standard reference materials for saxitoxin are obtained through the National Institute of Standards and Technology (NIST) and are accompanied by a Report of Investigation. The standard reference saxitoxin used in the RBA is the same as that employed with the MBA. The 3H-STX is obtained through American Radiolabeled Chemicals, Inc., and is accompanied by a Technical Data Sheet with lot specifications.

Appendix 1

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish

Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration. All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(**a**) *Traditional or microplate scintillation counter*.

(**b**) *Micropipettors*.—1–1000 µL variable volumes and disposable tips.

(**c**) *Eight channel pipettor*.—5–200 µL variable volume and disposable tips.

(**d**) *96-Well microtiter filter plate*.—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA, USA; Cat. No. MSFB N6B 50).

(**e**) *MultiScreen vacuum manifold*.—Millipore; Cat. No. NSVMHTS00.

(**f**) *Vacuum pump*.

(**g**) *Centrifuge tubes*.—15 and 50 mL, conical, plastic.

(**h**) *Mini dilution tubes in 96-tube array*.

(**i**) *Reagent reservoirs*.

(**j**) *Ice bucket and ice*.

(**k**) *Vortex mixer*.

(**l**) *Sealing tape*.—Millipore; Cat. No. MATA HCL00.

(**m**) *Volumetric flask*.—1 L.

(**n**) *–80°C freezer*.

(**o**) *Refrigerator*.

For traditional scintillation counter only:

(**p**) *MultiScreen punch device*.—Millipore; Cat No. MAMP 096 08.

(**q**) *MultiScreen disposable punch tips*.—Millipore; Cat. No. MADP 196 10.

(**r**) *MultiScreen punch kit B for 4 mL vials*.—Millipore; Cat. No. MAPK 896 0B.

(**s**) *Scintillation vials*.—4 mL.

For sample extraction:

(**t**) *Pipets*.

(**u**) *Centrifuge tubes*.—15 mL, conical, plastic.

(**v**) *Vacuum pump or house vacuum*.

(**w**) *pH meter or pH paper*.

(**x**) *Hot plate*.

(**y**) *Graduated centrifuge tubes*.—15 mL.

(**z**) *Centrifuge and rotor for 15 mL tubes*.

C. Reagents

(**a**) *[^{3}H] STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, USA, or International Isotopes Clearinghouse, Leawood, KS, USA).

(**b**) *STX diHCl*.—NIST RM 8642 (www.nist.gov).

(**c**) *3-Morpholinopropanesulfonic acid (MOPS)*.—Sigma (St. Louis, MO, USA; Cat. No. M3183-500G), or equivalent.

(**d**) *Choline chloride*.—Sigma (Cat. No. C7527-500G), or equivalent.

(**e**) *Rat brain membrane preparation*.—*Appendix 1* [*J. AOAC Int*. (future issue)].

For traditional counter:

(**f**) *Scintiverse BD liquid scintillation cocktail*.—Fisher Scientific (Waltham, MA, USA; Cat. No. SX-18), or equivalent.

For microplate counter:

(**g**) *Optiphase liquid scintillation cocktail*.—PerkinElmer Life Sciences (Downers Grove, IL, USA; Cat. No. 1200-139), or equivalent.

For sample extraction:

(**h**) *Hydrochloric acid (HCl)*.—1.0 and 0.1 M.

(**i**) *Sodium hydroxide*.—0.1 M.

(**j**) *Water*.—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in

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Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

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Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230[a]	1149[a]	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

[a] Outlier; not used in calculation.

receptor assay.

E. Preparation of Stock Solutions and Standards

(**a**) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(**b**) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(**c**) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(**d**) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(**e**) *Rat brain membrane preparation*.—Prepare rat brain membrane preparation in bulk [*Appendix 1*; *J. AOAC Int*. (future issue)] and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(**a**) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_o on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(**b**) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to

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Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Laboratory	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(**c**) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8″ Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter*.—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC50)\,\text{Hill slope}}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; *x* axis is the log concentration of STX; and *y* axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (Graph Pad Software, Inc.) is recommended. For the microplate counter users, receptor assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD, USA).

(**a**) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl

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Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	-0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

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Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1980**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	690	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

equiv./kg shellfish, using the following formulas:

$$(\text{nM STX equiv.}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv. in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCl equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

(**a**) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(**b**) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(**c**) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(**d**) QC check should be 3 nM STX ± 30% (in-well concentration).

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

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Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

Microplate row	Microplate column											
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [*Note:* The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

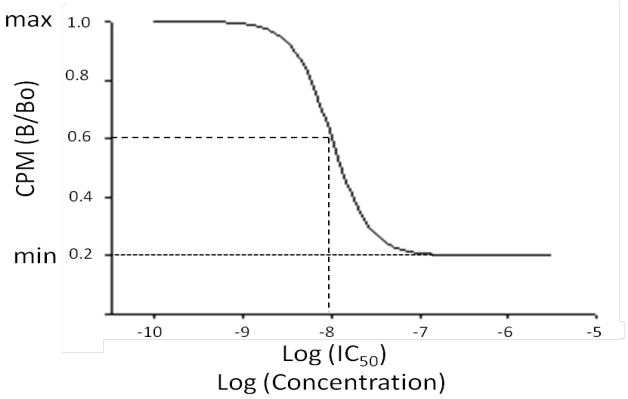


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC50.

Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(**a**) Sample quantification should be done only on dilutions that fall within B/B_o of 0.2–0.7. In the event that all sample dilutions fall below B/B_o 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_o > 0.7$), the sample is reported as below LOD. If more than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(**b**) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int*. (future issue)

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Appendix 2

FOOD CHEMICAL CONTAMINANTS

Single-Laboratory Validation of the Microplate Receptor Binding Assay for Paralytic Shellfish Toxins in Shellfish

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A single-laboratory validation (SLV) study was conducted for the microplate receptor binding assay (RBA) for paralytic shellfish poisoning (PSP) toxins in shellfish. The basis of the assay is the competition between [^{3}H]saxitoxin (STX) and STX in a standard or sample for binding to the voltage dependent sodium channel. A calibration curve is generated by the addition of 0.01–1000 nM STX, which results in the concentration dependent decrease in [^{3}H]STX-receptor complexes formed and serves to quantify STX in unknown samples. This study established the LOQ, linearity, recovery, accuracy, and precision of the assay for determining PSP toxicity in shellfish extracts, as performed by a single analyst on multiple days. The standard curve obtained on 5 independent days resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX $\pm$ 0.3 (RSD = 10.8%) with a slope of 0.96 $\pm$ 0.06 (RSD = 6.3%) and a dynamic range of 1.2–10.0 nM. The LOQ was 5.3 μg STX equivalents/100 g shellfish. Linearity, established by quantification of three levels of purified STX (1.5, 3, and 6 nM), yielded an r^2 of 0.97. Recovery from mussels spiked with three levels (40, 80, and 120 μg STX/100 g) averaged 121%. Repeatability (RSD_r), determined on six naturally contaminated shellfish samples on 5 independent days, was 17.7%. A method comparison with the AOAC mouse bioassay yielded r^2 = 0.98 (slope = 1.29) in the SLV study. The effects of the extraction method on RBA-based toxicity values were assessed on shellfish extracted for PSP toxins using the AOAC mouse bioassay method (0.1 M HCl) compared to that for the precolumn oxidation HPLC method (0.1% acetic acid). The two extraction methods showed linear correlation (r^2 = 0.99), with the HCl extraction method yielding slightly higher toxicity values (slope = 1.23). A similar relationship was observed between HPLC quantification of the HCl- and acetic acid-extracted samples (r^2 = 0.98, slope 1.19). The RBA also had excellent linear correlation with HPLC analyses (r^2 = 0.98 for HCl, r^2 = 0.99 for acetic acid), but gave somewhat higher values than HPLC using either extraction method (slope = 1.39 for HCl extracts, slope = 1.32 for acetic acid). Overall, the excellent linear correlations with the both mouse bioassay and HPLC method and sufficient interassay repeatability suggest that the RBA can be effective as a high throughput screen for estimating PSP toxicity in shellfish.

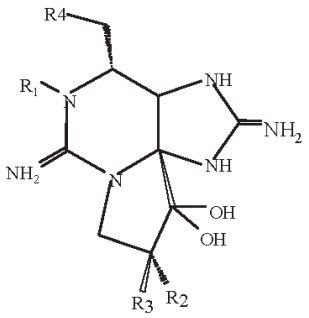
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Paralytic shellfish poisoning (PSP) is a seafood intoxication caused by the consumption of shellfish tainted with saxitoxins (STXs) produced by certain species of harmful algae. Saxitoxins are a suite of heterocyclic guanidinium toxins, of which currently more than 21 congeners are known (Figure 1). These congeners occur in varying proportions in the dinoflagellates that produce them and are further metabolized in shellfish that accumulate them, making analytical determination of PSP toxins in shellfish complex. The long-standing regulatory method for PSP toxins is the AOAC mouse bioassay (1), with a regulatory limit of 80 μg/100 g shellfish generally applied. Increasing resistance to whole animal testing has driven the need to develop alternative methods suitable for use in a high throughput monitoring or regulatory setting. In the past decade, several alternatives to the mouse bioassay have been developed and validated to various degrees. The precolumn oxidation HPLC method (2) has received First Action approval by AOAC as an Official Method for PSP (**2005.06**; 3) and has been accepted into the European Food Hygiene Regulations as an alternative to the mouse bioassay and further refined to optimize its use in the United Kingdom Official Control monitoring of PSP toxins in mussels (4). However, although the HPLC method performs well quantitatively, it is quite time consuming for high throughput screening needed by many monitoring programs. A qualitative lateral flow antibody test for PSP toxins with a detection limit of 40 μg/100 g, developed by

Jellett Rapid Testing Ltd (Chester Basin, NS, Canada), has been approved in the United States by the Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration (FDA) as a screening method. This method performed well in a comparison study with the mouse bioassay, with a false-positive rate of 6% and a false-negative rate of <0.1% (5), but it has not been put through a full AOAC collaborative trial, and does not provide quantitative analysis. To date, a suitable quantitative, high throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The current study establishes the single laboratory performance characteristics of the microplate receptor binding assay (RBA) for PSP toxins in shellfish and identifies it as a candidate for fulfilling the requirements of high throughput, quantitative analysis that measures a composite toxic potency in a manner analogous to the mouse bioassay.

STX elicit their paralytic effects by binding to site 1 on the voltage dependent sodium channel, thereby blocking the transmission of neuronal and muscular action potentials. Because all STX congeners bind to site 1 with affinities proportional to their mouse intraperitoneal (IP) toxicity (6), a receptor binding competition assay can be used to measure the integrated toxic potency of STX congeners in a sample, independent of which toxin congeners are present. Moreover, any toxin metabolites originating in the shellfish matrix will also be detected by the assay according to their affinity for the sodium channel receptor. In this binding competition assay, [^{3}H]STX competes with unlabeled STX and/or its derivatives for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H]STX is removed by filtration and bound [^{3}H]STX is quantified by liquid scintillation counting. The percent reduction in [^{3}H]STX binding in the presence of unlabeled toxin is directly proportional to the amount of unlabeled toxin present. A standard curve is established using increasing concentrations of unlabeled STX, and the concentration of PSP toxins in an unknown sample is quantified using this standard curve.

The assay tested in this single laboratory trial is a modification of the method of Doucette et al. (7) to a 96-well microplate format described by Van Dolah et al. (8). Application of microplate scintillation counting to the PSP assay was first reported by Powell and Doucette (9), who applied it to phytoplankton analysis. The use of the microplate format, in conjunction with microplate scintillation counting, makes the assay suitable for use in a high throughput monitoring or regulatory setting. Several versions of the PSP receptor binding assay have undergone method comparisons in different laboratories with favorable correlations to the mouse bioassay and/or other assays for PSP toxins in shellfish. Suarez-Isla and Valez (10) showed excellent linear correlation ($r^2 = 0.97$) between the RBA and mouse bioassay of 41 shellfish extracts between 40 and 10 000 μg STX equivalents/100 g. Llewellyn et al. (11) found that the sodium channel receptor assay compared well to three other methods of analysis for PSP toxins in shellfish (HPLC, mouse bioassay, and N2A cytotoxicity assay). Ruberu et al. (12) optimized the microplate format assay for use in the Packard Top Count microplate scintillation counter (a single channel counter; GMI, Inc., Ramsey, MN), compared results with the same assay performed on the Wallac microplate counter (a two-channel coincidence counter; Perkin Elmer Wallace, Gaithersburg, MD), and provided further correlation data with



		R1	R2	R3	R4	MU/μmol
Carbamate	STX	H	H	H	$OCONH_2$	2483
	Neo STX	OH	H	H	$OCONH_2$	2295
	GTX1	OH	OSO3-	H	$OCONH_2$	2468
	GTX2	H	OSO3-	H	$OCONH_2$	892
	GTX3	H	H	OSO3-	$OCONH_2$	1584
	GTX4	OH	H	OSO3-	$OCONH_2$	1803
Sulfocarbamoyl	GTX5 (B1)	H	H	H	OCONHSO3-	160
	GTX6 (B2)	OH	H	H	OCONHSO3-	-
	C1	H	OSO3-	H	OCONHSO3-	15
	C2	H	H	OSO3-	OCONHSO3-	239
	C3	OH	OSO3-	H	OCONHSO3-	33
	C4	OH	H	OSO3-	OCONHSO3-	143
Decarbamoyl	dcSTX	H	H	H	OH	1274
	dcNeoSTX	OH	H	H	OH	-
	dcGTX1	OH	OSO3-	H	OH	-
	dcGTX2	H	OSO3-	H	OH	1617
	dcGTX3	H	H	OSO3-	OH	1872
	dcGTX4	OH	H	OSO3-	OH	-
Deoxydecarbamoyl	doSTX	H	H	H	H	-
	doGTX2	H	H	OSO3-	H	-
	doGTX3	H	OSO3-	H	H	-

Figure 1. Structures and toxic potency of 21 saxitoxin congeners. Toxic potency is listed as mouse units (MU)/μmole, where a mouse unit is defined as the minimum amount required to kill a 20 g mouse in 15 min when administered by IP injection. The table is modified from ref. 15.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200
B	10^{-7}	10^{-7}	10^{-7}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
C	3 x 10^{-8}	3 x 10^{-8}	3 x 10^{-8}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
D	10^{-8}	10^{-8}	10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
E	3 x 10^{-9}	3 x 10^{-9}	3 x 10^{-9}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
F	10^{-9}	10^{-9}	10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
G	10^{-10}	10^{-10}	10^{-10}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
H	10^{-11}	10^{-11}	10^{-11}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			

U = unknown sample

Figure 2. Standardized plate layout recommended for the microplate RBA for PSP toxins in shellfish extracts. U = unknown sample.

the mouse bioassay. Usup et al. (13) utilized the microplate RBA method to compare predicted toxicity values in samples spiked with different STX congeners as assayed by the mouse bioassay and the RBA. Llewellyn (14) defined the competitive behavior of PSP toxin mixtures in receptor binding assays, using both the sodium channel and saxiphilin receptors, which explains their composite toxicity. However, none of these previous studies fully characterized assay performance according to AOAC single-laboratory validation (SLV) criteria that are the underpinning required for proceeding with an AOAC collaborative trial. Therefore, the current study was carried out to fulfill those requirements.

Experimental

Apparatus

(**a**) *Microplate scintillation counter*.—Wallac Microbeta, GMI Inc. (Ramsey, MN).

(**b**) *Microplate filtration manifold*.—Millipore (Bedford, MA).

(**c**) *Hot plate*.—Fisher Scientific (Suwannee, GA).

(**d**) *Countertop centrifuge*.—For 15 mL tubes, capable of 3000 × *g* (Fisher Scientific).

(**e**) *Microtiter filter plates (96 well) with 1.0 μm pore size type FB glass fiber filter/0.65 μm pore size Durapore support membrane*.—Cat. No. MSFB N6B 50 (Millipore Corp., Billerica, MA).

(**f**) *Microplate sealing tape*.—Cat. No. MATA HCL00 (Millipore Corp.).

(**g**) *Vortex mixer*.—Daigger Vortex Genie II (Daigger Scientific, Vernon Hills, IL).

(**h**) *Teflon/glass tissue homogenizer*.—Wheaton (Millville, NJ).

(**i**) *Polytron homogenizer*.—Brinkmann Instruments (Westbury, NY).

Reagents

(**a**) *Hydrochloric acid (HCl)*.—0.1 M.

(**b**) *[^{3}H]STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (International Isotopes Clearinghouse, Leawood, KS).

(**c**) *STX diHCl*.—FDA reference standard (Office of Seafood, Laurel, MD) or National Research Council (NRC) of Canada Institute of Marine Biosciences (Halifax, NS, Canada).

(**d**) *Assay buffer*.—75 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cat. No. H9136]/140 mM NaCl, pH 7.5 (Sigma, St. Louis, MO).

(**e**) *Liquid scintillation cocktail*.—Optiphase (PerkinElmer Life Sciences, Downers Grove, IL).

Preparation of Samples (0.1 M HCl Extraction)

Shellfish samples were shucked and homogenized according to the AOAC mouse bioassay protocol (1). For the HCl extraction method, 5.0 (±0.1) g of tissue homogenate was transferred to a tared 15 mL conical polypropylene centrifuge tube. A 5.0 mL volume of 0.1 M HCl was added, and the sample was mixed on a Vortex mixer. The pH was checked to

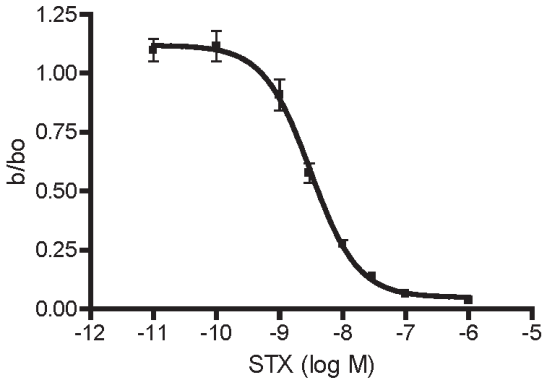


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

confirm it was between 3.0 and 4.0 in order to avoid alkalinization and destruction of the toxin, and adjusted with 1 M HCl or 0.1 M NaOH as needed. Tubes were placed in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Following removal from the boiling water bath, samples were allowed to cool to room temperature, and the pH was again confirmed to be between 3.0 and 4.0. The entire contents were then transferred to a graduated cylinder, diluted volumetrically to 10 mL, and centrifuged for 5 min at 1000 × *g*. The supernatant was transferred to a clean tube.

Preparation of Samples (Acetic Acid Extraction Method)

In a 50 mL plastic centrifuge tube, 5.0 ± 0.1 g homogenate was mixed with 3.0 mL 1% acetic acid on a vortex mixer. Tubes were capped loosely to avoid pressure buildup and placed in a boiling water bath for 5 min. Following removal from the water bath, samples were cooled in a beaker of cold water for 5 min, and then centrifuged for 10 min at 3000 × *g*. The supernatant was transferred to a 15 mL graduated conical test tube. A 3 mL amount of 1% acetic acid was added to the original tube with solid residue, mixed well on a vortex mixer, and centrifuged again for 10 min at 3000 × *g*. The second supernatant was combined with the first and diluted to 10 mL with water.

Preparation of Stock Solutions, Standards, and Reagents for Assay

(**a**) *Radioligand solution.*—[^{3}H]STX stock is provided in 50 μCi ampules, 24 Ci/mmol, 0.1 mCi/mL (4.17 μM). A 15 nM working stock of [^{3}H] STX was prepared fresh daily in 75 mM HEPES/140 mM NaCl (for 2.5 nM final in-well concentration).

(**b**) *STX standard curve.*—FDA STX dihydrochloride reference standard (100 μg/mL or 268.8 μM) used to prepare a bulk standard curve made up in advance and stored at 4°C for up to 1 month. The stock standard curve was made consisted of eight concentrations of STX in 0.003 M HCl [6×10^{-6}, 6×10^{-7}, 1.8×10^{-7}, 6×10^{-8}, 1.8×10^{-8}, 6×10^{-9}, 6×10^{-10}, 6×10^{-11}, and 0.003 M only HCl (reference)], which when diluted 1:6 in the assay, resulted in a standard curve of 0.01 nM–1000 nM STX. The reference provided a measure of total [^{3}H]STX binding in the absence of unlabeled STX.

Table 1. RBA measurements of calibration standards for assay linearity assessment (nM STX; *n* = 5)

Nominal	Mean	SD	RSD
1.5	1.7	0.16	10
3.0	3.0	0.52	17
6.0	6.0	0.34	6

(**c**) *Calibration standard (QC check).*—A reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) was prepared in 0.003 M hydrochloric acid, aliquotted in 1 mL volumes, and stored at 4°C for routine use (stable up to 1 month). On the day of the assay, 200 μL of each standard were pipetted into mini-dilution tubes for ease of pipetting into the microplate using an eight-channel pipettor.

(**d**) *Rat brain membrane homogenate.*—Cerebral cortices from 6-week-old male Holzman rats (Harlan Bioproducts, Indianapolis, IN) were homogenized on ice in a glass/Teflon tissue homogenizer in 75 mM HEPES/140 mM NaCl, pH 7.5, containing 0.1 mM PMSF (phenylmethanesulfonylfluoride;12.5 mL/brain) at 385 rpm for 10 strokes. Pooled homogenates were centrifuged at 20 000 × *g* for 15 min at 4°C and the pellet was resuspended in HEPES buffer (12.5 mL/brain) and rehomogenized on ice using a Polytron homogenizer set at 70% power for 20 s to ensure a fine suspension. The brain homogenate was aliquotted 2 mL/tube in cryovials and stored at –80°C. The protein concentration of the brain homogenate was determined using the Micro bicinchoninic acid (BCA) Assay (Pierce, Rockford, IL). For each assay, an aliquot of brain homogenate was thawed on ice and diluted with ice cold 75 nM HEPES/150 mM NaCl, pH 7.5, to yield a final protein concentration of 0.5 mg/mL in the assay.

Table 2. Recovery of analyte from spiked samples (μg STX equiv./100 g)

Nominal	Mean	SD	Measured RSD_r	Recovery, %
0	<dl[a]			
40	47	8.6	18.7	115
80	103.7	21.8	21	129
120	145.5	15.2	10.5	121

[a] <dl = Less than LOQ (5 μg STX equiv./100 g).

Table 3. Comparison of receptor binding assay (RBA; *n* = 5) with AOAC mouse bioassay (MBA) of naturally contaminated shellfish (μg STX equiv./100 g)

Sample	MBA	RBA mean	SD	RSD
LP1	340	438	74	17
LP2	534	715	96	13
LP3	1158	1533	329	21
LP4	65	91	7	9
LP5	350	608	150	25
LP6	462	518	114	22

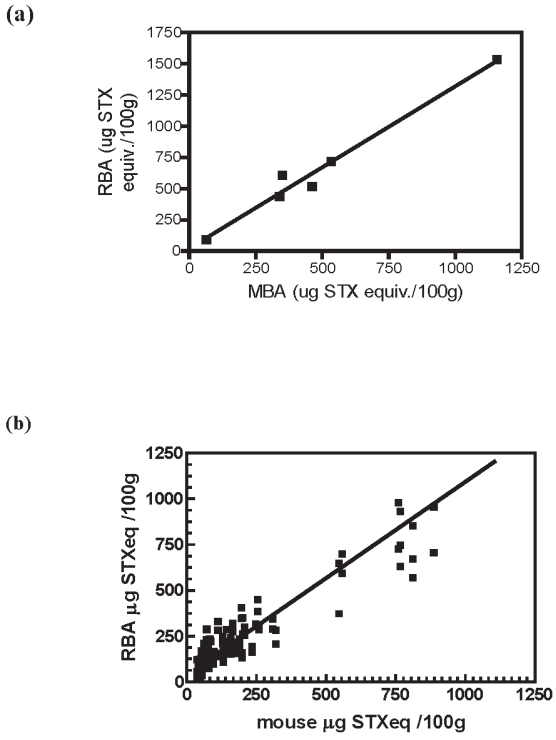


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days (r^2 = 0.98, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with a slope of 1.32.

Assay Procedure

(**a**) *Plate setup and incubation.*—A standardized plate layout was used for all assays (Figure 2). All standards, reference, QC check, and shellfish extracts were run in triplicate wells. For shellfish extracts, a standardized dilution series was run for each sample (1:10, 1:50, and 1:200), which ensured that at least one dilution would fall on the linear part of the competition curve for shellfish that contains between approximately 5 and 1500 μg STX equiv./100 g. Reagents were added in the following order: 35 μL STX standard or sample, then 35 μL [^{3}H]STX, followed by 140 μL brain homogenate. The addition of brain homogenate was carried out with sufficient force to ensure mixing of the well contents, but without risk of splashing. The plate was then covered and incubated at 4°C for 1 h.

(**b**) *Assay filtration and counting.*—The plate was filtered using a microplate vacuum filtration manifold, and each well rinsed twice with 200 μL ice-cold HEPES buffer at a filtration rate that ensured all wells were dry within 2–5 s. The microplate was then placed in a microplate scintillation counter cassette, and the bottom was sealed with plate sealing tape. Lastly, 50 μL scintillation cocktail was added to each well, and the top of the plate was sealed with sealing tape. The plate was allowed to sit for 30 min to ensure impregnation of the filters with scintillant prior to counting for 1 min/well in the microplate scintillation counter.

Data Analysis

Curve fitting was performed using a four-parameter logistic curve fitting model for a one-site receptor binding using Wallac Multicalc software. The software reports the in-well sample concentration in nM equiv. STX. Sample concentration was then calculated in μg STX equialents/100 g shellfish using the following formulas:

$$(\text{nM equiv. STX}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM equiv. STX in extract}$$

$$(\text{nm equiv. STX in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX equiv./mL}$$

$$\mu\text{g STX equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish extracted}} \times 100 = \mu\text{g STX equiv./100 g shellfish}$$

Critical Control Points

(*1*) For a ligand that interacts specifically at one receptor site, the slope of the resulting competition curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptable range of 0.8–1.2, linearity of the assay will be compromised, and quantification of the unknowns will be incorrect. Therefore, the assay should be re-run.

(*2*) The QC check standard should fall within ±30% of the stated value (3.0 nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.

Table 4. RBA-determined toxicities of nine naturally contaminated shellfish homogenates extracted using the 0.1 M HCl extraction method or the 1% acetic acid extraction method (μg STX equiv./100 g)

	HCl			Acetic acid		
Sample	Mean	SD	RSD	Mean	SD	RSD
1	11	4	36	19	7	39
2	600	143	24	488	104	21
3	690	142	21	584	167	29
4	136	8	6	131	41	31
5	152	27	18	167	21	13
6	302	87	29	270	72	27
7	340	88	26	264	63	24
8	262	79	30	252	48	19
9	63	26	41	54	19	34

(*3*) Sample quantification should be done only on dilutions that on the linear part of the curve [b/b_o = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and B_o is the maximum CPM)]. The RSD of the CPM must be <30%.

(*4*) For a given sample, if none of the sample dilutions falls within the linear range (i.e., the concentration is too high, $b/b_o < 0.2$), further dilutions must be made and the sample reanalyzed if a quantitative value is desired. If the sample concentration is too low to be quantified (i.e., $b/b_o > 0.7$) at sample dilution 1:10, the sample must be reported as below the LOQ.

Mouse Bioassay and HPLC Procedures

Shellfish samples extracted in parallel using the HCl and acetic acid extraction methods described above were analyzed using the standard protocols prescribed by the AOAC methods for mouse bioassay (1) or precolumn oxidation HPLC method (2).

Results and Discussion

Calibration Curve

To establish the dynamic range and repeatability of the calibration curve, five assays were performed by one analyst on separate days. The composite curve (Figure 3) resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%). Using the linear part of the curve (0.2–0.7 b/b_0) for quantification, a dynamic range of approximately one order of magnitude, 1.2–10.0 nM STX, was observed, as expected for a one-site binding assay. A QC check sample (3.0 nM STX) run in each assay averaged 3.0 ± 0.5 nM (RSD_r = 17.3%), with a recovery of 99.3%.

LOQ

Shellfish extracts were diluted a minimum of 10-fold prior to analysis to minimize matrix effects that can result in false positives. The LOQ was empirically determined as the concentration, in a 10-fold diluted sample, that results in a b/b_o of 0.7. This is a more conservative cutoff than the 0.8 b/b_o frequently used in receptor assays and was used because quantification was unacceptably variable above this b/b_o cutoff. This results in an LOQ of approximately 5 μg equiv. STX/100 g shellfish, which provides a more than one order of magnitude margin relative to the regulatory limit of 80 μg/100 g.

Linearity

Linearity was assessed by five independent assays of three calibration standards that were expected to fall on the curve between 0.2 and 0.7 b/b_o: 1.5, 3.0, and 6.0 nM STX prepared from FDA STX diHCl standard. Expected and measured values are listed in Table 1. Linear regression yielded a slope of 0.98 and an r^2 of 0.97.

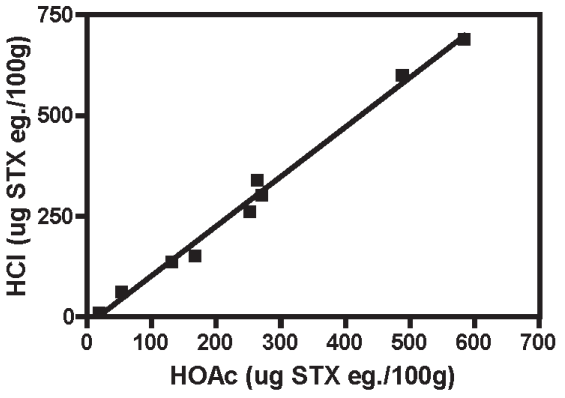


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

Table 5. HPLC analysis of nine naturally contaminated samples (1–9) extracted using 0.1 M HCl[a]

Sample	STX	NEO[b]	GTX1,4[c]	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HCl-1	3.5	0.0	0.0	7.3	0.0	0.0	10.8	6
HCl-2	231.6	23.9	42.6	324.5	28.3	249.7	900.4	412
HCl-3	220.8	53.7	74.9	436.3	43.4	338.1	1167.2	494
HCl-4	48.3	2.7	8.6	85.1	10.7	17.1	172.5	90
HCl-5	86.5	1.1	0.0	64.7	14.9	11.3	178.5	113
HCl-6	114.5	0.0	0.0	166.6	15.1	36.8	333.0	180
HCl-7	96.4	10.1	72.9	398.7	9.3	36.1	623.5	304
HCl-8	84.6	6.0	32.8	225.7	4.9	18.5	372.5	197
HCl-9	11.2	0.0	6.1	47.9	0.0	0.0	65.2	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.
[b] NEO = Neosaxitoxin.
[c] GTX = Gonyautoxin.

Recovery

Mussel tissue homogenates obtained from a local market were spiked with FDA STX diHCl standard at four levels bracketing the regulatory limit (0, 40, 80, and 120 μg/100 g) followed by thorough homogenization using a Polytron blender. Aliquots of spiked homogenate were stored at –80°C until extraction in 0.1 M HCl according to the protocol in the *Experimental* section. Extracts were analyzed in five assays performed on independent days. The mean recovery was 121% (Table 2).

Comparison of RBA-Reported Toxicity with the AOAC Mouse Bioassay

Six naturally contaminated shellfish samples were extracted in 0.1 M HCl according to the protocol in the *Experimental* section, and analyzed in five assays on independent days (Table 3). Three shellfish species were represented: clam *Mya arenaria* (whole) LP1, LP4; mussel *Mytilus edulis* (whole) LP2, LP3; and scallop *Plactopecten magellanicus* (viscera) LP5, LP6. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 of 0.98 was obtained relative to the mouse bioassay, with a slope of 1.29 (Figure 4a).

A separate study of 110 naturally contaminated shellfish samples, extracted using the 0.1 M HCl method, and analyzed by RBA and mouse bioassay, yielded similar results with an r^2 of 0.88 and a slope of 1.32 (Figure 4b).

Effect of Extraction Method on RBA-Reported Toxicities

The recent approval of the precolumn oxidation HPLC method for PSP toxins as AOAC Official Method **2005.06** (3) and its potential recognition as a reference method for PSP

Table 6. HPLC analysis of the same nine naturally contaminated samples (1–9) extracted using 1% acetic acid[a]

Sample	STX	NEO	GTX1,4	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HOAc-1	3.4	0.0	0.0	7.3	0.0	0.0	10.7	6
HOAc-2	187.6	13.1	21.7	280.7	25.1	248.9	777.1	329
HOAc-3	175.2	35.6	79.2	335.9	37.2	237.7	900.9	393
HOAc-4	33.4	3.1	11.3	61.8	6.0	15.5	131.1	68
HOAc-5	59.3	3.1	0.0	67.6	10.8	19.3	160.0	89
HOAc-6	100.8	0.0	0.0	158.0	11.8	28.4	299.0	162
HOAc-7	67.4	11.2	42.7	228.4	5.2	15.6	370.5	192
HOAc-8	71.0	8.3	34.4	190.3	4.3	12.6	320.8	173
HOAc-9	11.2	0.0	11.7	38.1	0.0	61.0	122.1	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.

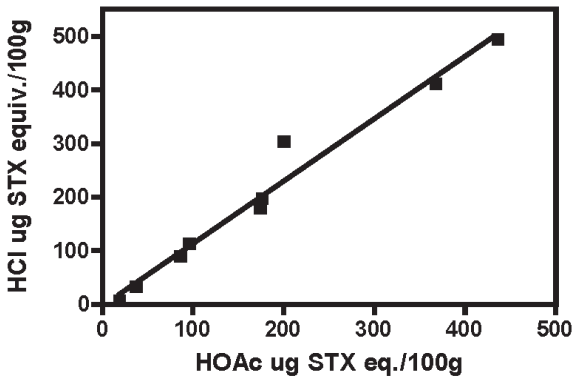


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

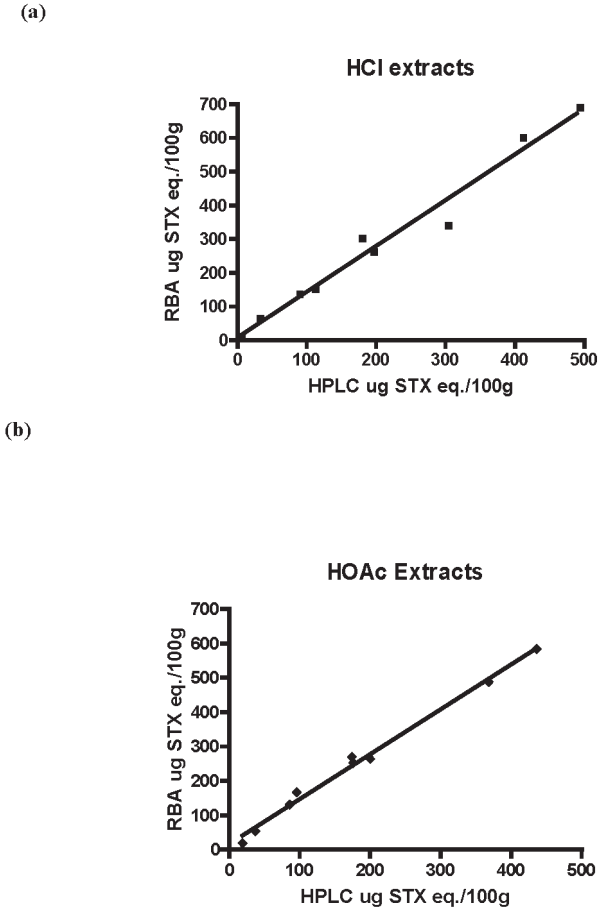


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

toxins prompted an investigation of the effects of extraction method on toxicity values reported by the RBA. Whereas the AOAC mouse bioassay prescribes shellfish extraction in 0.1 M HCl, the HPLC method uses extraction in 1% acetic acid. The 0.1 M HCl extraction procedure is known to result in the partial conversion of certain low-toxicity sulfocarbamoyl congeners to more highly toxic congeners in shellfish extracts, especially gonyautoxins, GTX5 and GTX6, to STX and neoSTX, and, thus, may result in somewhat higher toxicity values. To assess the effects of extraction procedure on RBA-reported toxicity, nine naturally contaminated shellfish samples (six blue mussel and three scallop) were homogenized and extracted independently using 0.1 M HCl and 1% acetic acid as described in the *Experimental* section. PSP toxicity in the extracts was then determined in four RBA assays run on independent days (Table 4). The between-assay RSD did not differ for samples prepared using the two extraction methods (25.8 and 26.3%, respectively). In general, the HCl extraction method resulted in slightly higher total toxicity values than reported for the acetic acid extracts (slope 1.23, r^2 = 0.99; Figure 5). The higher values reported for the HCl extracts are not explained by the conversion of sulfocarbamoyl toxins to more potent congeners in the HCl extracts, as can be seen in the toxin profiles determined by HPLC (Tables 5 and 6). Rather, the recovery of most congeners appears to be higher in the HCl extract. The higher concentrations reported in the HCl extract may reflect differences in the method by which volume is adjusted in the two extraction procedures. In the HCl method, final extract volume adjustment is made with the shellfish matrix present. In the acetic acid extraction, the matrix is first removed, the pellet re-extracted, the two extracts pooled, and then the final volume adjusted. HPLC analysis of the same samples showed a similar relationship between values reported for the HCl and acetic acid extracts (slope = 1.16, r^2 = 0.97; Figure 6) as seen in the RBA, with the HCl extracts containing greater STX equivalent/100 g.

Comparison of RBA with HPLC

The RBA showed good linear correlation with HPLC analysis of both HCl (r^2 = 0.98, slope = 1.39) and acetic acid (r^2 = 0.99, slope = 1.32) extracts, in both cases giving somewhat higher toxicities than the HPLC method (Figure 7). A number of factors may contribute to the difference in results for total toxic potencies by these two methods. The higher toxicity values given by the RBA may result in part from the fact that the HPLC method uses the STX free base molecular weight (300 Da), whereas the receptor assay (and mouse bioassay) uses the STX dihydrochloride molecular weight (372 Da) to calculate concentration, which would result in approximately 20% higher values in the RBA. Additional differences may result from the use of FDA as compared to the NRC saxitoxin standards in the RBA and HPLC methods, respectively. Higher RBA results may also result from the dominance of the more potent PSP congeners over the weaker congeners in mixtures competing for binding to the receptor, as detailed in ref. 13, which reflects their binding affinities. In

contrast to this complex behavior, the HPLC method adds linearly the concentrations of each congener based on toxic potencies determined by mouse bioassay for isolated congeners. In some cases, e.g., 11-hydroxysulfate epimers, the concentrations of separate epimers pairs are not resolved by HPLC, although their potencies differ widely as do their ratios in shellfish samples. Lastly, higher toxicity values reported by the RBA may reflect the presence of congeners or metabolites not reported by the HPLC method.

Ruggedness

Although formal ruggedness testing was not carried out during this SLV study, several steps in the procedure might be noted that can affect the precision and accuracy of the results. First, it is important to clarify shellfish extracts by centrifugation prior to running the assay, particularly if extracts are stored refrigerated or frozen before analysis, as precipitates in the extract may cause nonspecific binding that may result in overestimates of PSP toxin concentrations. Second, since the rat brain homogenate is a suspension, it is important to ensure that it remains evenly suspended by frequent vortex mixing or pipetting prior to and during its addition to the plate. The rate of assay plate filtration should ensure that the wells clear in 2–5 s, and the rinse buffer should be ice cold in order to minimize the rate of toxin release from the receptor. Lastly, following addition of liquid scintillant to the microplate wells, it is essential to allow a minimum of 30 min for the scintillant to penetrate the filters before counting. Counting prematurely can result in increased variability between wells and lower counts/well, thus increasing RSD. A count time of 1 min/well was chosen for this study as a compromise between optimum RSD and assay throughput. Increasing the count time to 5 min/well has been shown to improve the between-well RSD in this assay when using the Packard Top Count scintillation counter, a single detector instrument with somewhat lower efficiency than the Wallac Microbeta used in the current study (11).

Summary

This SLV and method comparison study demonstrates excellent linear correlation ($r^2 > 0.98$) between the microplate receptor binding assay and both the mouse bioassay and the precolumn oxidation HPLC method for the determination of PSP toxins in shellfish. The microplate format of the assay, when coupled with microplate scintillation counting, provides a quantitative high throughput screening tool for PSP toxin testing in shellfish. The tendency of the RBA to overestimate PSP toxicity relative to the reference methods minimizes the chance of returning false negatives. Where RBA-measured toxicity results in STX equivalent values close to the regulatory limit, confirmation with a reference method is necessary if a regulatory decision is being made. Nonetheless, application of the assay as a high throughput screen can alleviate the unnecessarily large numbers of animals used for the mouse bioassay on negative samples and, similarly, alleviate the lengthy analysis of samples by HPLC at very high or very low concentrations. We propose that this method be collaboratively tested to establish if it is robust enough to be used in monitoring and regulatory laboratories.

Acknowledgments

We thank Karen Mao and Steven Eaker for excellent technical support in the conduct of this study.

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Appendix 3

FOOD CHEMICAL CONTAMINANTS

Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study

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A collaborative study was conducted on a microplate format receptor binding assay (RBA) for paralytic shellfish toxins (PST). The assay quantifies the composite PST toxicity in shellfish samples based on the ability of sample extracts to compete with ^{3}H saxitoxin (STX) diHCl for binding to voltage-gated sodium channels in a rat brain membrane preparation. Quantification of binding can be carried out using either a microplate or traditional scintillation counter; both end points were included in this study. Nine laboratories from six countries completed the study. One laboratory analyzed the samples using the precolumn oxidation HPLC method (AOAC Method 2005.06) to determine the STX congener composition. Three laboratories performed the mouse bioassay (AOAC Method 959.08). The study focused on the ability of the assay to measure the PST toxicity of samples below, near, or slightly above the regulatory limit of 800 (µg STX diHCl equiv./kg). A total of 21 shellfish homogenates were extracted in 0.1 M HCl, and the extracts were analyzed by RBA in three assays on separate days. Samples included naturally contaminated shellfish samples of different species collected from several geographic regions, which contained varying STX congener profiles due to their exposure to different PST-producing dinoflagellate species or differences in toxin metabolism: blue mussel (*Mytilus edulis*) from the U.S. east and west coasts, California mussel (*Mytilus californianus*) from the U.S. west coast, chorito mussel (*Mytilus chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the west coast of the United States, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, from which only the hepatopancreas was homogenized. Among the naturally contaminated samples, five were blind duplicates used for calculation of RSD_r. The interlaboratory RSD_R of the assay for 21 samples tested in nine laboratories was 33.1%, yielding a HorRat value of 2.0. Removal of results for one laboratory that reported systematically low values resulted in an average RSD_R of 28.7% and average HorRat value of 1.8. Intralaboratory RSD_r, based on five blind duplicate samples tested in separate assays, was 25.1%. RSD_r obtained by individual laboratories ranged from 11.8 to 34.9%. Laboratories that are routine users of the assay performed better than nonroutine users, with an average RSD_r of 17.1%. Recovery of STX from spiked shellfish homogenates was 88.1–93.3%. Correlation with the mouse bioassay yielded a slope of 1.64 and correlation coefficient (r^2) of 0.84, while correlation with the precolumn oxidation HPLC method yielded a slope of 1.20 and an r^2 of 0.92. When samples were sorted according to increasing toxin concentration (µg STX diHCl equiv./kg) as assessed by the mouse bioassay, the RBA returned no false negatives relative to the 800 µg STX diHCl equiv./kg regulatory limit for shellfish. Currently, no validated methods other than the mouse bioassay directly measure a composite toxic potency for PST in shellfish. The results of this interlaboratory study demonstrate that the RBA is suitable for the routine determination of PST in shellfish in appropriately equipped laboratories.

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Paralytic shellfish poisoning (PSP) is caused by a suite of heterocyclic guanidinium toxins collectively called saxitoxins (STXs). Currently more than 21 congeners of STX are known; they occur in varying proportions in the dinoflagellates that produce them and may be further

Table 1. Shellfish homogenate samples analyzed for PSTs in the collaborative study[a]

Sample No.	Sample ID	Shellfish species and origin	Blind duplicate
1	MLV05	Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast	x
2	MLV06	California mussel (*Mytilus californianus*) from the U.S. west coast	x
3	MLV08	Green mussel (*Perna canaliculus*) from New Zealand	
4	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
5	MLV12	Blue mussel (*M. edulis*) east coast U.S., spiked with 200 µg/kg STX diHCl	
6	MLV14	Blue mussel (*M. edulis*) east coast U.S., spiked with 1200 µg/kg STX diHCl	
7	MLV16	Almeja clam (*Venus antique*) from Chile	
8	MLV01	Surf clam (*Spisula solidissima*) from the U.S. east coast	
9	MLV02	Chorito mussel (*M. chiliensis*) from Chile	
10	MLV04	Scallop (*Placopecten magellanicus*) from the U.S. east coast	
11	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
12	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
13	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
14	MLV13	Blue mussel (*M. edulis*) east coast U.S., spiked with 500 µg/kg STX diHCl	
15	MLV03	Chorito mussel (*M. chiliensis*) from Chile	
16	MLV05	Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast	x
17	MLV06	California mussel (*M. californianus*) from the U.S. west coast	x
18	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
19	MLV10	Butterclam (*Saxidomus gigantea*) from the U.S. west coast	
20	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
21	MLV15	Blue mussel (*M. edulis*) negative control, east coast U.S.	

[a] Sample number identifies the individual samples analyzed in the assays, with 1–7 analyzed in assay 1, 8–14 in assay 2, and 15–21 in assay 3. Sample identification (MLV for multilaboratory validation) describes the 16 unique samples, among which five were assayed as blind duplicates, to make a total of 21 samples. Blind duplicates, run in different assays, are identified by an "x."

metabolized in shellfish that accumulate them, making analytical determination of paralytic shellfish toxins (PST) in shellfish complex. The long-standing regulatory method for PST is the AOAC mouse bioassay (1; AOAC Method **959.08**), with a regulatory limit of 800 µg STX di HCl equiv./kg shellfish generally applied, but established at 400 µg STX diHCl equiv./kg in certain countries (e.g., the Philippines). However, at concentrations near the regulatory limit, the mouse bioassay can significantly underestimate PST in shellfish (2). This, in addition to increasing resistance to live animal testing in both the United States and the European Union (EU), has increased the need to develop alternative methods suitable for use in a high-throughput monitoring or regulatory setting.

In the past decade, several alternatives to the mouse bioassay have been developed. In the EU, the mouse bioassay remains the reference method for PST in shellfish, but European Commission (EC) Regulation 1664/2006 specifies that other internationally recognized methods may be used. Two HPLC methods, a precolumn oxidation method (3, 4; AOAC Method **2005.06**) and a postcolumn oxidation method (5; AOAC Method **2011.02**), have been approved by AOAC as *Official Methods*SM for PSP toxin analysis. The EC directive recognizes the precolumn oxidation HPLC method (AOAC Method **2005.06**) as an alternative to the mouse bioassay, but retains the mouse bioassay as the reference method in instances where results are challenged. HPLC methods separate and quantify individual STX congeners, which are then recombined according to their toxic equivalencies to yield a composite PST toxicity value. Although the HPLC methods perform well quantitatively, a high-throughput screening method capable of reporting toxic potency directly is still desirable for monitoring programs that often screen large numbers of negative samples. A qualitative lateral flow antibody test for PST with a reported detection limit of 400 µg STX equiv./kg was developed by Jellett Rapid Testing Ltd (Chester Basin, NS, Canada) and approved by the U.S. Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration as a screening method in specific circumstances. This method performed well in a comparison study with the mouse bioassay (6), but is not fully quantitative and has not been subjected to a full AOAC collaborative trial. To date, a suitable quantitative, high-throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The receptor binding assay (RBA) for PST is an excellent candidate for fulfilling the requirements of a high-throughput, quantitative assay that directly reports a composite toxic potency.

The basis of the RBA is the interaction between the toxins and their pharmacological target. All STX congeners bind to site 1 on the alpha subunit of the voltage-gated sodium channel with binding affinities proportional to their toxic potency (7). Therefore, an RBA can quantitatively measure the combined toxic potency of mixtures of STX congeners in a sample,

independent of the toxin congeners present (8). In the RBA for PST, tritiated STX ([^{3}H] STX) competes with unlabeled STX and/or its congeners for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H] STX is removed by filtration and receptor bound [^{3}H] STX quantified by liquid scintillation counting. The reduction in [^{3}H] STX binding is directly proportional to the amount of unlabeled toxin present. A standard curve is generated using increasing concentrations of nonradiolabeled STX standard from 10^{-10} to 10^{-6} M STX. The concentration of toxin in samples is determined in reference to the standard curve.

The assay being tested in this collaborative trial is a modification of the method of Doucette et al. (9) to incorporate a 96-well microtiter plate format, which increases sample throughput and minimizes error by reducing sample handling and pipetting steps. This microplate PST RBA was evaluated in a single-laboratory validation (SLV) study (10), which established an interassay repeatability (RSD_r) of 17.7% and good correlation with the mouse bioassay and precolumn oxidation HPLC methods. The toxin concentrations in shellfish tested in the SLV study ranged from near to well above the regulatory limit (approximately 900–15 000 μg STX diHCl equiv./kg). The current study focuses more specifically on the performance of the RBA in the critical range of shellfish toxicities below, near, and slightly above the regulatory limit (approximately 150–2400 μg STX diHCl equiv./kg).

The results of the collaborative study suggest that the RBA for PST is a suitable high-throughput screen for PST in shellfish. Although HPLC methods offer quantitative information on congener composition of samples, often the desired information is composite toxic potency, which requires the summation of individual congeners, corrected for their individual toxic equivalencies. The RBA provides a single integrated toxic potency value that reflects activity of all known and potential unknown congeners present in the sample. Use of the microtiter plate format, in conjunction with microplate scintillation counting, provides the ability to screen multiple samples simultaneously in a total assay time of less than 3 h. The assay format described in the current study provides for the quantitative determination of composite PST toxicity in seven shellfish extracts per 96-well microplate, each run in triplicate at three dilutions, covering toxicity ranges of approximately 35–6000 μg STX diHCl equiv./kg. In a high-throughput assay setting, multiple plates can be set up simultaneously, so that six assay plates can easily be accommodated each day by a single analyst, for a throughput of 42 samples/day. This compares favorably to an estimated throughput of 20–25 samples a day by the precolumn HPLC method (B. Niedzwiadek, Health Canada, personal communication) or 30–35 by mouse bioassay (B. Suarez, University of Chile, personal communication).

Collaborative Study

The focus of this study was to assess the performance of the RBA to determine PST toxicity in samples of commercially important shellfish at a range of concentrations below and above the regulatory limit. Twenty-one shellfish homogenates were included in the study, which represented 16 unique samples (Table 1). The homogenates included 12 naturally contaminated shellfish samples of different species collected from several geographic regions: blue mussel (*M. edulis*) from the U.S. east and west coasts, California mussel (*M. californianus*) from the U.S. west coast, chorito mussel (*M. chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from U.S. west coast, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, which included hepatopancreas only. Among the naturally contaminated samples, five were blind duplicates tested on separate days that were used for calculation of RSD_r. Samples run as duplicates are indicated in Table 1. Three samples consisting of STX-spiked mussel homogenate (*M. edulis*) at levels that bracketed the regulatory limits of 800 μg/kg (500 and 1200 μg/kg spike) and 400 μg/kg (200 μg/kg spike) were included to calculate recovery. One sample was the negative control homogenate of *M. edulis* to which the STX spikes were added. All homogenates were extracted by the study participants and the extracts analyzed by RBA in three assays on separate days.

Study Participants

Ten laboratories from seven countries agreed to carry out RBAs for this study, including the United States, Italy, Australia, New Zealand, Thailand, the Philippines, and South Africa. Participants included laboratories from regulatory authorities, as well as government and academic laboratories with monitoring needs. Five of the participating laboratories (Laboratories 1–5) have this method well established and may be considered routine users. Two laboratories had previous experience running this format of the PST RBA, but have not implemented it routinely. One laboratory had previous experience with receptor assays, but had not used the microplate filtration format of the assay. One laboratory had no previous experience with RBAs. Three laboratories from different countries, United States, Chile, and Thailand, carried out the AOAC official mouse bioassay method (AOAC Method **959.08**) on the same set of samples. All mouse bioassay laboratories were experienced regulatory authorities with monitoring responsibilities. One laboratory (Health Canada) performed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**).

Preparation of Homogenates

All shellfish samples were thoroughly homogenized using a polytron blender. For spiked samples, saxitoxin standard reference material (STX diHCl) was added to the specified concentration, and the sample was thoroughly rehomogenized to ensure homogeneity. The toxin congener profiles and concentrations of all samples were determined by the precolumn oxidation HPLC method (performed by Health Canada). STX equivalents were determined by mouse bioassay (performed by Maine Department of Marine Resources). Subsamples of each homogenate (12 g) were packaged in polycarbonate tubes and stored at –80°C until shipment to collaborating laboratories by courier. All samples were coded prior to distributing to collaborating laboratories, with the codes to each laboratory being unique, and provided blind. Coding consisted of two letters followed by a number in the form X A1-7, X B1-7, and

X C1-7, where the X indicated the laboratory, the second letter indicated the three assays to be conducted, and the numerical code indicated sample number within that assay. Three practice homogenates were similarly produced.

Shipment of Study Material

The following reagents were provided to the collaborating laboratories in a single shipment containing enough dry ice to keep the contents frozen for 5 days: [^{3}H] STX; STX diHCl standard; rat brain membrane preparation; 21 coded shellfish homogenates; three practice homogenates; and a QC check sample consisting of 18 nM STX diHCl. Sufficient homogenate (12 g) was provided to ensure an accurate weight of material could be removed from the storage vial if an additional extraction were necessary due to unexpected circumstances. The identity of the samples was not released to collaborators. All reagents were received frozen and in good condition. Each participant received electronically a detailed assay protocol, comprehensive instructions for conducting the study and data reporting, and data reporting forms.

Analysis

Participants extracted all homogenates using a modification of the 0.1 M HCl extraction method used in the AOAC standard mouse bioassay protocol (modified only by scale). They were asked to perform three RBAs, each on separate days. Each assay consisted of one 96-well plate that included a standard curve, QC check sample, and seven shellfish extracts. All samples and standards were tested in triplicate wells. All shellfish extracts were run at three dilutions (1/10, 1/50, and 1/200), which ensured that at least one dilution would fall on the linear part of the standard curve. Participants were instructed to analyze samples coded A, B, or C in the first, second, or third assay, respectively, in numerical order. The five blind duplicate samples were coded so that they were tested in two independent assays, with the combination of assays differing between duplicates. Before performing the official study, participants were asked to run a practice assay that included three shellfish homogenates in the same format to ensure that any unexpected problems were encountered and addressed prior to the official study. The practice samples consisted of a negative control mussel homogenate (MLV15), and two naturally contaminated samples that were also included in the full study (MLV05 and MLV11). The identity of the practice samples was not made known to participants. Results of the practice run were submitted by e-mail to the coordinating laboratory for review before proceeding with the full study.

For the mouse bioassay, participants followed the AOAC official mouse bioassay method (AOAC Method **959.08**), with the exception of a modified 0.1 M HCl extraction protocol used in the RBA protocol, which was modified only by scale so that 5 mL 0.1 M HCl was added to 5 g of shellfish homogenate, with all other aspects of the extraction protocol being identical. The HPLC laboratory followed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**); however, final concentrations in µg/kg and µg STX equiv./kg were calculated using the formula weight of STX diHCl [372 daltons (da)], as opposed to the free base (299.3 da) in the standard HPLC protocol, to more directly compare with the RBA.

Data Analysis and Reporting

Participants were asked to report whether they used a standard or microplate scintillation counter for the study and, if a microplate counter was used, which model, because of differences in inherent counting efficiency between current commercially available counters. For data analysis, participants were instructed to use GraphPad Prism software (La Jolla, CA) or the on-board curve-fitting software provided with their microplate scintillation counter e.g., PerkinElmer Wallac MultiCalc (Gaithersburg, MD) or Packard Top Count software (Packard Instrument Co., Meriden, CT), and to report what software was used. For analysis, a four parameter logistic fit, also known as a sigmoidal dose response with variable slope, or Hill equation, was prescribed. Participants presented their analyzed data on the spreadsheet template provided, including assay quality parameters (slope, IC_{50}, and quantification of the QC check sample), between-well CVs for each sample dilution that fell within the linear part of the standard curve (0.2–0.7 B/B_o), and calculated values for these samples in the well (nM), in the extract (µg STX equiv./mL), and in the shellfish tissue (µg STX equiv./kg). Participants were also asked to report all raw count data so that all results could be analyzed by the coordinating laboratory using identical software (GraphPad Prism 4.0) to assess whether systematic differences in quantification arose from using different curve-fitting software. All data were reported via e-mail to the coordinating laboratory.

The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in dilutions and calculations and for use of the prescribed curve-fitting model. Obvious errors were corrected and the participant laboratory was consulted for concurrence. The reviewed results were then used for evaluation in the collaborative study.

Statistical Evaluation of the Collaborative Study

For each sample analyzed, outliers were first determined using the Grubbs test at a probability value of 1% (www.graphpad.com), with no more than one outlier removed, so that valid data remained from a minimum of eight laboratories. The mean, S_R, and RSD_R, and HorRat values were then calculated for each sample. For blind duplicates, the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0, was used to further evaluate for outliers and determine S_r and RSD_r. GraphPad Prism was used to determine correlation among the RBA, mouse bioassay, and HPLC results.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration.

All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(**a**) *Traditional or microplate scintillation counter*.

(**b**) *Micropipettors*.—1–1000 µL variable volumes and disposable tips.

(**c**) *Eight channel pipettor*.—5–200 µL variable volume and disposable tips.

(**d**) *96-Well microtiter filter plate*.—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50).

(**e**) *MultiScreen vacuum manifold*.—Millipore; Cat. No. NSVMHTS00.

(**f**) *Vacuum pump*.

(**g**) *Centrifuge tubes*.—15 and 50 mL, conical, plastic.

(**h**) *Mini dilution tubes in 96-tube array*.

(**i**) *Reagent reservoirs*.

(**j**) *Ice bucket and ice*.

(**k**) *Vortex mixer*.

(**l**) *Sealing tape*.—Millipore; Cat. No. MATA HCL00.

(**m**) *Volumetric flask*.—1 L.

(**n**) *–80°C freezer*.

(**o**) *Refrigerator*.

For traditional scintillation counter only:

(**p**) *MultiScreen punch device*.—Millipore; Cat No. MAMP 096 08.

(**q**) *MultiScreen disposable punch tips*.—Millipore; Cat. No. MADP 196 10.

(**r**) *MultiScreen punch kit B for 4 mL vials*.—Millipore; Cat. No. MAPK 896 0B.

(**s**) *Scintillation vials*.—4 mL.

For sample extraction:

(**t**) *Pipets*.

(**u**) *Centrifuge tubes*.—15 mL, conical, plastic.

(**v**) *Vacuum pump or house vacuum*.

(**w**) *pH meter or pH paper*.

(**x**) *Hot plate*.

(**y**) *Graduated centrifuge tubes*.—15 mL.

(**z**) *Centrifuge and rotor for 15 mL tubes*.

C. Reagents

(**a**) *[^{3}H] STX*.—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, or International Isotopes Clearinghouse, Leawood, KS).

(**b**) *STX diHCl*.—NIST RM 8642 (www.nist.gov).

(**c**) *3-Morpholinopropanesulfonic acid (MOPS)*.—Sigma (St. Louis, MO; Cat. No. M3183-500G), or equivalent.

(**d**) *Choline chloride*.—Sigma (Cat. No. C7527-500G), or equivalent.

(**e**) *Rat brain membrane preparation*.—See *Appendix*.

For traditional counter:

(**f**) *Scintiverse BD liquid scintillation cocktail*.—Fisher Scientific (Waltham, MA; Cat. No. SX-18), or equivalent.

For microplate counter:

(**g**) *Optiphase liquid scintillation cocktail*.—PerkinElmer Life Sciences (Downers Grove, IL; Cat. No. 1200-139), or equivalent.

For sample extraction:

(**h**) *Hydrochloric acid (HCl)*.—1.0 and 0.1 M.

(**i**) *Sodium hydroxide*.—0.1 M.

(**j**) *Water*.—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in receptor assay.

E. Preparation of Stock Solutions and Standards

(**a**) *Assay buffer*.—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(**b**) *Radioligand solution*.—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230[a]	1149[a]	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

[a] Outlier; not used in calculation.

buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(**c**) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(**d**) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(**e**) *Rat brain membrane preparation*.—Prepare rat brain membrane preparation in bulk (*see Appendix: Rat Brain Membrane Preparation*) and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(**a**) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_o on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(**b**) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(**c**) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8″ Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Lab	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log \cdot EC50\ \text{Hill slope})}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; *x* axis is the log concentration of STX; and *y* axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (GraphPad Software, Inc.) is recommended. For the microplate counter users, receptor

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	–0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD).

(**a**) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl equiv./kg shellfish, from the in-well nM concentration obtained from the curve fitting software using the following formulas:

$$(\text{nM STX equiv}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCL equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1980**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	690	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

(**a**) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(**b**) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(**c**) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(**d**) QC check should be 3 nM STX ± 30% (in-well concentration). Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(**a**) Sample quantification should be done only on dilutions that fall within B/B_o of 0.2–0.7. In the event that all sample dilutions fall below B/B_o 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_o > 0.7$), the sample is reported as below LOD. If more

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

	Microplate column											
Microplate row	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [*Note:* The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(**b**) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int.* **95**, 795(2012)

Results and Discussion

Sample Characterization

All shellfish homogenates (MLV1–16) were analyzed by HPLC using the precolumn oxidation method (AOAC Method **2005.06**) to determine toxin congener profiles and quantify total PST as μg STX diHCl equiv./kg prior to initiation of the study (Table 2). It is noteworthy that the clear majority of samples, irrespective of shellfish species and location, were dominated largely by STX and GTX2,3 whereas the N1-hydroxylated congeners NEO and GTX1,4 were virtually absent, except in blue mussel from the U.S. west coast. The most unusual profile was observed in green mussel, which was dominated by the weakly toxic N-sulfo-carbamoyl congeners C1,2. The samples were analyzed by the AOAC mouse bioassay (AOAC Method **959.08**) by three laboratories that routinely perform the mouse bioassay for regulatory purposes (Table 3). The mouse bioassay detection limit is approximately 400 μg STX diHCl equiv./kg (one laboratory reported values as low as 290 μg STX equiv./kg). Because the study design included samples that bracketed the lower regulatory limit of 400 μg STX diHCl equiv./kg, several samples were reported as being below the mouse bioassay detection limit. For samples in which all values were above the detection threshold, the between-laboratory RSD_R of the mouse bioassay was 18.9%.

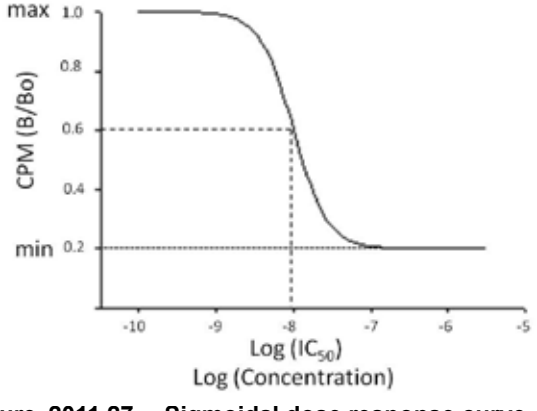


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

Data Reporting and Initial RBA Data Review

Nine of the 10 laboratories that received the study materials completed the study and reported results. All nine carried out the practice assay and reported results to the coordinating laboratory, which evaluated the results and provided feedback to the participating laboratories before initiating the full study. Following completion of the full study, the participating laboratories provided all raw and calculated data for each of

the three assays performed via e-mail to the coordinating laboratory. The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in sample dilutions and calculations, and for the use of the prescribed curve-fitting model. One laboratory used a sigmoidal curve-fitting model with the slope set to 1 (one-site binding curve in Prism), rather than the prescribed four-parameter logistic fit. In this case, the raw data were reanalyzed by the coordinating laboratory using the prescribed method. Obvious errors in calculation were corrected, such as accounting for the two-fold sample dilution resulting from the extraction process. In some cases, the participating laboratory carried out a fourth assay due to variability or inconsistency among dilutions for selected samples. In these cases, the value reported from the repeat (fourth) assay was used. One laboratory had consistent disagreement between the 1/50 and 1/200 dilutions when both fell within B/B_0 0.2–0.7. In all cases the 1/200 dilution overestimated almost two-fold relative to the 1/50 dilution, suggesting a systematic dilution error. In standard practice, these samples should be rerun. However, the instructions did not direct the participants to do so. Therefore, where there was corroborative evidence for the value reported by the 1/50 dilution, based on the 1/10 dilution, the 1/200 dilution was omitted. Where there was no basis on which to exclude the 1/200 value, an average value was calculated. This tended to result in an overestimate, and in two cases resulted in statistical outliers.

Overall Performance of the Method: Reproducibility

Table **2011.27A** summarizes the results obtained for 21 individual shellfish samples analyzed in three RBAs, determined by nine participating laboratories. Samples 1–7 were analyzed in the first assay, samples 8–14 in the second assay, and samples 15–21 in the third assay. Among these samples were five blind duplicates, treated here as individual unknown samples. One sample (marked by an footnote a in Table **2011.27A**) had a high variability in CPM between wells that was not attributable to any known cause, and was, therefore, omitted from analysis. Outliers identified by Grubbs test ($P < 0.01$) were excluded from the analysis (marked by footnote b in Table **2011.27A**). The overall RSD_R among all 21 independent samples was 33.2%, resulting in an average HorRat value of 2.0 (Table **2011.27A**). The HorRat values on individual samples ranged from 1.4 to 3.3, with a median value of 1.8. There was no apparent trend in reproducibility according to sample concentration or among shellfish species. If only the laboratories that are routine users of the RBA for PST (Laboratories 1–5) are included in the analysis, the average RSD_R is 23.1%, resulting in an average HorRat value of 1.4. Laboratory 9 tended to report the lowest values among the participating laboratories (14 of 21 samples), and although its individual sample values were not found to be statistical outliers, removing the results of this laboratory reduces all but one HorRat value (which remains unchanged), yielding an average HorRat value of 1.8 (range 1.0–2.8; Table **2011.27A**). Removal of any other single laboratory's results does not appreciably change the overall study performance. The reason for the systematically low values reported by Laboratory 9 is not clear, since the assay parameters fall well within those reported by the other laboratories. Given that assay parameters are within normal range, one possible source of systematic error could be incomplete extraction or pH adjustment of extracts, either of which would result in lower toxicity values.

A comparison of the RBA reproducibility with that of existing AOAC *Official Methods* is instructive. The AOAC collaborative study of the mouse bioassay (11), which entailed the analysis of seven samples representing three levels of STX-spiked shellfish by 11 participating laboratories, yielded a similar average RSD_R of 22%. More recent proficiency tests of the mouse bioassay performed in European regulatory laboratories report RSD_R of 2.3–38.3% on three samples run by eight laboratories (2) and RSD_R of 18.1–44.8% on two samples run by 20 laboratories (12). The mouse bioassay RSD_R values obtained in the current study ranged from 1.1 to 46.3% (average 19%) for three laboratories. The collaborative studies of the HPLC methods report reproducibility values for individual PST congeners, but do not report reproducibility of the composite toxic potency values. Collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an average RSD_R of 27.0% and HorRat value of 1.3 (range 0.8–2.1) for STX following C18 cleanup, but the reproducibility of other congeners varied considerably, with the maximum HorRat value (4.7), exceeding the highest HorRat value obtained by RBA (3.3).

Because composite toxic potency values were not reported in the studies of the HPLC methods, it is uncertain how this variability influences the composite toxic potency calculated from these methods. The average and ranges of HorRat values obtained for different congeners were: neoSTX–1.7 (range 1.2–2.5); dcSTX–1.1 (range 0.6–2.1); GTX1,4–1.9 (range 1.1–4.2), GTX2,3–1.4 (range 0.8–1.9); B1–1.1 (range 0.7–1.9); and C1,2–1.6 (range 0.9–4.5). Because of the variability obtained in neoSTX, GTX1,4, C3,4, and B2, AOAC Method **2005.06** calls for a second SPE-COOH cleanup of samples suspected of containing these congeners, after which reproducibility improved somewhat: neoSTX–1.8 (range 1.3–2.1); GTX1,4–1.3 (range 1.0–2.1); and C3,4–1.2 (range 0.8–1.8). The postcolumn oxidation HPLC method (AOAC Method **2011.02**) reported an average HorRat value of 0.6 for STX. In this method, neoSTX with an average HorRat of 1.9 (range 0.6–4.0) and GTX4 with an average HorRat of 1.6 (range 1.0–2.9) had reproducibility values that may affect the overall composite potency values. The maximum HorRat value (4.0) reported in this study also exceeded the maximum value reported in the RBA.

In summary, with the removal of Laboratory 9, the overall reproducibility of the RBA falls within the performance measures achieved by the established AOAC *Official Methods* for PST. The difference in reproducibility achieved by the laboratories that are routine users of the assay and participants who are not routine users of the method highlights the importance of training if this method were to be implemented in a regulatory setting.

Within-Laboratory Repeatability

Within-laboratory variability (RSD_r) was determined on five samples that were provided as blind duplicates. Participants were unaware that blind duplicates were included among the coded samples received. The duplicate samples were coded so that they were analyzed in separate assays, with different duplicate pairs falling into different assays (Table 1). One outlier was found among the results of the blind duplicates by Cochran's

test, $P < 0.025$ (Laboratory 7, sample MLV11) using the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0. An overall RSD_r of 25.1% was observed, with an RSD_R of 32.9%, yielding a HorRat value of 2.0, similar to that of the overall study (Table **2011.27B**). When the performance of individual laboratories was evaluated separately, the average RSD_r was 22.2%, with individual laboratories varying from 11.8 to 34.4% (Table **2011.27C**). Routine users of the microplate format of the PST RBA (Laboratories 1–5) obtained an average RSD_r of 17.1%, which is similar to that obtained in the SLV study (10), and lower than that obtained by nonroutine users (Laboratories 6–9), which averaged 26.1% and ranged as high as 34.4%. The AOAC collaborative study of the mouse bioassay (11) did not report RSD_r; however, analysis of the data from that study using AOAC INTERNATIONAL's Interlaboratory Study Workbook for Blind Duplicates results in an average RSD_r of 16.5% for three STX-spiked samples. Proficiency testing of the mouse bioassay performed in eight French laboratories reported an average RSD_r of 8.3% on three samples (2). The analysis of blind duplicates in the collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an RSD_r of 15.2% for STX following SPE C18 cleanup and an average RSD_r of 16.4% across all congeners, which ranged from 6.0 to 31.7%. Following SPE–COOH cleanup, repeatability was similar, with RSD_r of 17.2% across all congeners. The intralaboratory repeatability values obtained in the postcolumn oxidation HPLC method (AOAC Method **2011.02**) averaged 6.4% for STX; most other congeners were similar, with neoSTX being the only congener that showed a somewhat higher RSD_r of 23.3%.

In summary, the within-laboratory repeatability of the RBA was found to be acceptable, with all but two laboratories achieving an RSD_r of 23.3% or less, and the routine users of the assay achieving an average RSD_r of 17.1%.

Spike Recovery

Three samples included in the study were homogenates of blue mussel spiked with STX diHCl at concentrations intended to bracket the regulatory limits of 800 μg STX equiv./kg used by most countries and 400 μg STX equiv./kg imposed in the Philippines. Nominal concentrations in the spiked samples were 200, 500, and 1200 μg STX equiv./kg. Also included in the study was the blue mussel homogenate to which the STX spikes had been added, which was determined to be negative for STX by the precolumn oxidation HPLC method. The negative control homogenate was reported as nondetectable by eight of nine laboratories. Recovery of spiked STX by the RBA was 84.4, 93.3, and 88.1%, respectively, for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, and yielded a slope of 0.87 and r^2 of 0.86 (Figure 2). In the current study, the mouse bioassay reported < detection limit, and 68.6 and 40.5% recovery for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels. The AOAC collaborative study of the mouse bioassay (11) reported recoveries of 62.3% at spike levels similar to those in the current study (equivalent to 1000 μg STX diHCl equiv./kg) but higher recoveries of 81.5 and 96.0% were achieved at higher spike levels equivalent to 4000 and 8000 μg STX diHCl equiv./kg.

The observed poor recovery in the mouse bioassay at concentrations near and below the regulatory limit has been observed in other studies (2), and has been attributed to a salt or protective effect of the shellfish matrix, which, for concentrations at or below the regulatory limit of 800 μg/kg, is injected undiluted into the mouse. The spike recovery observed in the precolumn HPLC method in this study is also somewhat low, with 54.0, 62, and 51.5% recovery at the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, respectively. The AOAC collaborative study of the precolumn HPLC method reported 74.4–76.8% at similar spike levels following SPE C18 cleanup and 63.7–68.2% following SPE-COOH cleanup (3, 4). In comparison, the postcolumn HPLC method reported 88–104% recovery of STX spiked at levels somewhat lower than the current study. The higher recovery of the RBA than the HPLC method in the current study may reflect the use of the 0.1 M HCl extraction method in the RBA as compared to the acetic acid extraction used in the HPLC methods.

We previously established in the SLV study that the RBA performs well with shellfish extracted using either method (10). In that study, the RBA reported slightly higher toxicity values for shellfish extracts made using the 0.1 M HCl method than the acetic acid extraction, yielding a correlation of 0.99 with a slope of 1.23 (10). The higher toxicity reported by the RBA in 0.1 M HCl extracts may reflect the hydrolysis of less toxic congeners to more toxic congeners.

Assay Parameters and Quality Metrics

Table **2011.27D** summarizes the assay parameters and quality metrics for all laboratories. Eight of nine laboratories used microplate scintillation counters. Laboratory 4 used the manual counting method in which the microplate well filters are punched out, using an eight-place punch system, into traditional 4 mL scintillation vials and counted. Its performance using the manual counting method (RSD_r 17.4%) was similar to or better than that of the laboratories using the microplate method, indicating that using the manual counting method does not affect the performance of the assay. Similarly, there was no apparent difference in assay parameters when the Packard Top Count (single detector) was used, compared to the Wallac Microbeta (coincidence detector), although the reference CPM values obtained on the Top Count generally were somewhat lower due to differences in counting efficiency inherent in the differences in detector geometry. Eight of nine laboratories used GraphPad Prism for curve-fitting, while only Laboratory 5 used Wallac MultiCalc software. Values reported by Laboratory 5 fell well within the range of values reported by laboratories using Prism.

All assays resulted in slopes between –0.8 and –1.2, as specified in the protocol. This specification reflects the fact that in a competitive binding assay for a ligand that interacts specifically at a single receptor site, the slope of the resulting standard curve should theoretically be 1.0. Although curve-fitting software packages often include a one-site binding curve that fixes the slope at 1.0, we specified in the protocol the use of the four-parameter logistic fit (also known as sigmoidal dose-response with variable slope), because it more readily identifies problems with the standard curve that may skew results. Laboratory 9 reported results using a one-site binding curve fit; in this case, the coordinating laboratory recalculated their raw data using the four-parameter logistic fit. The protocol also calls for RSD% < 30 on all standards. Most analysts did not experience variability problems in the standard wells. Infrequent high RSDs were most often associated with the well

Table 2. Congener profiles in shellfish homogenates included in the collaborative study[a]

Sample name	Species	STX	NEO	dcSTX	GTX1,4	GTX2,3	dcGTX2,3	B1	C1,2	C3,4	Total PSP	µg STX diHCl equiv./kg
MLV01	Surf clam	639.8		74.0		226.2	207.0				1146.9	894.3
MLV02	Almeja clam	298.3				1290.1		266.6			1855.0	802.1
MLV03	Chorito mussel	77.6				310.4					388.0	195.5
MLV04	Atlantic sea scallop	831.6				2785.6					3617.3	1890.2
MLV05	Atlantic sea scallop	193.8				576.2					770.0	412.8
MLV06	California mussel	912.8		10.9		0.0		233.8			1157.5	931.3
MLV07	Blue mussel, U.S. east coast	548.2				1097.3					1645.5	965.2
MLV08	Green mussel	164.2		63.5			272.3	454.8	3629.0		4419.6	340.8
MLV09	Blue mussel, U.S. west coast	432.3	124.9	8.7	353.7	727.8		506.4			2153.9	1070.9
MLV10	Butter clam	1763.5		40.6		533.2		203.5			2540.8	2000.9
MLV11	Almeja clam	159.1		12.2		185.5					356.8	236.9
MLV12	Blue mussel spike	108.4									108.4	108.4
MLV13	Blue mussel spike	310.2									310.2	310.2
MLV14	Blue mussel spike	618.5									618.5	618.5
MLV15	Blue mussel blank										0.0	0.0
MLV16	Chorito mussel	389.8		14.3		754.1					1158.1	684.9

[a] Values for individual congeners are in µg/kg. Values for composite toxicity are in µg STX diHCl equiv./kg. Abbreviations for congeners are as follows: STX – saxitoxin; NEO – neosaxitoxin; dcSTX – decarbamoyl saxitoxin; GTX1,4 – gonyautoxin 1 and gonyautoxin 4; GTX2,3 – gonyautoxin 2 and gonyautoxin 3; B1 – gonyautoxin 5 (also known as sulfocarbamoyl STX B1); C1,2 – sulfocarbamoyl STX C1 and sulfocarbamoyl STX C2; C3,4 – sulfocarbamoyl STX C3 and sulfocarbamoyl STX C4.

Table 3. Mouse bioassay results on collaborative study samples from three laboratories[a]

Sample No.	Sample ID	MBA Lab A	MBA Lab B	MBA Lab C	MBA Avg.	MBA s_R	MBA RSD_R, %
1	MLV05	400	415	340	385	39.7	10.3
2	MLV06	550	597	540	562	30.4	5.4
3	MLV08	440	<dl[b]	370	405	49.5	12.2
4	MLV09	670	612	760	681	74.6	11.0
5	MLV12	<dl	<dl	<dl	—	—	—
6	MLV14	489	489	480	486	5.2	1.1
7	MLV16	585	585	470	547	66.4	12.1
8	MLV01	750	716	600	689	78.6	11.4
9	MLV02	670	1115	590	792	282.9	35.7
10	MLV04	2040	<dl	1080	1560	678.8	43.5
11	MLV07	1480	748	670	966	446.8	46.3
12	MLV09	—	594	670	602	11.3	1.9
13	MLV11	380	379	<dl	380	—	—
14	MLV13	<dl	343	<dl	343	—	—
15	MLV03	400	364	<dl	382	—	—
16	MLV05	—	396	370	383	18.4	4.8
17	MLV06	—	702	630	666	50.9	7.6
18	MLV07	—	<dl	690	690	—	—
19	MLV10	1320	890	870	1027	254.2	24.8
20	MLV11	—	364	290	327	52.3	16.0
21	MLV15	<dl	<dl	<dl	—	—	—

[a] Values are in µg STX diHCl equiv./kg.

[b] dl = Detection limit.

in column 1 of the 96-well plate. Most analysts removed the suspect well from the curve-fitting process. When the RSD for a given standard was near the stated cutoff (e.g., 31–33%), and left in the curve-fitting process, there was no apparent effect on the curve parameters listed as criteria for assay acceptance.

The average IC_{50} among all 27 assays was 1.9 + 0.45 nM (RSD_R 23.5%). The other assay quality metric called for by the protocol is the analysis of the QC check sample, which should be 3 ± 0.9 nM STX (30% RSD, in-well concentration). Four of the 27 assays had QC values outside the stated limits, with no obvious error responsible for the variability. Among these, Laboratory 7 reported 6.5 nM for the QC check in assay 3 and an IC_{50} of 3.4 nM, which was outside the norm. Similarly, Laboratory 8 reported a QC of 1.5 nM in assay 2 and a low IC_{50} of 1.4 nM, which is at the lower edge of acceptability. In general practice, these values would trigger repeating the assay. However, because of the minimal number of laboratories participating in the study, both of these assays were retained in the study. In neither case were the reported sample values systematically higher or lower than those reported in the other assays.

LOD and LOQ

The LOD was calculated based on the measurement of the negative control shellfish matrix (MLV15) using the blank + 3×SD approach according to Eurachem guidelines (13), as recently applied to AOAC Method **2006.02**, an ELISA for domoic acid in shellfish using a similar four-parameter logistic curve (14). All laboratories reported <dl for this sample using the prescribed cutoff of B/B_0 <0.7 for quantification, with the exception of Laboratory 8, which was removed as an outlier as determined by Grubbs test ($P < 0.01$). If these samples are instead quantified using the B/B_0 values obtained, a mean of 5.5 ng/mL is obtained with an SD of 5.7 ng/mL, resulting in an LOD of 45 µg STX diHCl equiv./kg. Using the blank + 10×SD definition, an LOQ of 126 µg STX di HCl equiv./kg is thus obtained. We previously established empirically that a 1/10 dilution of shellfish extracts is sufficient to remove matrix effects in the RBA (10), when a quantification cutoff of B/B_0 <0.7 is used. This is the basis for the ten-fold minimum sample dilution used in the current study. The IC_{70} values (B/B_0 0.7) for all standard curves run in the study are presented in Table **2011.27D**. An average of 0.80 ± 0.188 nM STX diHCl was obtained across all assays, following the removal of one outlier based on the Grubbs test ($P < 0.01$). Applying the blank + 3×SD to this value, an LOD of 64 µg STX diHCl equiv./kg is obtained; applying the blank + 10×SD to this value results in an LOQ of 131 µg STX diHCl equiv./kg for a sample diluted 1/10 and extracted as indicated in the study, in fair agreement with the value calculated above.

Correlation with HPLC and Mouse Bioassay

Comparison of the RBA results with the mouse bioassay

Nominal	Avg	S_R	RSD_R,%	Recovery, %
200	169	58	34.6	84.4
500	466	133	28.5	93.3
1200	1057	228	21.7	88.1

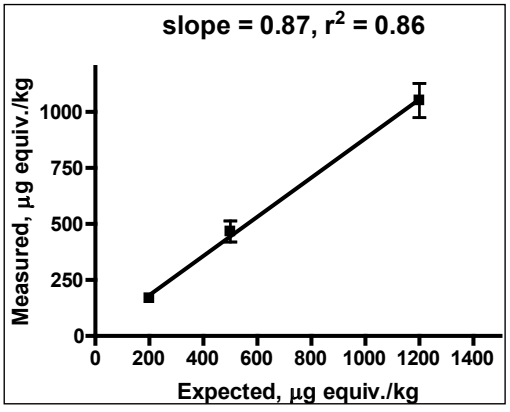


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

results yielded an r^2 of 0.84 and a slope of 1.64, indicating that the RBA reports somewhat higher STX equivalents in shellfish, relative to the mouse bioassay (Figure 3). This overestimate has been previously reported for both RBA and HPLC methods (2, 9) at the STX levels near or below the regulatory limit, which are the focus of the current study. Consistent with these findings, the HPLC method also reported higher values than the mouse bioassay in this study, with a slope of 1.33 and an r^2 of 0.84. RBA results correlated better with the precolumn oxidation HPLC method, with a slope of 1.20 and an r^2 of 0.92.

RBA Yielded No False Negatives Relative to the Regulatory Limit

When the data from the three methods were sorted by increasing µg STX diHCl equiv./kg as reported by the mouse bioassay, the RBA did not report any false negatives when compared to the regulatory limit of 800 µg STX equiv./kg (Table **2011.27E**). When compared with the precolumn oxidation HPLC method, only Laboratory 9 reported values lower than the HPLC method. The fact that the RBA reports somewhat higher toxicity than the mouse bioassay or HPLC at levels near or below the regulatory limit is beneficial from a food safety standpoint. The higher values reported presumably arise from better recoveries, as demonstrated above. From a shellfish producer's perspective, the improved detection limits relative to the mouse bioassay and better recovery of low toxin levels compared to the HPLC can help to provide advance warning of developing toxicity, allowing producers to harvest early, delay harvest, or move cultures, as appropriate.

Participants' Comments

Laboratory 6 participated in the study without previous experience running receptor assays, and in doing so, identified several points needing clarification that have since been added to the proposed *Official Method* as enumerated in this report: (*1*) The vacuum required for filtration was not specified at 4–8″ Hg, which is critical because insufficient vacuum pressure results in too slow a clearance of the wells, whereas too much pressure results in an airlock and no filtration at all. (*2*) Scintillation counting time for the microplates is 1 min/well. (*3*) Instructions have been added regarding how to calculate sample concentration if more than one dilution falls within B/B_o 0.2–0.7; specifically, an average value should be calculated from all sample dilutions falling within B/B_o 0.2–0.7. When corrected for dilution, serial sample dilutions should yield similar quantification. The absence of linearity between sample dilutions indicates either error in dilution or sample matrix interference; however, at the minimum sample dilutions recommended in the proposed *Official Method*, matrix effects from shellfish homogenates have not been encountered (10). In the current study, the nonlinearity of dilutions experienced in several samples by Laboratory 8 was not observed by the other laboratories, suggesting a systematic sample dilution issue rather than a sample matrix problem. Although experienced in RBAs in general, Laboratory 8 had not previously run the microplate filtration format of the assay for PST.

Laboratory 9, which reported generally lower values than the other laboratories, although familiar with the assay, had not performed it in more than a year. The lower values reported do not appear to be associated with conduct of the assay, or scintillation conduct of the assay, or scintillation counting, since the assay metrics are well within the averages reported by the other laboratories. Insufficient boiling or pH adjustment of sample extracts are a possible explanation. These points identified by the study participants should be added to the critical steps identified in the SLV study (10) that can affect precision and accuracy of the assay results, including: (*1*) ensure that the water is strongly boiling during extraction; (*2*) carefully adjust pH of extracts; (*3*) ensure even distribution of the membrane preparation across the microplate by frequent vortex-mixing or pipetting before and during its addition to the plate; (*4*) the wells must clear within 2–5 s during filtration; (*5*) the wash buffer should be ice-cold to minimize the rate of toxin release from the receptor; and (*6*) following addition of scintillant to the wells, incubate a minimum of 30 min to ensure that the scintillant fully penetrates the filters before counting.

Recommendations

The collaborative study of the RBA for PST was completed by nine laboratories representing six countries. Collaborators quantified PST as a composite toxicity value reported in µg STX di HCl equiv./kg in a variety of shellfish species from different regions of the world, containing varied toxin congener profiles. The study included laboratories with extensive experience as well as others with little or no previous experience. The study also included both microplate and scintillation counters as end points, because either instrument type could potentially be used by test laboratories. The study demonstrates that the RBA yields adequate repeatability, reproducibility, and recovery for routine determination and monitoring of PST in shellfish. The greater precision attained by laboratories that received prior training on the RBA and routinely implement this assay suggests that

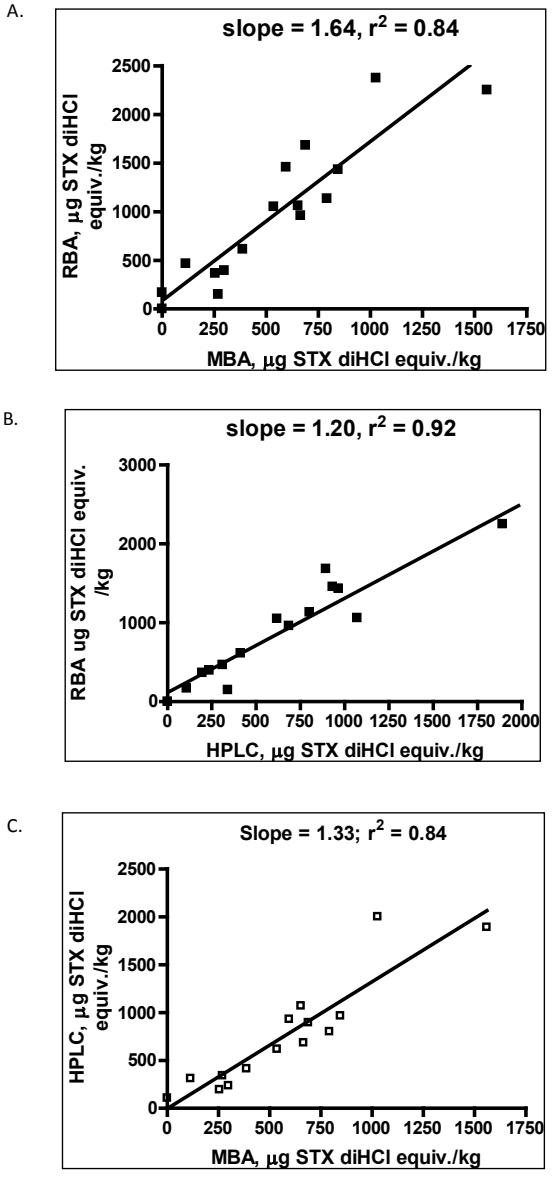


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

the overall interlaboratory reproducibility can be further improved. It is recommended that this method be accepted by AOAC INTERNATIONAL as Official First Action for the determination of PST in shellfish.

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Appendix: Rat Brain Membrane Preparation

The rat brain membrane preparation used in this assay can be produced in bulk, aliquotted, and stored at –80°C until use. Under this storage condition, the preparation is stable for a minimum of 6 months. The following protocol provides sufficient membrane preparation for a minimum of 125 plates and can be scaled up or down as needed.

A. Apparatus

(**a**) *Teflon/glass homogenizer*.—Motorized tapered Teflon pestle and glass tube, 15 mL.

(**b**) *Motorized tissue homogenizer*.—Polytron or small hand-held blender.

(**c**) *High-speed centrifuge and fixed angle rotor.*—Capable of 20 000 × *g* (rcf).

(**d**) *Centrifuge tubes*.—12–15 mL rated for >20 000 × *g* (rcf).

(**e**) *Plastic cryovials*.—2 mL.

(**f**) *Graduated beaker*.—300 or 500 mL.

(**g**) *Pipets*.—Disposable 5 and 10 mL.

(**h**) *Forceps*.

B. Reagents

(**a**) *20 Rat brains*.—Male, 6-week-old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA; http://hilltoplabs.com) or equivalent.

(**b**) *MOPS*.—pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G).

(**c**) *Choline chloride*.—100 mM (Sigma; Cat. No. C7527-500G).

(**d**) *Phenyl methylsulfonyl fluoride (PMSF)*.—Sigma; Cat. No. P7626.

(**e**) *Isopropanol*.

C. Procedure

(*1*) Prepare 1 L 100 mM MOPS buffer, pH 7.4, containing 100 mM choline chloride (detailed protocol in **E**, above) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol; dissolve 0.174 g PMSF in 10 mL isopropanol to make 100 mM stock. Aliquot and store at –20°C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh on the day of use.

(*2*) Remove medulla and cerebellum from each brain using forceps and discard. Place the cerebral cortex (*see* Figure 1) in a small amount of ice-cold buffer and place on ice.

(*3*) Place one cerebral cortex in 12.5 mL MOPS/choline Cl/PMSF, pH 7.4, in glass/teflon homogenizer (two brains in 25 mL buffer will fit into 30 mL homogenizer tube). Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes (more if necessary to homogenize brain; there should be no visible chunks remaining in the homogenate). Keep tube in ice at all times. Pour homogenized tissue into 250 mL beaker on ice and repeat procedure with remaining cortices.

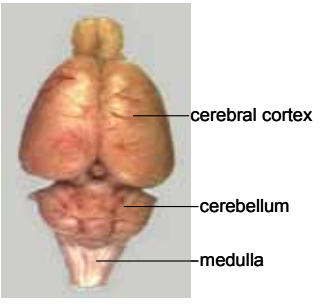


Figure 1. Rat brain.

(*4*) Transfer pooled homogenized tissue to centrifuge tubes, balance the tubes (pairwise; use ice-cold buffer to balance), and centrifuge at 20 000 × g for 15 min at 4°C.

(*5*) Aspirate the supernatant and resuspend the pellets in ice-cold MOPS/choline Cl/PMSF buffer, using an adequate amount (~5 mL) to fully resuspend the pellet (can use clean glass stir rod to break up pellet), not exceeding 10 mL per brain.

(*6*) Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume to 200 mL total (keep on ice).

(*7*) Keeping the beaker on ice, Polytron (or use a small hand-held blender at low speed) at 70% full speed for 20 s to obtain a consistent homogenate.

(*8*) Aliquot 2 mL/tube into cryovials. It is critical to keep the preparation well mixed while dispensing, e.g., prior to each aliquot to ensure equal allocation of protein/receptors to each vial. Keep cryotubes on ice.

(*9*) Freeze and store at –80°C. This preparation is stable for at least 6 months. Use a permanent marker to label the preparation date on the storage container.

D. Protein Assay

(**a**) Determine protein concentration of membrane preparation using Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) protein assay kit or equivalent protein assay (Thermo Fisher, Rockford, IL). The above protocol should yield 6–8 mg protein/mL of rat membrane preparation.

(**b**) Determine membrane dilution needed for the assay. The protein concentration in the daily working stock for the assay should be 1 mg/mL (this is diluted in the assay to yield 0.5 mg/mL in-assay concentration). Based on the protein concentration determined in the protein assay, determine the dilution needed to achieve 1 mg/mL. This is the dilution used in section **E**(**e**) above for all assays using this lot of membrane preparation. The protocol above typically yields a protein concentration that requires a dilution of 1/6–1/8. (Do not use less than 1/4 dilution or filtration wells may become clogged.) Protein concentration will need to be determined for each new batch of membrane preparation.

Appendix 4

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Evaluation of variability and quality control procedures for a receptor-binding assay for paralytic shellfish poisoning toxins

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The receptor-binding assay (RBA) method for determining saxatoxin (STX) and its numerous analogues, which cause paralytic shellfish poisoning (PSP) in humans, was evaluated in a single laboratory study. Each step of the assay preparation procedure including the performance of the multi-detector TopCount® instrument was evaluated for its contribution to method variability. The overall inherent RBA variability was determined to be 17%. Variability within the 12 detectors was observed; however, there was no reproducible pattern in detector performance. This observed variability among detectors could be attributed to other factors, such as pipetting errors. In an attempt to reduce the number of plates rejected due to excessive variability in the method's quality control parameters, a statistical approach was evaluated using either Grubbs' test or the Student's *t*-test for rejecting outliers in the measurement of triplicate wells. This approach improved the ratio of accepted versus rejected plates, saving cost and time for rerunning the assay. However, the potential reduction in accuracy and the lack of improvement in precision suggests caution when using this approach. The current study has recommended an alternate quality control procedure for accepting or rejecting plates in place of the criteria currently used in the published assay, or the alternative of outlier testing. The recommended procedure involves the development of control charts to monitor the critical parameters identified in the published method (QC sample, EC_{50}, slope of calibration curve), with the addition of a fourth critical parameter which is the top value (100% binding) of the calibration curve.

Keywords: receptor-binding assay; paralytic shellfish poisoning; saxitoxins; variability

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Introduction

Coastal regions with a history of the occurrence of paralytic shellfish poisoning (PSP) toxins present unique challenges to the organisations responsible for protecting public health. The agencies responsible for monitoring these toxins in shellfish (e.g., mussels, oysters) and other seafood species have traditionally relied on the mouse bioassay (MBA) (American Public Health Association (APHA) 1970). Until recently this live animal assay has been the only method recognised by the National Shellfish Sanitation Program (NSSP) as administered by the US Food and Drug Administration (USFDA). The MBA has served these monitoring programmes well over the decades, but the continued use of live animals for toxin testing presents practical and ethical concerns. The MBA is also recognised as having relative poor accuracy and precision due to matrix effects at low dilutions and inherent differences in response among animals. As a result there has been a considerable amount of work and progress in the development of alternative methods including a receptor-binding assay (RBA) method (Doucette et al. 1997; Powell and Doucette 1999; Ruberu et al. 2003) and HPLC methods (Lawrence et al. 2005; van de Riet et al. 2009). The latter HPLC method has recently been accepted by the Interstate Shellfish Sanitation Conference (ISSC) and USFDA for use within the NSSP. The RBA method has recently been issued as an Official Method of Analysis (OMA) (number 2011-27) by the Association of Official Analytical Chemists (AOAC), but has yet to be presented to the ISSC for acceptance.

Most, if not all, monitoring programmes have similar requirements with respect to an acceptable replacement method for the MBA. Analytical turn-around time and sample throughput are critical factors for getting data into the hands of managers quickly, so decisions can be made regarding quarantines and notification of the public. Shellfish sample collection and shipment to an accredited laboratory can introduce significant time delays, often 24–48 h, placing the responsible agency at an immediate disadvantage in its efforts to protect consumers. Therefore, there is a need for a method that can provide data within hours of

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sample arrival. The preferred method must also be capable of automation to accommodate a significant influx of samples when PSP levels begin increasing in a region. It is also highly preferable that the methodology be simple enough to be carried out by a trained technician, as opposed to the more technically sophisticated methods that require an experienced analyst with an advanced degree. Other desirable features include improved accuracy, precision and sensitivity relative to the current MBA. The lack of precision of the MBA creates ambiguity when results are close to the action level (80 μg of saxitoxin (STX) equivalents per 100 g of shellfish tissue, abbreviated as 80 μg/100 g). Replication would help alleviate this ambiguity but is usually impractical when large numbers of samples are being assayed and throughput time must be minimised.

The van de Riet HPLC method may be the alternative method of choice for some regulatory laboratories. One seemingly minor but very important practical consideration in this regard is the administrative location of the current MBA work. If this work is presently conducted in a laboratory section in which chemical instrumental analyses are also conducted (e.g., HPLC methods), then the adoption of the HPLC method for PSP toxins could be a relatively simple transition both technically and organisationally. However, if the MBA work is currently conducted in a microbiology setting, a number of obstacles may preclude adoption of a chemical instrumental method in favour of an assay format more familiar to the microbiologist, such as an immunoassay or receptor assay. The potential obstacles in these times of reduced resources include retraining or hiring new staff, purchasing of expensive equipment or transferring resources from one department to another. Although HPLC technology includes automation via autosamplers, other factors such as time for careful filtration makes the analytical time spent per sample long enough that results for many of the samples in the queue are not available until the following workday. Furthermore, at present there are standards commercially available through the National Research Council of Canada for 12 of the more than 30 analogues of STX. The cost of these standards, and the lack of a domestic supply, may be of concern for a regulatory laboratory that processes thousands of samples per year. The detailed, compound-specific information provided by the current HPLC methods will provide valuable insight into the toxin profile(s) present along a coastal region, but may not be essential for routine monitoring purposes. A quick and reliable estimate of total toxicity is what is typically needed by the public health manager.

An alternative method that may satisfy the criteria listed above is the RBA. This competitive binding assay (Doucette et al. 1997; Ruberu et al. 2003) uses the same AOAC sample extraction procedure used for the MBA. The 96-well plate format of the RBA allows testing of up to seven samples in triplicate, with three dilutions per sample to ensure the proper concentration range is represented. Multiple plates can be queued on the plate reader, with results from several successive plates available on the same day. In fairness, the MBA will likely provide results faster for the first several samples assayed, but will fail to meet the high throughput requirements during a major event due to the lack of automation. The RBA procedures are straightforward and can easily be performed by a trained technician. The reporting limit established in our laboratory for the RBA is significantly lower (4 μg/100 g tissue) than the detection limit of the MBA (35 μg/100 g tissue in the CDPH laboratory), illustrating the high sensitivity of the RBA method. Another advantage of the RBA is that it does not require careful filtration of samples prior to analysis as is the case with the HPLC method, reducing the time required for sample preparation. The majority of reagents are commonly available and relatively inexpensive, the exception being the tritiated STX needed for competitive binding. This reagent is not readily available through government services such as the National Institute of Standards and Technology (NIST), but is currently available commercially within the United States. Reliance on proprietary materials is always a point of concern for regulatory laboratories if there are no alternative sources available. A possible source of error in the RBA is the rat membrane synaptasome preparation. Not only is it a very inconvenient preparation procedure to carry out, but also due to its heterogeneity this membrane can be associated with high assay variability. One way to overcome this would be to have it available commercially as a standardised reagent.

Our previous experience with the RBA (Ruberu et al. 2003) was encouraging relative to the criteria mentioned above, and the precision of the method in our laboratory was found to be 10%. However, more recent work in our laboratory has suggested that method precision was no better than the MBA. Therefore, it was determined that a more detailed investigation into the various components of this assay was warranted in the hopes that method precision could be improved, facilitating the decision-making process for public health managers.

Materials and methods

Chemicals and reagents

- ^{3}H-STX diacetate in methanol (Lot #040616, 0.1 m Ci ml^{-1}, specific activity = 18.0 Ci mmol^{-1}) (American Radiolabeled Chemicals Inc., St. Louis, MO, USA).

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- FDA reference standard, STX dihydrochloride (Lot #088 100 μg ml^{-1} in 20% ethanol–water at pH 3.5) (USFDA, Office of Seafood, Laurel, MD, USA).
- Rat membrane synaptosome: the rat membrane preparation containing sodium channel receptors was composed of 20 brains from 6-week-old male Hotsman rats (Harlan Bioproducts, Indianapolis, IN, USA) and prepared according to the methodology of Doucette (Doucette et al. 1997). This preparation was divided into 2 ml aliquots and frozen at −70°C. A single aliquot was thawed for each RBA plate preparation.
- All reagents, standards and dilutions were prepared in 100 mM MOPS/100 mM choline Cl buffer at pH 7.4. To prepare this buffer, 20.9 g of MOPS (3-morpholinopropanesulfonic acid) and 13.96 g of choline chloride were dissolved in 900 ml of water, the pH adjusted to 7.4 and the final volume brought to 1 L with water.

Instrumentation

Scintillation counting was performed on a PerkinElmer Life and Analytical Sciences instruments TopCount® Model B. MicroScint-20 cocktail (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA) was used as the scintillant for all RBA work.

Receptor binding assay (RBA) protocol

The RBA procedure involved the addition of 35 μl of MOPS/choline Cl buffer, 35 μl of unknown sample (or STX standard), 35 μl of ^{3}H-STX, and 105 μl of a 1:6 diluted synaptosome preparation, in this order, to a 96-well microtitre filtration plate. A typical plate outline is given in Figure 1. All calibration standards, QC samples, reference samples and shellfish sample extracts are run in triplicate on each plate. The first three columns of each plate were used to generate a calibration curve. Saxitoxin dihydrochloride standard was used for the calibration curve in the following final in-assay molar concentrations: 1×10^{-6}, 1×10^{-7}, 3×10^{-8}, 1×10^{-8}, 3×10^{-9}, 1×10^{-9}, 1×10^{-10} and 1×10^{-11}. Three wells per plate served as a reference blank, containing the material and reagents described above but omitting a source of non-radiolabelled STX. The reference blank establishes the maximum binding (B_{max}) for each plate. A quality control (QC) sample yielding an in-assay concentration of 3.0×10^{-9} M STX standard, independently made, was used as a daily QC check. All pipetting was carried out using a certified, calibrated eight-channel pipette. To achieve equilibrium binding, the plate was incubated for 1 h at 4°C, then filtered using a MultiScreen vacuum manifold system and rinsed with 200 μl of ice-cold (4°C) MOPS/choline Cl buffer to remove unbound toxin. To each well 50 μl of the scintillant (MicroScint®) were added, and the top of the plate sealed with tape. The prepared plate was placed inside the TopCount scintillation counter for 30 min. This allowed the scintillant to dark adapt and the contents to mix, prior to counting the receptor-bound ^{3}H-STX.

Criteria that must be met for assay acceptance are as follows: (1) the slope of the standard curve must be between 0.8 and 1.2, (2) the relative standard deviation (RSD) of counts per minute (CPM) for each standard must be <30%, and (3) the QC check must be ±30% of the in-assay concentration of 3.0×10^{-9} M STX. Criteria for sample acceptance and quantification are: (1) $B/B_0 = 0.3$–0.7 and (2) RSD of the sample CPM must be <30%.

	1	2	3	4	5	6	7	8	9	10	11	12
A	$1X10^{-6}$	$1X10^{-6}$	$1X10^{-6}$	Ref Blank	Ref Blank	Ref Blank	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200
B	$1X10^{-7}$	$1X10^{-7}$	$1X10^{-7}$	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
C	$3X10^{-8}$	$3X10^{-8}$	$3X10^{-8}$	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
D	$1X10^{-8}$	$1X10^{-8}$	$1X10^{-8}$	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
E	$3X10^{-9}$	$3X10^{-9}$	$3X10^{-9}$	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
F	$1X10^{-9}$	$1X10^{-9}$	$1X10^{-9}$	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U4 1:200	U7 1:50	U7 1:50	U7 1:50
G	$1X10^{-10}$	$1X10^{-10}$	$1X10^{-10}$	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
H	$1X10^{-11}$	$1X10^{-11}$	$1X10^{-11}$	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50	QC	QC	QC

Figure 1. Layout of a typical 96-well plate used in RBA. The first three columns are used to generate the calibration curve. Six wells are used for quality control samples (QC) spiked at 3.0×10^{-9} M in assay concentration, three wells are used to determine maximum binding (ref blank) (B_{max}), and the rest of the wells are used for unknown samples (U).

Statistical analysis

MedCalc statistical software (MedCalc Software, Mariakerke, Belgium; Windows Version 10.4.8.0; http://www.medcalc.org) was used for all statistical analyses. Analysis of variance (ANOVA) was used to evaluate the significance of variability of mean counts among sequential plate readings and among detectors for a given plate reading. Plates found to have a significant difference among either sequential readings or detectors were subjected to post-hoc significance testing with the Student–Newman–Keuls (SNK) test for all pairwise comparisons. Prism (Graph Pad Software, Inc., La Jolla, California, USA) was used to generate the STX binding curves.

Results and discussion

Method variability study of RBA

Our laboratory has been following the RBA protocol as developed by Doucette et al. (1997) and later modified by Ruberu et al. (2003) for the detection of STXs in shellfish matrices. This methodology involves the competitive binding between STX analogues (in sample) and tritiated saxitoxin (^{3}H-STX) reagent. As we gained more experience with the assay it became clear that there were sources of variability that were not well understood. This involved unacceptable levels of variance among triplicate samples at a given dilution, variability in reference samples, and in QC standards placed at the beginning and end of each plate. The RBA requires pipetting of small volumes of reagents (35–100 µl) and is comprised of several independent steps, each of which is a potential source of variability. Our previous work (Ruberu et al. 2003) determined the RSD for assays of environmental samples to be 10%. In order to study the overall variability of the RBA with the goal of improving the method's precision, it was necessary to deconstruct the assay to its simplest components, then "rebuild" step by step, evaluating each step for its contribution to overall assay variability. Identified in this study are inherent differences among wells of the plate for replicate samples, heterogeneity of the rat membrane (binding sites) in each well, and the competitive binding process in each well. Given that each well acts as an independent experiment within a single plate, some amount of variability was expected for the measured CPMs among wells.

Another factor that can contribute to assay variability is the instrument's inherent variability among its 12 detectors, each of which reads a total of eight wells per plate. Detector normalisation is performed as part of routine maintenance of the instrument to minimise the variability that may exist among detectors. To understand detector variability it is important to know how the detectors are set up and which order the detectors read the wells. The TopCount® instrument has two rows of six detectors each. The plate is read starting from the top row A to bottom row H (Figure 1). When a plate is read, the first set of six detectors measure wells A1, A3, A5, A7, A9 and A11, then move down to read wells B1, B3, B5, B7, B9 and B11. Subsequently, wells C1, C3, C5, C7, C9, C11 and A2, A4, A6, A8, A10 and A12 are read simultaneously by both sets of detectors. This continues until the set of wells G2, G4, G6, G8, G10 and G12 and the last set of wells H2, H4, H6, H8, H10 and H12 have been read by the second set of detectors. Not all wells are read simultaneously. As such, with a 5-min count time per well, the time difference between the measurement of the first and last wells is about 50 min. This can be a substantial period with respect to dissolution between sample and cocktail. To evaluate this potential source of variability to the assay, the count data for the series of plates studied were grouped by detector and statistically analysed by ANOVA to determine if there was a significant difference among the 12 detectors and, if so, which detectors were responsible for this variability.

Instrument background plate

To determine the inherent background variability in counts among the wells of a single plate, all 96 wells were filled with 50 µl of MicroScint® cocktail and counted three times in succession with a 30-min dark adapt delay period prior to each measurement. Background counts ranged from 8 to 36 CPM, from 7 to 27 CPM, and from 8 to 27 CPM for the three consecutive readings with average counts of 17.9, 17.8 and 16.5 CPM respectively. The standard deviation (SD) for the three count cycles ranged between 4 and 5 CPM. Figure 2 shows the CPM variability of the instrument background plate with respect to each

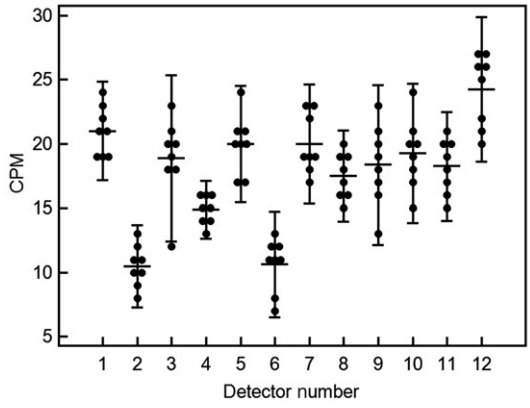


Figure 2. Plot of the instrument background plate second count cycle depicting the randomness of CPMs in the 96 wells. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

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detector. Since there is no mixing of reagents involved in this plate, the variation seen here is attributed solely to counting statistics and to differences between the 12 detectors themselves. There was no significant pattern of variability observed for any single detector or to the time at which a well was counted. Although detectors 2 and 6 exhibited lower cpm values than the other ten detectors (Figure 2), the pattern of detector performance varied among the three sequential plate readings.

Blank plate

The next step was to determine the variability in counts among wells when a source of tritium was present. For this study all 96 wells were filled with 35 µl of ^{3}H-STX followed by 50 µl of MicroScint® cocktail. This blank plate was counted five times in succession with a 30-min dark adapt delay period prior to each counting cycle. Potential contributors to variability such as rat membrane preparation, competing non-labelled toxin, the competitive binding process itself and the washing/filtering step were absent.

Results showed a gradual increase in average CPM for the five sequential readings (Figure 3) with the greatest increase between the first ($CPM_{average} = 700$; RSD = 19%) and second ($CPM_{average} = 869$; RSD = 17%) measurements. The CPM stabilised with the next three readings ($CPM_{average} = 915$, 939, 954; RSD = 17%, 16%, 16%). A one-way ANOVA determined that there was a significant difference among the mean CPMs ($p < 0.001$) for the five counting cycles. SNK post-hoc significance testing for all pairwise comparisons determined that the first two plate readings were significantly different from one another ($p < 0.05$) and both were significantly different from plate readings three through five. It also showed that the last three plate readings were not significantly different from one another ($p > 0.05$). From these observations it is clear that the increase in CPM is due to mixing of the aqueous phase with the cocktail. An additional time of 3 h is needed to reach equilibrium in mixing. This is not a practical concern for the present assay because subsequent steps involve rinsing and filtering of each well prior to adding the cocktail, resulting in a single phase in each of the wells at the time of counting. However, shaking the RBA plate prior to the incubation step would make certain that all the reagents in the wells are properly mixed. These results give insight into the two-phase mixing process in a plate format. Unlike conventional liquid scintillation counting methods where 20 ml vials are vigorously shaken to obtain homogenous mixing prior to counting, in the plate format mixing can be an issue in obtaining reproducible results when assays with two phases are involved. This is further supported by looking at the first set of wells measured at the start of the count and the last set of wells measured (about 50 min later) within a single plate. The former (row A) had a $CPM_{average}$ of 447 while the latter (row H) had a $CPM_{average}$ of 723.

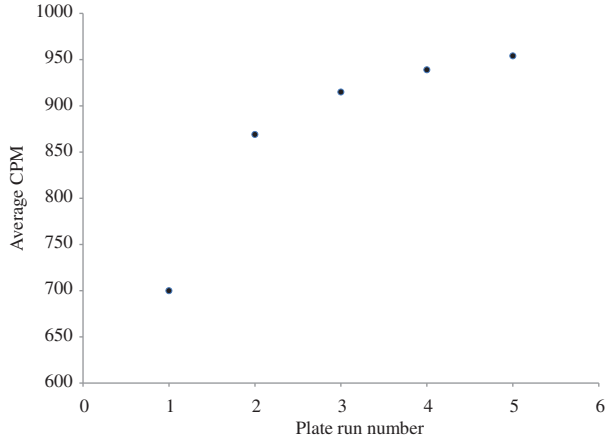


Figure 3. Graph of the blank plate five sequential readings showing a gradual increase in average CPM.

Reference plate (non-competitive binding)

The next step in reconstructing the assay involved introduction of binding sites for the ^{3}H-STX, i.e. non-competitive binding. In practice such a reference sample is run in triplicate on each RBA plate and the average CPM is used to determine maximum binding, B_{max}. This B_{max} value is used as the baseline and is compared with the sample CPM to generate the actual binding of samples. The reference plate was prepared by adding reagents in the following order: 35 µl of MOPS buffer, 35 µl of ^{3}H-STX and 105 µl of rat membrane preparation, then processed following the standard RBA protocol described above. This plate was measured three successive times.

The reference plate had a higher average CPM (1196 CPM) compared with the blank plate, with an RSD of 19%. Theoretically, the reference plate CPM values are expected to be lower than the blank plate, because the membrane binding sites would not retain all of the available ^{3}H-STX, with the excess being removed during the filtration step. The lower CPM of the blank plate is attributed to incomplete mixing of the ^{3}H-STX with the scintillation cocktail rather than the amount of tritiated toxin present. Since there is no aqueous phase in the reference plate, mixing does not become an issue. When the CPM values of the wells counted first (row A) are compared with those counted last (row H) there was no significant difference, which supports that phase mixing is absent. The comparable RSDs for the blank plate and the reference plate suggest that the addition of the rat membrane preparation, and the subsequent rinsing and filtering steps, do not contribute a significant amount of variability to

the assay. A one-way ANOVA determined that there was no significant difference among mean CPMs ($p > 0.3$) for the three sequential plate readings.

The reference plate, however, exhibited a significant difference among detectors (two-way ANOVA, $p < 0.001$). The same pattern in detector performance was observed for all three plate readings and the SNK pairwise comparisons determined that detector #12 was significantly different from all other detectors ($p < 0.001$) (Figure 4). The data from detector #12 were omitted and the statistical analysis repeated. The removal of this detector's data did not change the ANOVA outcome for sequential plate readings or detector variability.

QC plate (competitive binding)

To evaluate the added variance component associated with competitive binding, a non-labelled STX standard was added to compete with the ^{3}H-STX, creating a competition for binding sites. For the non-labelled STX, a solution at 1.8×10^{-8} M, with a final concentration of 3.0×10^{-9} M in assay, was used. The standard RBA plate configuration contains triplicates of this solution and their average CPM is used as the plate's QC sample. The reagents added per well for the QC plate were identical to the reference plate described above, with the addition of 35 µl of QC sample prior to the addition of 35 µl of the ^{3}H-STX. This plate was measured three successive times.

As expected, due to the introduction of competitive binding, the mean CPM of the QC plate was considerably lower than that of the reference plate (825 and 1196 CPM, respectively). Fewer binding sites for the radiolabelled toxin resulted in lower activity in the well after the rinsing and filtering steps. Triplicate counting of this plate gave an RSD of 17%. There appeared to be a slight decline in counts over the three successive plate readings (Figure 5). There was a slightly significant difference among successive plate readings ($p = 0.04$), which was due to a significant difference between the first and third plate readings ($p < 0.05$).

Consistent with the results of the previous plate, there was a significant difference among detectors (two-way ANOVA, $p < 0.001$). The SNK pairwise comparisons of detectors did not identify a single detector to be different from all others, however detector #11 differed significantly from seven other detectors ($p < 0.05$) and detector #12 differed significantly from five other detectors ($p < 0.05$).

Overall assay variability

Introduction of the heterogeneous rat membrane preparation increased RSD only slightly for triplicate readings from 16% (blank plate) to 19% (reference plate). This demonstrates that the number of receptor sites in each aliquot of the membrane preparation is fairly uniform and does not affect assay precision significantly. With the introduction of competitive binding (QC plate) the RSD remained in the same range: 17%. Overall, an inherent variability of approximately 17% is associated with this assay, which is independent of the addition of the membrane preparation or the non-radiolabelled STX and subsequent competitive binding process. When assay variability was evaluated with respect to individual detectors, there was no reproducible pattern in detector performance, although there were frequent occurrences of one or more detectors having significantly different CPM than the rest of the detectors for a given plate. The detectors with the lowest and highest levels of variability were different from plate to plate. Removal of data for a detector that was found to be significantly different from a majority of the remaining detectors did not change the outcome of the ANOVA for any of the series of plates. This detector variability observed

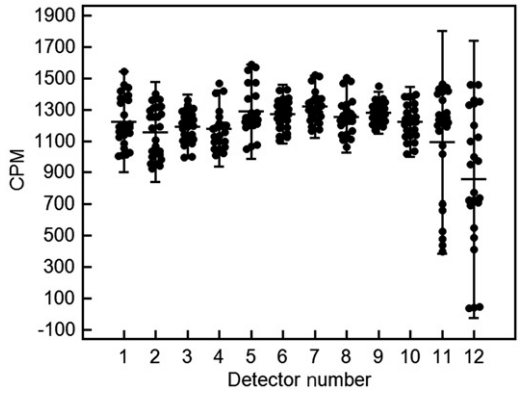


Figure 4. Scatter plot of the reference plate, first reading showing detector variability. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

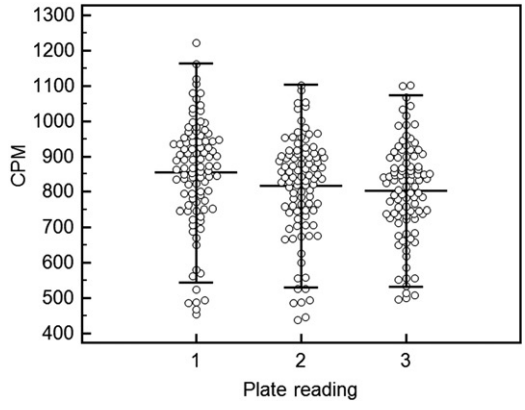


Figure 5. Results of the QC plate showing the variability of CPM in the three successive readings. For each run the mean and error bars at 2 SDs per plate are shown.

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could be attributed to pipetting error along single rows. Since an eight-channel micropipette is used to add reagents to single rows, an error in one such addition will show up as a single detector inconsistency. This would erroneously label one or more detectors as being different to other detectors. Although our initial work (Ruberu et al. 2003) on the RBA showed an RSD of 10%; a more recent study (van Dolah et al. 2009) demonstrated an RSD of 17.7% comparable with the current finding of 17%. It should be noted that our initial work was conducted using a six-detector instrument and with more experienced analysts, which could be the reason for the lower RSD for that study.

RBA acceptance criteria

In a typical RBA plate, each sample (calibration standard sample, QC sample, unknown sample, reference sample) is run in triplicate and the average CPM value is used for further calculations. Triplicate samples, rather than duplicates, are run to improve the accuracy of this assay. According to the acceptance criteria of the RBA assay (van Dolah et al. 2012), a given set of triplicate sample data are rejected if the RSD exceeds 30%, requiring reanalysis of the rejected sample. On average about 10% of the samples analysed in our laboratory are rejected due to the high variance among the triplicate values. In addition, if the QC sample CPM has $>30\%$ RSD, then the entire plate must be rejected. This loss of data results in the need to prepare and run a new plate, increasing both the turnaround time for results and the cost of the assay. One possible way of preventing samples from being rejected is to identify and remove outliers within a set of replicates. By eliminating outliers, the variability of replicates may be reduced to an acceptable level ($<30\%$ RSD), preventing invalidation of the entire plate or of individual samples. Therefore, we investigated a statistical approach to eliminate outliers methodically.

Grubbs' test and Student's t-test

A comparison of statistical outlier tests concluded that the Grubbs' test (Grubbs 1969) and the Student's *t*-test (Sokal and Rolf 1981) were best suited for determining an outlier within a triplicate dataset. The Grubbs' test compares the suspected outlier to the mean of all replicates, including the suspected value. The Student's *t*-test compares the potential outlier to the mean of the remaining values. The Grubbs' test is therefore more conservative in approach and it would be expected that this test would identify fewer outliers than the Student's *t*-test. The Grubbs' test for triplicates determines that a value is an outlier if the calculated value (G) is greater than the critical value (Z) of 1.153 at a 95% confidence interval ($\alpha = 0.05$). The Student's *t*-test determines that a value is an outlier in a triplicate dataset if the calculated *t*-value is greater than the critical *t*-value of 12.706 ($\alpha = 0.05$). By running the triplicate sample data values through these statistical tests, an outlier can be determined in an unbiased fashion, possibly avoiding the rejection of the entire sample.

The two outlier tests were evaluated by analysing data of each plate reading for the experiments presented above. For example, statistical analysis of the second plate reading of the QC plate, which had an RSD of 17.5% with an average CPM value of 817, resulted in both tests identifying a total of four outliers (Table 1). The results calculated after the four outliers were removed gave an average of 823 CPM with a 17% RSD. Removing the outliers did not improve the RSD significantly but showed a slight increase in CPM. Other plates tested for outliers had similar results, with an insignificant lowering of the sample RSD and slight increases or decreases in CPM. Since the CPM values directly relate to STX concentration of a sample, it is possible that the removal of outliers could have an impact on the accuracy of the assay results.

In order to evaluate how outlier testing would impact results of actual shellfish samples, 17 samples were spiked with STX concentrations that ranged from 5 to 1000 µg/100 g shellfish tissue and were evaluated for outliers using both statistical tests. Each triplicate set of data were assessed in two ways: raw data (no outliers removed) and data with outliers removed. Each test identified the same outliers and, as expected, the *t*-test identified additional outliers that were not detected by the Grubbs' test. Removal of the outliers kept these samples from being rejected ($<30\%$ RSD in

Table 1. Results from two outlier tests for three successive readings of the QC plate.

	Raw data			Grubbs test outliers removed			Student's *t*-test outliers removed		RSD % change		
Plate reading	Mean CPM	SD	RSD	Mean CPM	SD	RSD	Mean CPM	SD	RSD	Grubbs	*t*-test
1	854	155	18	859	152	18	859	152	18	0.03	0.03
2	817	143	18	823	140	17	823	140	17	0.03	0.03
3	803	135	17	810	135	17	809	136	17	0.01	0.00

triplicate wells), however there was no significant improvement in the precision of the assay. The average recovery of STX was 118% and 113% for raw data and data with outliers removed, respectively. Although the assay accuracy was improved on average, some plates showed a decrease in accuracy after outliers were removed, indicating that the suspected outlier was closer to the actual value than the remaining data points. In practice, outlier testing would likely reduce the number of samples and plates rejected, thereby reducing the time required to report results and lowering the per sample cost of the assay. The potential negative effect on method accuracy suggests caution with this approach in the absence of tangible evidence of analytical error during plate preparation.

Control charts for RBA

A better way of identifying erroneous data is through the use of control charts, which are based on a laboratory's acceptable and attainable performance criteria for precision and accuracy for a given method. A control chart enables the laboratory to monitor its performance visually by updating the chart with data from each subsequent analytical run. In this way a control chart for each critical parameter of a method tracks the detection of data outside of the acceptable performance limits. Control charts are prepared by plotting the date or run number as the abscissa and the value of interest, e.g. STX concentration of the QC check sample estimated on each plate, as the ordinate. Performance limits are established by averaging at least 20 measurements that have acceptable individual statistics, setting control limits and identifying the range of variability for that parameter. Rather than setting an arbitrary acceptance limit of ±30% for recovery of the QC sample, each laboratory can establish control limits based on their performance to determine whether or not an RBA plate is acceptable. Typical control limits are based on the number of SDs from the estimated mean. Once the mean and SD have been determined, the parameters from each subsequent assay are added to the appropriate control chart to maintain a continuous record of performance. In addition to the detection of erroneous values that would indicate an unacceptable plate, control charts allow tracking of systematic changes in method performance (e.g. due to degrading stock solutions, changes in materials like plate manufacturers, etc.) as well.

Figure 6 shows control charts for three RBA parameters acquired from our laboratory over a period of 1 year: (1) QC check standard, (2) slope of the binding curve and (3) EC_{50}. For each of the three control charts, control limits were based on the mean ±2 SD of the first set of 20 acceptable data. For example, the estimated mean for the QC check standard (3.0 nM theoretical concentration) from the first 20 plates was 2.94 nM and the calculated SD was 0.962 nM, resulting in control limits of between 1 and 4.5 nM (Figure 6(a)). Two data points on this plot had QC check standard values that were found to be outside of the control limits, requiring those two plates to be rejected and the assay run again for those samples. In contrast, the current RBA protocol of ±30% calculates an acceptance limit of between 2.1 and 3.9 nM for the QC check standard, which would result in 12 data points out of control, hence the rejection of 12 RBA plates. The latter criterion is arbitrary since the accuracy of the QC sample is method, instrument and analyst specific and must be established per individual laboratory.

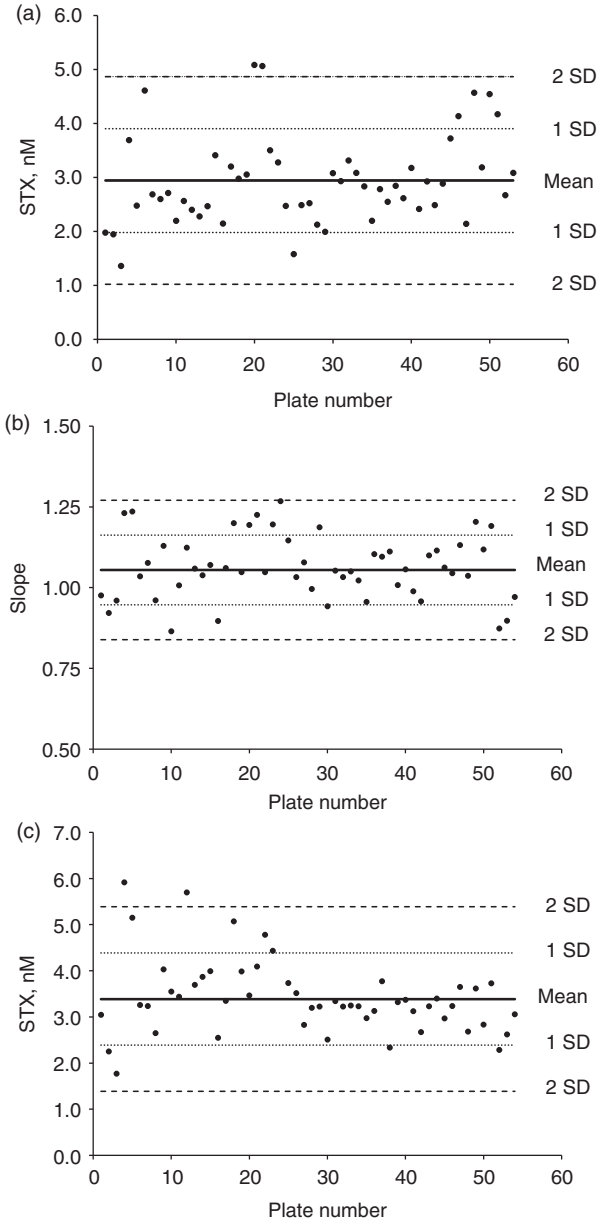


Figure 6. Control charts for (a) averaged daily QC samples on a plate, (b) slope of the binding curve and (c) EC_{50} per plate.

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When the established control limits are exceeded and a trend is observed, results are investigated for method bias and potential mistakes, allowing corrective actions to be taken to address the root cause to prevent recurrence of the error. Figure 6(b) shows the control chart for the slope of the standard curve. The slope was demonstrated to be a very stable parameter, with tight control limits ranging from 0.8 to 1.3 with no data points being rejected. The EC_{50} parameter is shown in Figure 6(c). In this case a high variability is seen at the beginning of the chart and as the analyst gains more experience with the assay the EC_{50} value becomes more consistent with less variation.

An additional parameter that has been inconsistent and highly variable in the RBA is the maximum binding, or top value of the binding curve. Ideally, the standard binding curve should plateau at 100% binding. However, often times we observe the plateau significantly below (80%) or above (120%) this value. Such a large shift in the curve significantly affects the final results of STX concentration in a sample. It is sometimes caused by one or more of the lowest three standards being out of control, thus dragging the curve in one direction. The top plateau was monitored using a control chart (Figure 7). The 1 SD control limit established for this data was between 0.9 and 1.1, which is ±10% binding. We have observed that this parameter can have a significant effect on the outcome of the results and therefore recommend developing a control chart to monitor its performance. Currently the importance of the top plateau is overlooked and not considered as an assay performance acceptance criterion.

Conclusions

Previous studies from our laboratory demonstrated the RBA method for the detection of STXs to be very reliable and to have the potential of being an alternate regulatory test method for PSP. Our current work focused on identifying sources of variability associated with the RBA and evaluating alternate QC approaches for validating test plates. The assay variability work included evaluation of each step of the assay by deconstructing its procedural steps, and also assessing the instrument's detector variability. The overall variability of the assay was determined to be 17%. Results discussed above show that the variability within a plate arises from several factors, such as counting statistics, analyst variability, mixing of well contents with cocktail, and the inherent measurement technique of the TopCount®. It is not known if the same variability would be observed in other instruments with different numbers of detectors or with detectors placed in a different array. A pipetting error along a row by the eight-channel micropipette would point to single detector variability as well and would be hard to identify. It is recommended periodically to evaluate individual detector performance with either a reference plate or a QC plate format similar to that used in the current study.

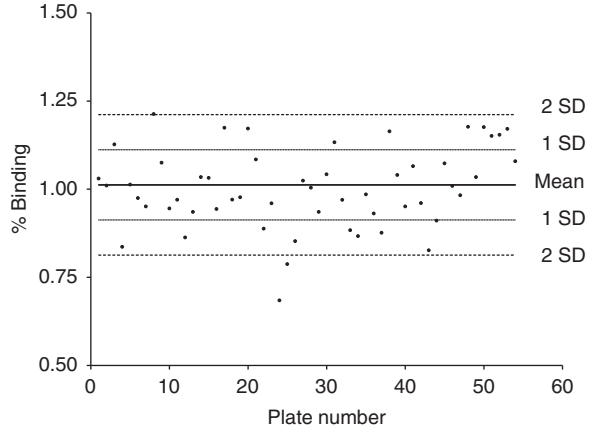


Figure 7. Control chart for the top plateau, maximum binding (B_{max}), of the calibration curve.

We have explored the use of two different outlier tests, Grubbs' test and Student's *t*-test, alone and in combination with the allowable procedure recommended in the NOAA protocol. Overall, removal of outliers lowers the RSD between replicate wells of a sample to $<30\%$, thus preventing that sample from being rejected. As a result it is expected that routine outlier testing would reduce the number of samples and plates rejected under the current QC criteria, which would help minimise the turnaround time between sample receipt and the reporting of results. The reduction in the number of rejected samples would also lower the cost per sample of the assay. Although some improvement in precision will be gained when an outlier is excluded, it is possible that accuracy will be diminished if the excluded value is closer to the actual target concentration. The potential negative effect on method accuracy suggests that the removal of suspected outliers should only be considered if it is suspected that there is an error associated with the sample(s) involved (e.g., a pipetting error).

Another avenue explored was the use of control charts for monitoring the three critical parameters of the RBA method, i.e. QC check standard, slope and EC_{50}. Establishing acceptable limits within each laboratory for respective parameters will ensure consistent performance over time, identify plates that must be rejected because one or more parameters are outside of the set control limits, and allow identifying and correcting process changes that would affect every assay. Establishing control limits for the maximum binding (B_{max}) as a fourth critical parameter for RBA performance is recommended. Such a development of associated control charts can be a part of the

laboratory's routine QC programme and is recommended as the primary quality control process for the RBA.

Acknowledgements

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Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Titan Fan, Ph.D
Affiliation	Beacon Analytical Systems, Inc
Email	titan@beaconkits.com, holly@beaconkits.com
Proposal Subject	Detection of ASP biotoxins in *Mytilus edulis* (Blue Mussel) shellfish by ELISA for Domoic Acid
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas, Table 2.
Text of Proposal/ Requested Action	SLV Proposal supporting the use of Beacon Domoic Acid Plate Kit as fit for purpose as an Approved NSSP Method for quantification of ASP toxins in Marine Biotoxin Monitoring Programs
Public Health Significance	Shellfish consumption can pose a mammal and bird health risk (1) when toxins produced by cyanobacteria present in water and shellfish growing areas, concentrate in shellfish meat due to their filter feeding system. A Closed Status for any growing areas with shellfish tissue levels of ASP of 2 mg/100 g (20 ppm) or more have been established to protect the consumer from exposure (2). The most common clinical signs of acute toxicity are gastrointestinal distress, confusion and neurological symptoms, disorientation, memory loss, coma and death (3). (1). M.Fernanda, F, Mazzillo, C. Pomeroy, J.Kuo, P. Ramondi,R. Prado, M.Silver. 2010. Aquatic Biol. 9:1-12. (2). NSSP Guide for the Control of Molluscan Shellfish: 2015 Rev. Sec.IV Chp. II., p 231. (3). Kathi A. Lefebvre, Alison Robertson, Toxicon, Vol. 56, Issue 2, 15 Aug. 2010, p. 218-230.
Cost Information	The price per sample is eight to nine dollars dependent upon the number of samples tested during one ELISA run, and/or the volume of kits purchased. There is an ELISA Plate Reader requirement. They can range in price from a low cost unit at approximately $2,600 to a higher cost of $15,000 USD unit depending upon complexity.
Action By 2017 Laboratory Committee	Recommended referral of Proposal 17-108 to an appropriate committee as determined by the Conference Chair.
Action By 2017 Task Force I	Recommended adoption of the Laboratory Committee on Proposal 17-108
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-108.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 17-108.
Action by 2019 Laboratory Committee	Recommended referral of Proposal 17-108 to an appropriate committee as determined by the Conference Chair.

Action by 2019 Task Force I	Recommended adoption of the Laboratory Committee on Proposal 17-108
Action by 2019 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-108.
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 17-108.
Action by 2023 Laboratory Committee	Recommended referral of Proposal 17-108 to an appropriate committee as determined by the Conference Chair.
Action by 2023 Task Force I	Recommended adoption of the Laboratory Committee on Proposal 17-108
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-108.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 17-108.

Single Laboratory Validation (SLV) Submission to the Interstate Shellfish Sanitation Conference (ISSC) in support of Method Approval as an Approved NSSP Method

Justification for New Method

For: Domoic Acid (ASP) Plate Kit, Cat. # 20-0249
Type of Method: Enzyme Linked Immunosorbent Assay (ELISA) utilizing a polyclonal antibody for detection of the ASP Biotoxin, Domoic Acid.
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Purpose of Intended Use of the Method. The purpose and intended use of this method is to provide a laboratory method for quickly establishing a quantified level of the ASP biotoxin, Domoic Acid, in *Mytilus edulis* (Blue Mussel) tissue as required for closing and opening of shellfish growing areas.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods. It appears there is a need for additional approved methods as an alternative to HPLC for the Biotoxin type: Amnesic Shellfish Poisoning (ASP). An ELISA method would enable monitoring laboratories to become proficient in operating a quantification method for ASP toxins levels at a lower equipment and reagent cost using a method that requires less training than HPLC to operate. This proposal demonstrates the equivalency of the HPLC and ELISA when using the same sample extract. This offers an additional benefit in that any confirmation testing could be completed using the same sample extract.

Method Limitations: This proposal offers supporting data for use of the method with *Mytilus edulis (blue mussel)* tissue only.

Method Documentation
Method Title: Domoic Acid (ASP) Plate Kit, Cat. # 20-0249
Method Scope: The method is a competitive enzyme linked immunosorbent assay (ELISA) for the quantification of domoic acid (DA) residues in *Mytilus edulis* shellfish tissue. Domoic acid is produced by some species of the diatom Pseudo-nitzschia which is the primary toxin associated with amnesic shellfish poisoning (ASP). Current legislation in the NSSP limits the amount of DA allowed in harvested shellfish to 2 mg/100 g (20 ppm) and will close shellfish growing areas to shellfish harvesting to protect consumers from exposure to the toxin. The test kit provides a tool to close and open shellfish growing areas by rapidly monitoring toxin levels as levels can quickly rise and fall.

References: (For HPLC Method) M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Sample. NRC Institute for Marine Biosciences, Technical Report #64 National Research Council Canada #33001.

ELISA Principle- The kit is a competitive ELISA method utilizing a plate coated with Rabbit polyclonal antibodies specific to the analyte Domoic Acid. The Domoic Acid–HRP (Horse Radish Peroxidase) Enzyme Conjugate competes with any toxin from the shellfish sample extract for binding with the antibody on the plate. After and incubation period of 30 min, the plate is washed with water to remove excess material and an enzyme substrate is added to react with any HRP Enzyme Conjugate bound to the plate. The amount of bound Conjugate is inversely proportional to the amount of DA toxin in the sample extract and can be visualized by development of a blue color. The ELISA is stopped at 30 min. with a 0.1 N HCl solution, and evaluated by reading the absorbance (OD) at 450 nm wavelength in a plate reader. The OD of the sample is compared to the Calibration Curve and multiplied by the total dilution factor of 4000 to obtain the concentration of toxin in the shellfish tissue sample.

Shellfish Sample Preparation: Fresh shellfish are externally washed and removed from the shell, approximately 15 single animals are combined. Composite sample is washed, drained then homogenized for ~20 seconds using a Waring blender with 16 oz. Mason jar fitted with ice crusher blade. Samples are aliquoted and can be frozen at -20^{o}C until use.

Shellfish Sample Extract Preparation: Composite mussel samples are extracted using a 4 X ratio of 50% methanol/water to tissue weight and mixed using a vortex mixer for 3 minutes. A sample of approximately 1 ml was aliquoted into a 1.5 ml Eppendorf tube and centrifuged for 5 min. at 12,000 rcf. Supernatants were diluted 1:1000 (as directed in the Test Kit Product Insert) into 10% acetonitrile/water (Sample Dilution Buffer). Diluted samples are used in the ELISA. The extraction and dilution procedure results in a total dilution factor of 4000 to be used in calculation of DA residues present in the original tissue sample.

Proprietary Aspects. Beacon Analytical Systems has developed the kit including antibodies and HRP enzyme conjugate.

Equipment: Microplate Reader with a filter for reading at 450 nm wavelength. Sample Preparation-blender, scale, extraction container with lid (10-20 ml), vortex mixer, microcentrifuge (12,000 rcf), disposable 1.5 ml centrifuge tubes, calibrated variable pipettes 1.0 ml and 0.010 ml with disposable tips, timer, and wash bottle.

ELISA Kit Reagents.

- **Plate** – (1) containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.
- **Domoic Acid Calibrators–** (4) vials each containing 2 ml with a concentration of 0, 0.5, 5, and 50 µg/L (ppb) Domoic Acid
- **Domoic Acid HRP Enzyme Conjugate** – (1) vial containing 12 ml
- **Substrate** – (1) vial containing 14 ml
- **Stop Solution** – (1) vial containing 14 ml (Caution! Contains 1N HCl. Handle with care.)
- **Product Insert** containing instructions for use.
- **Certificate of Conformity** (Specific to each Kit Lot#).

ELISA Test Procedures:

1. Allow reagents and sample extracts to reach RT prior to running the test
2. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
3. Using a pipette with disposable tips, dispense **100 µl** of the **Calibrator or sample extract** into the appropriate test wells. Please use a clean pipette tip for each sample addition.
4. Dispense **100 µl** of the **HRP Enzyme Conjugate** into each well.
5. Shake the plate gently for 30 seconds using a back and forth motion. Then incubate the wells for **30 minutes** at RT.
6. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with laboratory grade water and then decant. Repeat four times for a total of five washes.
7. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the water.
8. Dispense **100 µl** of the **Substrate** into each well. Shake the plate gently for 30 seconds using a back and forth motion.
9. Incubate the wells for **30 minutes** at RT.
10. Dispense **100 µl of the Stop Solution** into each well.
11. Measure and record the absorbance (Optical Density; OD) of the wells at 450 nm using a strip or plate reader. The OD correlates to a concentration of DA (ppb) based upon the Calibration Curve run with each set of samples.
12. To obtain the concentration of Domoic acid in the sample multiply the concentration results by the Total Dilution Factor of 4000.

Note: If the sample absorbance is higher or lower than the 0.5 or 50 ppb Calibrator results, the tissue levels should be expressed as less than or greater than the corresponding tissue levels (<2ppm or >200ppm DA). The sample dilution can be modified appropriately and retested along with another set of Calibrators.

Note: Running Calibrators and samples in duplicate will provide optimal assay precision and accuracy.

Quality Control:

Beacon Analytical Systems is ISO 9001:2015 approved for their quality systems for immunochemical test kit development, manufacturing and supporting activities.

Overview of Kit Quality Control - Each kit is tested following the ELISA procedure in the product insert. During manufacturing operation duplicates of the Kit Calibrator Reagents are run in order to meet established criteria prior to shipment.

Each Domoic Acid (DA) Calibrator's absorbance (OD) and binding characteristics (% B/B0) must be within a set of ranges. Ranges have been established for the Domoic Acid Plate Kit from historical data.

All plate components are tested for precision prior to using them in kits. The tolerance for variation within one lot of plates is less than or equal to 5%.

DA Calibration solutions are prepared using certified reference standard material purchased from the Canadian National Resource Council and are tested to be within 2% of the previous lot of control.

The R^2 correlation of the DA Kit Calibration Curve should be 0.99 or above.

All CV's must be less than or equal to 5%.

All QC data is kept electronically and backed up with hard copies at our manufacturing plant.

Single Laboratory Validation Criteria and Results
Section # 1 - Accuracy/ Trueness & Measurement Uncertainty
Section # 2 – Ruggedness
Section # 3 - Precision & Recovery
Section # 4 - Specificity
Section # 5 - Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity,
Section # 6 - Comparability (If intended as a substitute for an established method accepted by the NSSP).

Materials and Methods

Blank Mussel Tissue: Five different bags of mussels were purchased locally and screened on the ELISA for DA content. The ELISA screening did not find DA residues in these samples. They were used in spiking experiments (Table 1).

Table 1: Sample Type - Mussel (Blank) DA Blank Mussel Tissue used in validation.

ID	Harvest Date	Location	Type	Commercial Name	DA ELISA Screening Results*
A.	10/24/2016	Chebeague Island, ME	Aquaculture	Bangs Island Mussels	0 ppm
58	01/30/2017	Addison, ME	Natural	Moosabec Mussels	0 ppm
59	02/02/2017	Chebeague Island, ME	Aquaculture	Bangs Island Mussels	0 ppm
F.	04/18/2017	P.E.I., Canada	Natural	Cape Cod Shellfish Co.	0 ppm
E.	04/24/2017	Addison, ME	Natural	Moosabec Mussels	0 ppm

*All Blank tissue screening results were below the DA ELISA Level of Quantitation (LOQ).

Domoic Acid Standards

Certified Reference Material (CRM) – Certified Calibration Solution for Domoic Acid (CRM-DA-g, Lot# 20140730) purchased from National Research Council Canada. The certified concentration values and associated uncertainties for Domoic Acid and *epi*-Domoic Acid in this solution is 103.3 $\pm$ 3.4 ug/ml (at 20 ^{o}C). This is used in the manufacturing of Calibrator Solutions supplied with test kit.

Domoic Acid (DA) Standard used for sample spiking at ppm levels - Domoic Acid from *Mytilus edulis*, Calbiochem Catalog # 324378, Lot #2879693, 1 mg/vial. Reconstituted Solution adjusted for purity (98%).

Equivalency of CRM and DA Standards using HPLC Analysis: HPLC was used to confirm the concentration and equivalency of this standard to the reference material prior to the use in spiking experiments. The Calbiochem standard was diluted 1:40 into 10% acetonitrile/DI water (ELISA sample dilution buffer) or 50% methanol/50% DI water (Sample extraction solution) and each run in triplicate on HPLC. The concentration of the Calbiochem solution used for tissue spiking was assigned using the average of 6 replicates as 1.873 mg/ml.

Shellfish Sample Extract Preparation: Composite mussel samples are extracted using a 4 X ratio of 50% methanol/water to tissue weight. Samples were spiked with Calbiochem DA Standard at this time (if

required) and mixed using a vortex mixer for 3 minutes. A sample of approximately 1 ml was aliquoted into a 1.5 ml Eppendorf tube and centrifuged for 5 min. at 12,000 rcf. Supernatants were diluted 1:1000 (as directed in the Test Kit Product Insert) into 10% acetonitrile/water (Sample Dilution Buffer). Diluted samples were used in the ELISA resulting in a total dilution factor from the sample preparation of 4000.

Mussel Tissue- Certified Reference Material for Domoic Acid in Mussel tissue. - Certified Reference Material for Domoic Acid (CRM-ASP-MUS-d, Lot# 201112) purchased from National Research Council Canada. The concentration of DA and epi-DA is reported to be 49 $\pm$ 3 ug/g.

Extraction efficiency using Mussel Tissue CRM. Evaluation of Test method extraction and recovery was evaluated using this reference material (Table 2). Sample A was prepared by addition of CRM Reference mussel tissue to blank mussel tissue (Table 1) at a 1:1 ratio resulting in a DA tissue concentration of 24.5 ug/g, Sample F was prepared by blending the 4 gm standard with 9 gm of blank mussel tissue to obtain a tissue concentration of 15 ug/g. Both samples were extracted as described above and tested by ELISA. Recovery percentages at two different spiking levels were found to be 107 and 109 percent in mussel tissue by ELISA.

Table 2. – Mussel CRM extraction recovery results.

Sample Blank Mussel Spiked with DA Mussel CRM	DA ELISA Result (ppm)	% Recovery
Sample A spiked at 24.5 ppm DA	26.2 ppm	107 %
Sample F spiked at 15.0 ppm DA	16.3 ppm	109 %

Section 1: Accuracy/Trueness & Measurement Uncertainty (Table 3)

Working Range – Twenty samples of DA Blank Mussel Tissue were spiked with a low level (10 ppm), and twenty samples at a high level-20 ppm using the Calbiochem standard and extracted and evaluated by ELISA. Data and results are shown in Table 3.

Data Summary- Accuracy/Trueness

% Accuracy 10 ppm spike = 96.0 %

% Accuracy 20 ppm spike = 95.9 %

Data Summary – Measurement Uncertainty

Measurement uncertainty determined using a two-sided, 95% Confidence interval calculation

10 ppm spike = 0.662

20 ppm spike = 1.224

Table 3. Results of Accuracy/Trueness Testing of Blank and Spiked Mussel Tissue

Sample	Spiked Mussel (10 ppm)	% Accuracy (10 ppm)		Sample	Spiked Mussel (20 ppm)	% Accuracy (20 ppm)
1	10.70	107.0		1	21.06	105.3
2	8.06	80.6		2	23.97	119.8
3	8.07	80.7		3	16.87	84.3
4	10.29	102.9		4	19.07	95.3
5	11.15	111.5		5	16.27	81.4
6	8.18	81.8		6	17.18	85.9
7	8.43	84.3		7	16.80	84.0
8	11.26	112.6		8	18.62	93.1
9	11.42	114.2		9	16.31	81.5
10	8.81	88.1		10	22.74	113.7
11	8.91	89.1		11	20.10	100.5
12	9.51	95.1		12	18.06	90.3
13	97.7	97.7		13	21.85	109.3
14	10.60	106.0		14	17.25	86.2
15	8.63	86.3		15	17.55	87.8
16	12.20	122.0		16	22.39	111.9
17	9.42	94.2		17	19.16	95.8
18	8.39	83.9		18	17.06	85.3
19	8.02	80.2		19	22.27	111.4
20	10.18	101.8		20	18.96	94.8
Average	9.60	96.0 %			19.18	95.9 %
+/- SD	1.31				2.42	
Measurement Uncertainty @ 95% CI		0.662			Measurement Uncertainty @ 95% CI	1.224

Section 2: Ruggedness

Method: Composite mussel samples were spiked at 10 and 20 ppm, extracted with 50% methanol/water for 3 minutes, centrifuged and diluted in 10% acetonitrile/water with a total dilution factor of 4000. The diluted sample extract was evaluated on two different manufactured test kit lots. The data and results can be found in Table 4.

Data Summary:

Value for the test of symmetry of the distribution of Kit Lot 1	**0.153**
Value for the test of symmetry of the distribution of Kit Lot 2	**0.563**
Variance of kit Lot 1	**26.07**
Variance of kit Lot 2	**49.53**
Ratio of the larger to smaller variance of Lot 1 & Lot 2	**1.89**
Significant Difference between Lot 1 & Lot 2 based upon paired t-test	**No**

Table 4

Time of Analysis	Sample	Kit Lot 1	Kit Lot 2
Day 1	1A	8.43	8.81
	1B	6.94	8.91
	2A	11.26	9.51
	2B	11.42	9.77
Day 2	3A	9.51	9.25
	3B	10.6	9.42
	4A	8.63	8.39
	4B	12.2	7.86
Day 3	5A	10.48	8.77
	5B	10.18	9.99
Day 1	6A	17.18	22.74
	6B	16.8	29.36
	7A	18.62	23.97
	7B	16.31	25.41
Day 2	8A	22.39	16.87
	8B	19.16	19.07
Day 3	9A	21.06	16.27
	9B	17.23	24.37
	10A	20.77	17.4
	10B	22.27	19.1
Skewness		0.153	0.563

Variance	**26.07**	**49.53**
Ratio of variances	**1.89**	
P-Value (Paired t-test)	**0.546**	
Significant Difference	**No**	

Results: The data summary indicates the values of symmetry for kit lot 1 and 2 are within the range of -2 to +2, a non-significant degree of skewness in the distribution. The ratio of the variances between lot 1 & 2 is less than 2 indicating homogeneity of variance. A paired t-test used for data analysis results in a p-value of 0.546 which indicates there is no significant difference between Kit 1 and Kit 2.

Ruggedness continued - ELISA parameters

Method: Composite mussel samples were spiked at 10 and 20 ppm, extracted with 50% methanol/water for 3 minutes, centrifuged and diluted in 10% acetonitrile/water with a total dilution factor of 4000. The ELISA Standard Operating Procedure (SOP) parameters were modified in the ELISA then tested for an evaluation of the critical steps in procedure (Table 5).

1. Incubation time for the initial step of the ELISA is set at 30 min. The incubation time was modified to be a total of 15 minutes or 45 minutes. The spiked sample data was evaluated by Welsh's t-test and found not to be significant at either time point tested.
2. A wash step is required in the ELISA to remove unbound materials. The SOP wash is repeated 5 times with water. This was changed to a 4 time wash. The spiked sample data was evaluated by Welsh's t-test and found to be significant from the SOP data.
3. The SOP for test incubation temperature is that the ELISA should be run at RT (20-28 °C). The incubation temperature was modified to be 4 °C or 30 °C. The spiked sample data was evaluated by a paired t-test and found not to be significant at either temperature tested.
4. The kit reagents should be equilibrated to RT prior to running the ELISA. All kit reagents were removed directly from a 4 °C refrigerator and run in comparison to RT reagents. A t-test on the resulting data indicated no significant difference in the results.

Table 5

	ELISA Standard Operating Procedure	Definition of ELISA SOP		Variation Factor	Significantly different to SOP by t-test.		Variation Factor	Significantly different to SOP by t-test.
1.	**Primary Incubation Time is 30 min.**	**Incubation time for HRP Enzyme Conjugate, Sample Extract or Calibrator on plate**		**Incubation time changed to 15 min.**	**No**		**Incubation time changed to 45 min.**	**No**
2.	**Plate water Wash Step is Repeated 5 times.**	**Water wash step to remove unbound materials prior to**		**Wash Step is changed to repeat 4 times.**	**Yes**			

		Substrate addition						
3.	**Incubation Temperature done at room temperature**	**ELISA incubation steps run at RT (20-28 °C).**		**ELISA Incubation at 4 °C.**	**No**		**ELISA Incubation at 30 °C.**	**No**
4.	**Reagent Temperature**	**Kit reagents are equilibrated to RT prior to running test.**		**Reagent Temperature is cold (4 °C).**	**No**			

Section 3: Precision & Recovery

Precision

Method: Evaluation of mussel tissue spiked with a low (10 ppm), medium (20ppm) and high level (40 ppm) of DA was completed using the method of extract preparation and ELISA analysis previously outlined, to evaluate the method consistency over a range of concentrations.

Data Summary- Precision -The F value obtained in the evaluation between groups was less than the critical value of 2.39 (for 9 and 20 degrees of freedom) at 0.05 significance level indicating the mean values from the samples are not significantly different.

The F value obtained in the evaluation of different concentrations (subgroups within groups) is greater than the critical value of 1.93 (for 20 and 30 degrees of freedom) at the 0.05 significance level indicating the mean values of each concentration are significantly different. This is an expected result since there were three sample concentration used to generate the data (10, 20 and 40 ppm) which are quite different.

From this ANOVA analysis (Table 6) we can conclude that the precision of the method is consistent over the range of sample concentrations tested.

Table 6. **Fully nested/hierarchical random analysis of variance (ANOVA)**

Source of Variation	Sum Squares	DF	Mean Square
Between Groups	30.651802	9	3.405756
Between Subgroups within Groups	9,583.973276	20	479.198664
Residual	323.251852	30	10.775062
Total	9,937.87693	59	

F (VR between groups) = 0.316078 P = 0.9633

F (using group/subgroup msqr) = 0.007107 P > 0.9999

F (VR between subgroups within groups) = 44.472939 P < 0.0001

Recovery

Method: Evaluation of mussel tissue spiked with a low, medium and high level of DA was done using the method outlined, to evaluate the method consistency over a range of concentrations. The results are found in Table 7.

Data summary

The variance ratio for the component of concentration in sample is not significant at 95% CI.
Recovery Percentage over the average data set (10, 20 & 40 ppm) using spiked mussel tissue is 99.55%.

Table 7 RECOVERY

	Low Spike 10 ppm		Medium Spike 20 ppm		High Spike 40 ppm	
Sample	Average	Spike minus Average	Average	Spike minus Average	Average	Spike minus Average
1	8.62	1.38	19.96	0.04	37.00	3.00
2	7.93	2.08	23.08	-3.08	39.42	0.58
3	10.39	-0.39	21.30	-1.30	40.02	-0.02
4	10.60	-0.59	20.86	-0.86	37.39	2.61
5	9.38	0.62	19.63	0.37	39.00	1.00
6	10.01	-0.01	19.12	0.88	40.41	-0.41
7	8.51	1.49	18.67	1.34	43.41	-3.41
8	10.03	-0.03	20.80	-0.80	36.52	3.48
9	9.63	0.38	19.09	0.91	43.80	-3.80
10	10.09	-0.09	20.69	-0.69	41.52	-1.52

Anova: Single Factor

Source of variation	*df*	*SS*	*MS*	*F*	*P-value*	*F crit*
Concentration	2	3.24	1.62	0.55	0.59	3.35
Error	27	80.24	2.97			
Total	29	83.48				

Section 4: Specificity

Method: Four compounds were evaluated to challenge the specificity of the ELISA, three were included due to their similarity of structure to DA, glutamine & glutamic acid at 100 ppm and kainic acid at 20 ppm. Saxitoxin (20 ppm) was evaluated due to the potential that it may be present in the shellfish at the same time as DA. Shellfish extracts containing DA from extracted tissue levels of 0 (blank), 10 and 20 ppm DA were run on the ELISA. These same extracts were run in the presence of the suspected interfering compound to evaluate any significant change in the ELISA result.

Data Summary:
Using a two sided t-test at a 0.05 significance level it was determined that the average Specificity index (SI_{avg}) for the four compounds tested did not differ from 1 (Table 8).
Table 8

Interfering Compound	Conc. (ppm)	SI_{avg}	Significantly different from control by t-test.
Glutamine	100	1.15	No
Glutamic Acid	100	0.89	No
Saxitoxin	20	1.26	No
Kainic Acid	20	1.15	No

Section #5 Linear Range/Limit of Detection/Limit of Quantitation/Sensitivity
Method: Multiple blank mussel tissue samples were spiked with Calbiochem DA standard at the following levels: 2, 3, 5, 10, 15, 20, 30, 40 ug/g then extracted and evaluated by ELISA. To establish the linear range of response the data was evaluated and expressed in Fig. 1. The line of response falls within the bracketed 0.95-1.05 range with data from tissue concentrations from 3 ug/g to 40 ug/g. The data for 2 ug/g falls outside and is not considered within the linear range of the ELISA. The range of assay detection from 3-40 ppm is inclusive of the current NSSP criteria for closing of shellfish beds at 2 mg DA per 100 grams shellfish tissue.
Figure 2 plots the coefficient of variation for each concentration within the linear range which are all under 10%. We can calculate the limit of detection (LOD) of the method and the limit of quantitation (LOQ) shown below using this data.
Data Summary
Linear range of the method as implemented is 3-40 ppm DA in tissue (Fig.1).
The limit of detection (LOD) of the method as implemented is 0.91 ppm DA in tissue.
The limit of quantitation (LOQ) of the method as implemented is 3.0 ppm DA in tissue.
Linear Range Plot – Figure 1
The linear range of the ELISA is established to be from 3 to 40 ppm DA in mussel tissue (Figure 1).

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Figure 1: Linearity of Beacon DA ELISA

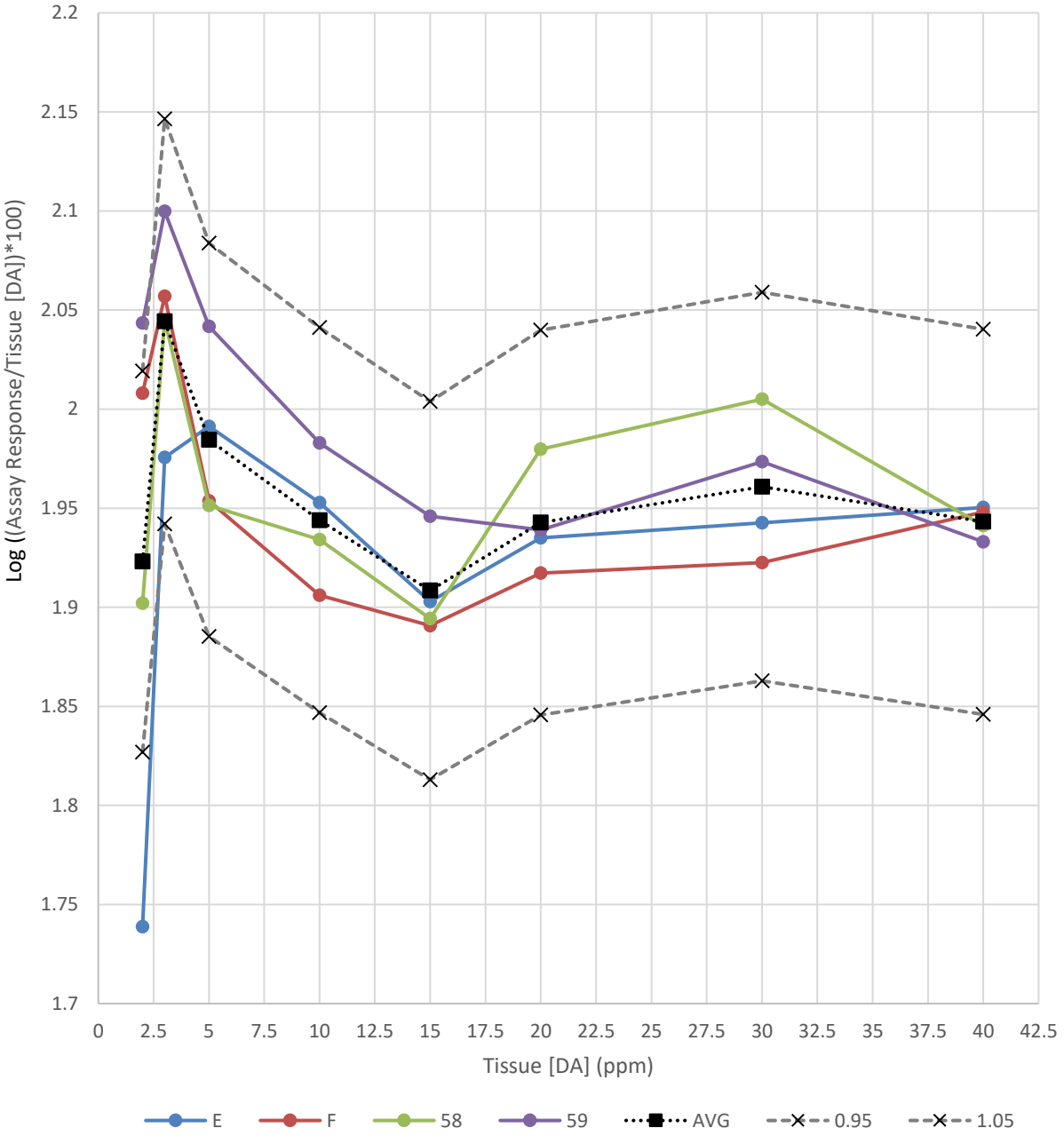
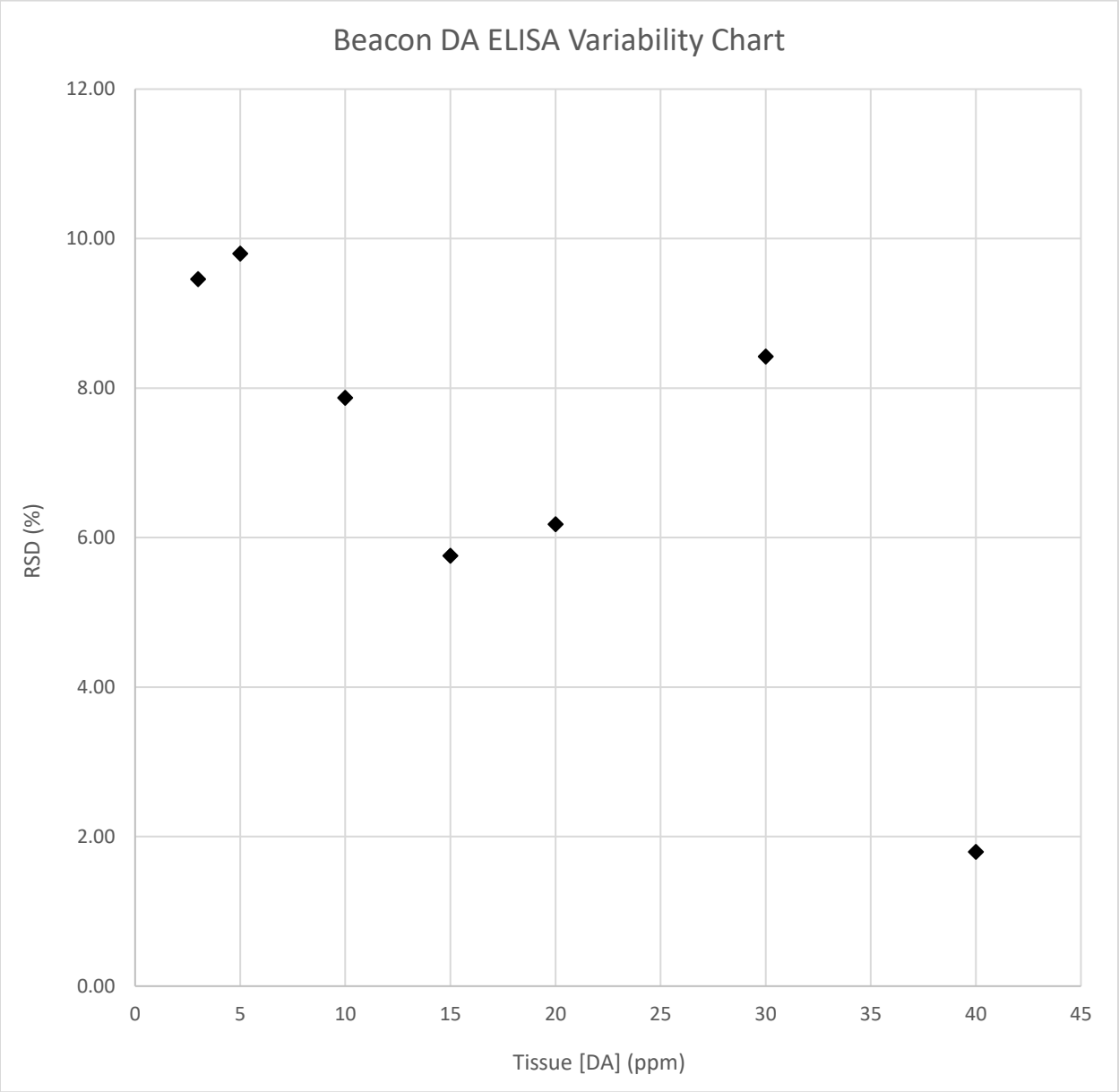


Figure 2: Limit of Detection & Limit of Quantitation/Sensitivity



Section # 6 - Comparability to NSSP Approved Method for Biotoxin Testing –HPLC

Reference from NSSP Guide for the control of Molluscan Shellfish 2015 Revision.
Table 2- Approved Methods for Marine Biotoxin Testing for ASP: M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Sample. NRC Institute for Marine Biosciences, Technical Report #64 National Research Council Canada #33001.
Method: The HPLC uses a C-18 reverse phase chromatography column with a mobile phase of 10% acetonitrile and 0.1% trifluroacetic acid. The mussel tissue extracts have been prepared using the sample extraction procedure for the ELISA which are then diluted 1:5 with 10% acetonitrile prior to injection into the HPLC system. The 2 procedures use the same sample extracts and the results are compared in Table 9. There were 14 mussel tissue samples tested of which 50% were naturally incurred samples and 50% were spiked with Calbiochem DA standard.

Data Summary for the comparison of the new method to the officially recognized method:
Value for the test of symmetry for the data by HPLC reference method: 0.93
Value for the test of symmetry for the data by the DA ELISA method: 1.45
Symmetry is within the range of -2 to +2 and is not a significant degree of skewness.

Variance of data generated by the HPLC reference method: 166.90
Variance of the data generated by the DA ELISA method: 675.73
Ratio of the larger to smaller of the variances: 4.05
This value indicates a lack of homogeneity of variance and indicates the use of a Welch's t-test for further data analysis to determine if there is a difference between the data means.
Based upon the Welch's t-test there no significant difference between these two analytical methods.

Table 9

Sample	Collection Date	HPLC Data	DA ELISA
Mytilus edulis		DA (ppm)	DA (ppm)
1	9/6/16	9.48	9.50
2	8/30/16	4.78	4.2
3	8/30/16	16.14	19.80
4	01/30/17	4.42	4.80
5	01/30/17	8.77	8.70
6	01/30/17	15.78	22.80
7	01/30/17	28.49	26.20
8	9/20/16	10.64	21.30
9	10/3/16	27.04	51.40
10	9/20/16	1.60	6.90
11	9/28/16	43.11	91.80
12	9/19/16	17.80	36.70
13	9/26/16	39.79	68.70
14	10/3/16	12.10	22.70

	SKEW	0.93	1.45
	VARIANCE	166.90	675.73
	Ratio of Variance		4.05
	Welch's T-test		-1.43
	df =		(19)
	T =		2.09
Conclusion: Means are not different between the 2 methods of analysis.			

Discussion and Summary

The results of this single laboratory validation demonstrate that the Beacon Domoic Acid (ASP) Plate Kit is an effective procedure for quantitative determination of DA residues in *Mytilus edulis* shellfish tissue. Data presented for ELISA performance meets the validation criteria for accuracy/trueness, measurement uncertainty, ruggedness, precision and recovery. The specificity of the test kit was challenged with four compounds of potential interference and was found to perform properly at DA levels of interest. The linear range of the ELISA was determined to be 3 to 40 ppm which brackets the NSSP established criteria of 20 ppm for the ASP biotoxin in shellfish beds. This linear range would allow for the continued use of the method should a lower criteria be established. The comparative data from the ELISA and the officially recognized HPLC method demonstrate good correlative performance.
The ability to use the same sample extract on the ELISA and HPLC confers ease of use for confirmatory testing. The sample throughput is high, while cost and training requirements are minimal. The Beacon Domoic Acid (ASP) Plate Kit is an appropriate tool for quantification of DA residues for use in biotoxin monitoring programs as it allows rapid sample analysis and turnaround time.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	**Domoic Acid (ASP) Plate Kit**
Name of the Method Developer	**Dr. Titan Fan**
Developer Contact Information	**Beacon Analytical Systems, Inc.** **82 Industrial Park Road** **Saco, ME 04072** **Tel. (207) 571-4302** **Fax (207) 602 6502** **Email: titan@beaconkits.com**

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for an inexpensive laboratory method with the ability to process multiple samples rapidly and quantify the domoic acid levels in mussel tissue.
2. What is the intended purpose of the method?		The method can be used in shellfish bed monitoring programs to document the quantifiable levels of domoic acid in mussel tissue.
3. Is there an acknowledged need for this method in the NSSP?		The method offers higher sample thruput and quantifiable results to monitor increasing or decreasing levels of domoic acid.
4. What type of method? i.e. chemical, molecular, culture, etc.		It is an immunochemical method utilizing an antibody specific to the toxin in an enzyme linked immunosorbent assay (ELISA).
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Domoic Acid (ASP) Plate Kit
Method Scope		Quantitative Analysis of Domoic Acid in mussel tissue
References		Performance Validation Report and Product Insert included with this submission.
Principle		Composite mussel samples are extracted and run on an ELISA Test Kit which quantifies Domoic Acid residues by comparison to an internally run calibration curve.
Any Proprietary Aspects		Yes, this is a commercial test kit.
Equipment Required		Sample Preparation- Blender, scale, extraction container with lid (10-20 ml), vortex mixer, centrifuge (12,000 rcf), Disposable centrifuge tubes. Calibrated variable pipettes 1.0 ml and 0.010 ml with disposable tips. Plate Reader, timer, wash bottle.
Reagents Required		Methanol and laboratory grade water in a 1:1 ratio. 10% Acetonitrile in laboratory grade water.
Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2-8°C before testing.
Safety Requirements		Protective safety items are indicated such as safety glasses, gloves and lab coat. Kit reagents including

		calibrators, conjugates and extracts should be handled with caution since they contain a toxic substance. The kit Stop Solution is a strong acidic solution (1 N Hydrochloric Acid) and needs to be safely handled and disposed of appropriately.
Clear and Easy to Follow Step-by-Step Procedure		Product Insert is included in each test kit and included in this submission package.
Quality Control Steps Specific for this Method		The Certificate of Conformity included with each kit documents the performance characteristics of the Test Kit Lot Reagents. This provides the test operators a reference to evaluate the results generated in their laboratory.
C. Validation Criteria		
1. Accuracy / Trueness		SLV - Section 1
2. Measurement Uncertainty		SLV - Section 1
3. Precision Characteristics (repeatability and reproducibility)		SLV - Section 3
4. Recovery		SLV- Section 3
5. Specificity		SLV- Section 4
6. Working and Linear Ranges		SLV - Section 5
7. Limit of Detection		SLV - Section 5
8. Limit of Quantitation / Sensitivity		SLV - Section 5
9. Ruggedness		SLV- Section 2
10. Matrix Effects		None observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		SLV - Section 6
D. Other Information		
1. Cost of the Method		The price per sample is eight to nine dollars dependent upon the number of samples tested during one ELISA run, and/or the volume of kits purchased.
2. Special Technical Skills Required to Perform the Method		Knowledge of GMP laboratory skills as well as proper pipetting technique, and safe handling of solvents.
3. Special Equipment Required and Associated Cost		An ELISA Plate Reader is required which can range in price from a low cost unit at approximately $2,600 to a higher cost of $15,000 USD unit depending upon complexity.
4. Abbreviations and Acronyms Defined		ASP-amnesic shellfish poisoning. DA-domoic acid. ELISA-Enzyme Linked Immunosorbent Assay. OD-Optical Density (Reader Output). HRP-horse radish peroxidase enzyme. % B/B0- percent of measured bound fraction in a test sample divided by the total bound from a blank Calibrator. ppm = parts per million, equivalent to mg/kg, ppb –parts per billion.
5. Details of Turn Around Times (time involved to complete the method)		One assay can be completed in under 90 minutes including sample preparation (12 samples). One plate can be used to test a maximum of 36 samples.
6. Provide Brief Overview of the Quality Systems Used in the Lab		Beacon Analytical Systems is ISO 9001:2015 approved for their quality systems for Immunochemical test kit development, manufacturing and supporting activities. Overview of Kit Quality Control - Each kit is tested following the ELISA procedure in the product insert. During manufacturing operation duplicates of the Kit Calibrator Reagents are run in order to meet established criteria prior to shipment. Each Domoic Acid (DA) Calibrator's absorbance (OD) and binding characteristics (% B/B0) must be within a set of ranges. Ranges have been established for the Domoic Acid Plate Kit from historical data. All plate components are tested for precision prior to using them in kits. The tolerance for variation within one lot of plates is less than or equal to 5%. -DA Calibration solutions are prepared using certified reference standard material purchased from the Canadian National Resource Council and are tested to be within 2% of the previous lot of control. - The R^2 correlation of the DA Kit Calibration Curve should be 0.99 or above. - All CV's must be less than or equal to 5%. - All QC data is kept electronically and backed up with hard copies at our manufacturing plant.

Submitters Signature 	Date: 06/30/2017
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:

Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.

14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery** - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** - the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** - the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range** - the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Beacon Analytical Systems Inc.®



Domoic Acid (ASP)

Plate Kit

Cat. # 20-0249
Product Insert

PLEASE READ COMPLETELY BEFORE USE

INTENDED USE

The Beacon Domoic Acid (ASP) Plate Kit is a competitive ELISA for the quantitative analysis of domoic acid in shellfish samples.

USE PRINCIPLES

The Beacon Domoic Acid (ASP) plate kit is a competitive enzyme-labeled immunoassay for the biotoxin which causes Amnesic Shellfish Poisoning (ASP). Shellfish sample extract(s) or calibrator solution(s) are pipetted into a test well followed by Domoic Acid HRP enzyme conjugate to initiate the reaction. During a 30 minute incubation period, domoic acid from the sample and domoic acid HRP enzyme conjugate compete for binding to the domoic acid antibody coated on the plate wells. Following this incubation, the wells are washed to remove any unbound domoic acid and HRP enzyme conjugate. After washing, a colorless substrate is added to the wells and any bound enzyme conjugate will convert the substrate to a blue color. Following another 30 minute incubation, the reaction is stopped with the addition of stop solution and the amount of color in each well is measured. The color of the unknown sample is compared to the color of the calibrators and the domoic acid concentration of the sample is derived. The color intensity is inversely proportional to the amount of domoic acid present.

MATERIALS PROVIDED

The kit in its original packaging can be used until the end of the month indicated on the box label when stored at 2 to 8 ºC.

- **Plate** – (1) containing 12 test strips of 8 wells each vacuum-packed in aluminized pouch with indicating desiccant.
- **Domoic Acid Calibrators–** (4) vials each containing 2 ml with a concentration of 0, 0.5, 5, and 50 µg/L (ppb) Domoic Acid
- **Domoic Acid HRP Enzyme Conjugate** – (1) vial containing 12 ml
- **Substrate** – (1) vial containing 14 ml
- **Stop Solution** – (1) vial containing 14 ml (Caution! Contains 1N HCl. Handle with care.)
- **Product Insert** containing instructions for use.
- **Certificate of Conformity** (Specific to each Kit Lot#).

MATERIALS REQUIRED BUT NOT PROVIDED

Acetonitrile,& Methanol (ACS grade)	Timer
Laboratory quality distilled or deionized water	Wash bottle
Variable volume pipettes with disposable tips capable of dispensing 10-100 microliters (µl), and up to 1000 µl.	Vortex mixer
Multi-channel pipette; 8 channel capable of dispensing 100 µl	Paper towels or equivalent absorbent material
Microwell plate or strip reader with 450 nm filter	Disposable micro centrifuge tubes
Microcentrifuge capable of a speed of 12,000 rcf. (x g)	Kitchen Blender for sample homogenization

SPECIFICITY

Domoic Acid (DA) is an amino acid similar in structure to kainic acid which naturally occurs in some seaweed. The % cross reactivity of several compounds relative to DA is shown in the table below.

Compound	% CR	Compound	% CR
Domoic acid	**100 %**	**Saxitoxin**	**< 0.1 %**
Glutamine	**< 0.1 %**	**Kainic acid**	**0.005 %**
Glutamic acid	**< 0.1 %**		

KIT HANDLING NOTES and PRECAUTIONS

- Store all kit components at 4 °C to 8 °C (39 °F to 46 °F) when not in use.
- Each reagent is optimized for use in the Beacon Domoic Acid (ASP) Plate Kit. Do not substitute reagents from any other manufacturer into the test kit. Do not combine reagents from other Beacon Plate Kits with different lot numbers.
- Dilution or adulteration of reagents or samples not called for in the procedure may result in inaccurate results.
- Do not use reagents after expiration date.
- Reagents should be brought to room temperature (RT), 20 to 28 ºC (62 to 82 ºF) prior to use. Avoid prolonged (> 24 hours) storage at room temperature.
- Domoic acid calibrators contain 10% acetonitrile and should be kept tightly capped to minimize evaporation.
- The Stop Solution is 1N hydrochloric acid, which is corrosive and an irritant. Avoid contact with skin and mucous membranes. Immediately clean up any spills and wash area with copious amounts of water. If contact should occur, immediately flush with copious amounts of water.
- Precise transfer of samples and reagents by using an appropriate and calibrated pipette is critical to obtain proper assay results. Please pipette carefully.
- If running more than two strips at once, the use of a multichannel pipette is required.
- In one assay a maximum of four strips (32 wells) is recommended, for example –4 calibrators in duplicate (8 wells), and 12 sample extracts in duplicate (24 wells).

SAMPLE DILUTION BUFFER PREPARATION- 10% ACETONITRILE/Water

- Mix 1 part ACS grade acetonitrile with 9 parts distilled or deionized (DI) water to make the Sample Dilution Buffer for the shellfish samples.
- Transfer to a clean glass container with tight-fitting lid and swirl to mix. Store tightly sealed to minimize evaporation.

SAMPLE EXTRACTION BUFFER PREPARATION- 50% METHANOL/Water

- Mix 1 part ACS grade methanol with 1 part distilled or deionized (DI) water to make the Sample Extration Buffer.
- Transfer to a clean glass container with tight-fitting lid and swirl to mix. Store tightly sealed to minimize evaporation.

SAMPLE PREPARATION - Shellfish Tissue Extract –Mytilus edulis (Blue Mussel)

1. Remove shellfish tissue (12-15 animals) from shell, wash, drain dry and homogenize using a kitchen blender.
2. Weigh 2 g of homogenized tissue and add 8 ml of a 50% Methanol/Water solution.
3. Mix for 3 minutes using Vortex mixer (4 X dilution)
4. Transfer 1 ml into a microcentrifuge tube and centrifuge at 12,000 x g for 5 minutes. Extracts can be stored at -20°C.
5. Prepare a 1:1000 dilution of the supernatant with Sample Dilution Buffer using the following procedure:

A. 1:10 dilution - 50 microliters of supernatant layer avoiding any particulates, into 450 microliters Sample Dilution Buffer, Mix.

B. 1:100 dilution – 10 microliters of dilution A. into 990 microliters Sample Dilution Buffer, Mix,

6. Use B. in ELISA. – Total Dilution Factor (TDF) = 4000

Shellfish Analysis:

- ✓ EU Screening Level = 20 ppm (20 mg/kg) Assay Dilution Factors are set to detect 20 ppm Domoic Acid,

Extraction of Shellfish Tissue and Preparation for ELISA	
Dilution of shellfish homogenate in water (2 g homogenized tissue with 8 ml 50% MEOH/DI H_2O)	**4 X** Dilution
*Secondary Dilution into Sample Dilution Buffer	**1000 X Dilution**
Total Dilution Factor (TDF) to obtain Tissue Levels of Domoic Acid	**4000 X**
✓ Assay Range of Detection in Tissue	2 mg / kg to 200 mg / kg
Domoic Acid Plate Kit Calibrators ug / L (ppb)	Predicted Tissue Levels (X 4000 TDF)
Negative Control (Blank)	0
0.5	2 ppm
5.0	20 ppm
50.0	200 ppm

ASSAY PROCEDURE

(Note: Running Calibrators and samples in duplicate will provide optimal assay precision and accuracy.)

1. Allow reagents and sample extracts to reach RT prior to running the test.
2. Place the appropriate number of test wells into a micro well holder. Be sure to re-seal unused wells in the zip-lock bag with desiccant.
3. Using a pipette with disposable tips, dispense **100 µl** of the **Calibrator or sample extract** into the appropriate test wells. Please use a clean pipette tip for each sample addition.
4. Dispense **100 µl** of the **HRP Enzyme Conjugate** into each well.
5. Shake the plate gently for 30 seconds using a back and forth motion. Then incubate the wells for **30 minutes** at RT.
6. Decant the contents of the wells into an appropriate waste container. Fill the wells to overflowing with laboratory grade water and then decant. Repeat four times for a total of five washes.
7. Following the last wash, tap the inverted wells onto absorbent paper to remove the last of the water.
8. Dispense **100 µl** of the **Substrate** into each well. Shake the plate gently for 30 seconds using a back and forth motion.
9. Incubate the wells for **30 minutes** at RT.
10. Dispense **100 µl of the Stop Solution** into each well.
11. Measure and record the absorbance (Optical Density; OD) of the wells at 450 nm using a strip or plate reader.
12. To obtain the concentration of Domoic acid in the sample multiply the results by the Total Dilution Factor of 4000.

Note: If the sample absorbance is higher or lower than the 0.5 or 50 ppb Calibrator results, the tissue levels should be expressed as less than or greater than the corresponding tissue levels (<2ppm or >200ppm DA). The sample dilution can be modified appropriately and retested along with another set of Calibrators.

CALCULATE RESULTS

1. Semi-quantitative results can be derived visually by simple comparison of the sample color to the color of the Calibrator wells. Samples containing less color than a Calibrator will have a concentration of Domoic Acid greater than the tissue correlated concentration of the Calibrator. Samples containing more color than a Calibrator will have a concentration less than the tissue correlated concentration of the Calibrator.
2. It is preferred for quantitative results to be determined using commercially available software for ELISA evaluation such using a 4-Parameter curve fit. Alternatively, a semi-log curve fit can be used if 4-Parameter software is not available. Samples with OD's greater than the lowest calibrator, or lower than the highest calibrator will need to be diluted accordingly and repeated with and calibrators in an additional run.
3. Beacon can supply a spreadsheet template which can be used for data reduction. Please contact Beacon for further details.

SAMPLE CALCULATIONS

Well Contents	OD	Average OD ± SD*	%RSD	%B/Bo**
Negative Control	2.033 1.994	2.014 ± 0.027	1.4	100
0.5 ppb Calibrator	1.610 1.671	1.640 ± 0.043	2.7	81
5 ppb Calibrator	1.095 1.155	1.125 ± 0.042	3.8	56
50 ppb Calibrator	0.501 0.482	0.492 ± 0.013	2.7	24

Actual values may vary; this data is for example purposes only.

* Standard deviation

**B/Bo% equals the average sample absorbance divided by the average 0 ppb Calibrator absorbance multiplied by 100.

TECHNICAL ASSISTANCE

For questions regarding this kit or for additional information about Beacon products, call (207) 571-4302 or contact us at info@beaconkits.com.

Safety- To receive complete safety information on this product, contact Beacon Analytical Systems, Inc. and request Safety Data Sheets. Stop Solution is 1N hydrochloric acid. Handle with care.

General Limited Warranty

Beacon Analytical Systems, Inc. ("Beacon") warrants the products manufactured by it against defects in materials and workmanship when used in accordance with the applicable instructions for a period not to extend beyond a product's printed expiration date. BEACON MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. The warranty provided herein and the data, specifications and descriptions of Beacon products appearing in published catalogues and product literature may not be altered except by express written agreement signed by an officer of Beacon. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and, if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Beacon's sole obligation shall be to repair or replace, at its option, any product or part thereof that proves defective in materials or workmanship within the warranty period, provided the customer notifies Beacon promptly of any such defect. The exclusive remedy provided herein shall not be deemed to have failed of its essential purpose so long as Beacon is willing and able to repair or replace any nonconforming Beacon product or part. Beacon shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by a customer from the use of its products. However, in some states the purchaser may have rights under state law in addition to those provided by this warranty.

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REV.ISSC 06302017HL

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Michael Hickey, Jeff Kennedy, Diane Regan
3. Affiliation	Massachusetts Division of Marine Fisheries
4. Address Line 1	836 S Rodney French Blvd
5. Address Line 2	
6. City, State, Zip	New Bedford, MA 02744
7. Phone	(508) 990-2860
8. Fax	(508) 990-0449
9. Email	Michael.hickey@mass.gov
10. Proposal Subject	Conditionally Conforming Laboratory Status
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter I. Shellfish Sanitation Program Requirements for the Authority @.03 B. 1. b. Section II. Model Ordinance Chapter III. Laboratory @.01 Section II. Model Ordinance Chapter XV. Depuration .03 J. (4)
12. Text of Proposal/ Requested Action	The requested action is to create a NSSP laboratory status of conditionally conforming. This status is based on a demonstrated proficiency of laboratory method performance. Laboratories that are found to conditionally conform for a laboratory analysis may support the NSSP. **MO Chapter 1.@.03 B. 1. b.** v. Performance Evaluation: Conditionally Conforms. Tto be deemed conditionally conforming under the NSSP, a laboratory must meet one of the following laboratory performance criteria: (a) Complete an appropriate ISSC Accepted SLV; or (b) Complete a Method Verification Study, Section IV. Chapter II. .20 that successfully transfers; or (c). Successfully complete a proficiency and/or inter-laboratory study approved by the FDA Shellfish LEO or State certified Shellfish LEO. (d) This laboratory status will remain in effect until an technical FDA Shellfish LEO or FDA certified State Shellfish LEO Evaluation occurs as in @.03 B. **MO Chapter III. @.01 Quality Assurance** A. NSSP Conformance Required for all laboratories supporting the NSSP. All laboratory analyses shall be performed by a laboratory found to conform, conditionally conform or provisionally conform by the FDA Shellfish LEO or FDA certified State Shellfish LEO in accordance with the requirements established under the NSSP. **MO Chapter XV. .03 J. (4)** (a) Are analyzed by a laboratory which has been evaluated and found to conform or conditionally conform to the NSSP pursuant to the requirements in Chapter III, using an NSSP-Approved Method;
13. Public Health Significance	A technical Laboratory evaluation, as outlined in MO Chapter 1.@.03B.1.b.ii, is conducted to verify that conditions are present *in the laboratory* which **should**

	result in the accurate outcome of method data. A performance evaluation **verifies** that the method data produced *by the laboratory and for all analysts* is accurate. A technical evaluation does not examine the quality of a laboratory's method data for validity, standardization or for individual analysts. If a laboratory has successfully passed a proficiency study, SLV or MV, and statistically confirmed method data results, the laboratory can be assumed to have technically performed the method correctly. Under current interpretation a laboratory may have completed and had accepted by the conference a method SLV with accompanying checklist yet not be able to support the NSSP with data until a FDA Shellfish LEO or FDA certified State Shellfish LEO conducts a technical inspection at their laboratory using the laboratory's own checklist. If a laboratory has proven its ability to perform a method, then the laboratory should be able to conditionally support the NSSP with data. A cooperative goal of the NSSP, FDA and the SSCA is to assure that a laboratory's data is accurate, verified and standardized. Method based performance evaluations confirm data which results in standardization across laboratories. Method based performance evaluations statistically verify data accuracy. Performance Evaluations therefore support the legal defensibility of the laboratory's Laboratory Quality Management System.
14. Cost Information	Cost of conducting SLV, MV or Proficiency Participation
Action by 2019 Laboratory Committee	Recommended no action on Proposal 19-101. Rationale: This issue is addressed by Proposal 19-301.
Action by 2019 Task Force I	Recommended adoption of Proposal 19-101 as submitted.
Action by 2019 General Assembly	Recommended referral of Proposal 19-101 to an appropriate committee as determined by the Conference Chair.
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 19-101.
Action by 2023 Laboratory Committee	Recommended referral of Proposal 19-101 to an appropriate committee as determined by the Conference Chairperson
Action by 2023 Task Force I	Recommended referral of Proposal 19-101 as amended to the Laboratory Committee with the provision that a recommendation for interim approval be provided at the 2023 Fall Executive Board Meeting. **MO Chapter 1.@.03 B. 1. b.** v. Performance Evaluation: Conditionally Conforms. Tto be deemed conditionally conforming under the NSSP, a laboratory must meet one of the following laboratory performance criteria: (a) Complete an appropriate ISSC Accepted SLV; or (b) Complete a Method Verification Study, Section IV. Chapter II. .20 that successfully transfers; or ~~(c). Successfully complete a proficiency and/or inter-laboratory study approved by the FDA Shellfish LEO or State certified Shellfish LEO.~~ (c~~d~~)

	This laboratory status will remain in effect until an technical FDAShellfish LEO or FDA certified State Shellfish LEO Evaluation occurs as in @.03 B. **MO Chapter III. @.01 Quality Assurance** A. NSSP Conformance Required for all laboratories supporting the NSSP. All laboratory analyses shall be performed by a laboratory found to conform, conditionally conform or provisionally conform by the FDA Shellfish LEO or FDA certified State Shellfish LEO in accordance with the requirements established under the NSSP. **MO Chapter XV. .03 J. (4)** (a) Are analyzed by a laboratory which has been evaluated and found to conform or conditionally conform to the NSSP pursuant to the requirements in Chapter III, using an NSSP-Approved Method;
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 19-101.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 19-101.
Action by 2024 Laboratory Committee	Recommended Proposal 19-101 as substituted and submit to the 2024 Executive Board for interim approval. **Chapter III** **@.01 Quality Assurance** C. FDA Responsibilities. The FDA will ensure that all laboratories generating data in support of the NSSP will be evaluated at a minimum frequency of once every three (3) years. (1) Evaluations will be conducted by either an FDA Shellfish LEO or an FDA certified State Shellfish LEO as appropriate. Normally the initial evaluation of a laboratory will be conducted by FDA. (2) Evaluations are ~~generally~~ conducted onsite; however, evaluations may be conducted ~~but can under certain circumstances~~ be ~~by~~ desk/virtual audit when scheduling or extenuating circumstances will not permit a timely onsite evaluation. ~~(evaluation follow-up,~~ action ~~plan monitoring, nonconformity corrections, major changes in personnel, workload or facilities, etc.).~~ (3) When there is an immediate and critical need, and: (a) no NSSP conforming or provisionally conforming laboratory exists to perform a specific Approved NSSP Method, or (b) no NSSP conforming or provisionally conforming laboratory is accessible (i.e. due to distance, etc.) to perform specific Approved NSSP method, or (c) following a request for emergency consideration as defined in Section II, Ch. 1 @.01 I. The following alternative laboratories may implement that Approved ISSC Method in support of the NSSP:

	(a) A laboratory that has not been evaluated or assigned an NSSP operational status, provided that: i. the ISSC Executive Board is notified within a reasonable period of time regarding the laboratory used; and, ii. the appropriate FDA Office is notified within a reasonable period of time regarding the laboratory used; and, iii. the laboratory meets the following laboratory performance criteria: 1. completed the SLV study which resulted in the Approved NSSP method; or 2. maintains a recognized laboratory accreditation status (e.g., ISO 17025:2017, LAAF, etc.), with documentation of accreditation provided to the FDA prior to method implementation; and 3. successfully completed a Method Verification Study, as outlined in Section IV, Chapter II, .20, and the method verification data package has been approved by an FDA Shellfish LEO. If an FDA Shellfish LEO is unavailable to review the method verification data package in a timely manner, an FDA certified State Shellfish LEO may review and approve the data package instead. State Shellfish LEOs will only review method verification data packages pertaining to methods for which they have documented proficiency and evaluation standardization. (b) An existing NSSP conforming or provisionally conforming laboratory that has not been evaluated for the specific Approved NSSP method to be used, provided that: i. the ISSC Executive Board is notified within a reasonable period of time regarding the laboratory used; and, ii. the appropriate FDA Office is notified within a reasonable period of time regarding the laboratory used; and, iii. the laboratory has successfully completed a Method Verification Study, as outlined in Section IV, Chapter II, .20, and the method verification data package has been approved by an FDA Shellfish LEO. If an FDA Shellfish LEO is unavailable to review the method verification data package in a timely manner, an FDA certified State Shellfish LEO may review and approve the data package instead. State Shellfish LEOs will only review method verification data packages pertaining to methods for which they have documented proficiency and evaluation standardization. If the immediate and critical need for analysis becomes an ongoing or recurring need, the laboratory implementing the method will work with the FDA

	Shellfish LEOs to promptly initiate the evaluation process. D. Wet Storage and Post-Harvest Processors. For any laboratory providing analytical testing services for depuration, wet storage or PHP, initial and subsequent triennial evaluations will be required and conducted in accordance with @.01 and @.02 of this Chapter by an FDA Shellfish LEO or an FDA certified State Shellfish LEO ~~as appropriate~~. It is understood that academic laboratories involved in PHP Validation or Verification have special circumstances such as extended periods of inactivity resulting from university schedules or funding constraints; however, written documentation of Quality Control practices will be required for time periods in which they are preparing for or actively participating in a PHP Validation or Verification. Times in which the lab is inactive can be explained with a not applicable notation.
Action by 2023 Executive Board	Adopted the recommendation of 2024 Laboratory Committee. Interim Approval on October 30, 2024.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Robert Rheault
Affiliation	ECSGA
Email	bob@ecsga.org
Proposal Subject	Aquaculture Seed Shellstock
Specific NSSP Guide Reference	Section II Model Ordinance, Chapter VI. Shellfish Aquaculture, Requirements of the Authority @.02
Text of Proposal/ Requested Action	**@ .02 Seed Shellstock** A. The Authority shall establish the maximum seed size for each species of shellfish that can be produced in prohibited waters. In determining the maximum seed size Authorities shall establish sizes that require a minimum of 60~~120~~ days of growing with water temperatures over 50 degrees F to reach market size. B. For states that have not established a minimum market size, the Authority shall establish record-keeping protocols to track seed sourced from prohibited waters to ensure seed have at least 60 days of growing with water temperatures above 50 degrees F before sale for human consumption. C. ~~B.~~ The Authority shall establish appropriate corrective actions for ~~when~~ seed that exceeds the maximum seed size when it is being cultured in ~~has been~~ ~~produced in~~ waters classified as prohibited. ~~C.~~ All sources of seed produced or collected in prohibited waters shall be sanctioned by the Authority
Public Health Significance	Existing language does not describe how the Authority should establish maximum seed size in states that have no minimum market size. Further the existing language does not require that shellfish from prohibited waters are held in waters above 50 degrees to ensure that the animals are metabolically active. Shellfish seed collected or cultured in prohibited waters have been shown through repeated sampling not to accumulate heavy metals at levels that exceed EPA alert levels. (John Mullen RI DOH, unpub. Data, Rheault unpubl. Data, Rice unpub. Data, Leavitt unpub. Data). A period of one month is typically adequate to purge bacterial contaminants provided water temperatures are high enough to maintain active metabolic activity (above 50 degrees F or 10 degrees C) (Richards 1988). Several studies have demonstrated that viral contamination in relayed or depurated shellfish is reduced to non-detect levels in 30-40 days (McLeod et. Al. 2017 and Choi and Kingsley 2016). The Authority has the option to deny seed culture in any area, or to require additional testing for deleterious substances, or to require longer purge periods as they deem necessary based on potential sources of

	contaminants. References Cited: Richards, G. (1988), Microbial Purification of Shellfish: A Review of Depuration and Relaying, J. Food Protection 51(3)218-251. A. McLeod et. Al. (2017) Depuration and Relaying: A Review on Potential Removal of Norovirus from Oysters. Comprehensive Reviews in Food Science and Food Safety, Vol.16, pp. 692-706 Choi, C. and D. H. Kingsley. Temperature-Dependent Persistence of Human Norovirus within Oysters (Crassostrea virginica). Food and Environmental Virology, 8:141-147. 2016 Supporting Information: RI DOH metals data, (oyster seed grown in Billington Cove Marina) Unpublished data from Rd. Dale Leavitt: (clam seed grown in Warwick Cove Marina)
Cost Information	Proposal would not impact the enforcement costs for the authority and would simplify management for growers.
Action by 2019 Task Force I	Recommended referral of Proposal 19-108 to an appropriate committee as determined by the Conference Chairperson.
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 19-108
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 19-108
Action by 2023 Aquaculture Committee	Recommended no action on Proposal 19-108. Rationale: There is not sufficient data or need for action
Action by 2023 Task Force I	Recommended referral of Proposal 19-108 to an appropriate committee as determined by the Conference Chairperson
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 19-108.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 19-108.

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB #

SAMPLE COLLECTION FORM

210957 12AM10:09

DATE OF COLLECTION		DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
12/12/01	Anal B mus/Pet	12/20-12/21/01	12/21/01	PCE

CONDITION	SEALED	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
HOT FROZEN COLD OTHER	YES NO	OYSTER'S			61
EXP DATE	**SIZE**	**BRAND NAME**	**TEMP ESTAB**	**TEMP REC °C**	**TEMP ANALYSIS**
		Geib			
DATE OF SHIP	**FROM LOT OF**	**COLLECTED AT**			**REASON**
		Little Allen's HARBOR			01
		ADDRESS	**FOLLOW UP SAMPLE #1**		**SOURCE**
					10
SHIPPER/PACKER & ADDRESS					**ANALYSIS**
76 Quaker Dr West Warwick 02893			Metals		[illegible] 13
COMPLAINT*			**ORIGINAL CONTAINER YES NO**		**DATE PURCHASED**
NAME & LOCATION OF STORE WHERE PURCHASED					**PRODUCT USED YES NO**

HOW STORED FROZEN	AMBIENT COLD	IMPORT PRODUCT YES NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE ,TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

SIGNED FROM John Mellen	SIGNED TO [illegible]	DATE 12.12.01	TIME 10:15 AM	HOW STORED on ice	
AGENT # PRINT NAME 164 John Mellen	PRINT NAME THEODORE C. PLINKAS				
SIGNED FROM [signature]	SIGNED TO				
PRINT NAME Theodore C. Plinkas	PRINT NAME	12.17.01	10:20 Am		
SIGNED FROM	SIGNED TO				
PRINT NAME	PRINT NAME				

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb 4.34 ppm	36. Cd 0.53 ppm	37. Cu 61.7 ppm	38. Cr 0.98 ppm	39. Zn 385 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Samples from upwelling
B. Geib Lease
Water Temp 43°

LABORATORY NOTES:

Ni: 1.86 ppm
Fe: 244 ppm

RECEIVED
JAN - 2 2002
RI DEPARTMENT OF HEALTH
DIVISION OF FOOD PROTECTION

EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*

* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.

WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB # 15 13115 29 3 9:22

SAMPLE COLLECTION FORM

4/15/02 - 4/16/02 4/16/02 PLE

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
3/28/02 Paul By RE/ms	3.29.02	4.2.02	MP

CONDITION	SEALED	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
HOT FROZEN COLD OTHER	YES NO	OYSTERS			61
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS

DATE OF SHIP	FROM LOT OF	COLLECTED AT	REASON
		Geib lease	01
		ADDRESS	FOLLOW UP SAMPLE #1 SM 11 SM 17 SM 17 / SOURCE 10
SHIPPER/PACKER & ADDRESS			ANALYSIS 24(6,13)
COMPLAINT*			ORIGINAL CONTAINER YES NO / DATE PURCHASED
NAME & LOCATION OF STORE WHERE PURCHASED			PRODUCT USED YES NO

HOW STORED		IMPORT PRODUCT	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING
FROZEN COLD	AMBIENT	YES NO				

CHAIN OF CUSTODY - DATE, TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

SIGNED FROM John Miller	SIGNED TO Phuna Tun	DATE 3-29-02	TIME 9.30	HOW STORED in cooler	
AGENT* PRINT NAME 16 4 John Miller	PRINT NAME				
SIGNED FROM Phuna Tun	SIGNED TO Kerry Patterson	3-29-02	10:00	in refriguator 7	
PRINT NAME Phanca Tun	PRINT NAME Kerry PATTERSON				
SIGNED FROM Kerry Patterson	SIGNED TO Chris Ellis	4/2/02	10:40	Ref	
PRINT NAME Kerry PATTERSON	PRINT NAME Chris Ellis				

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb 0.28 ppm	36. Cd 0.30 ppm	37. Cu 21.4 ppm	38. Cr 0.40 ppm	39. Zn 360 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN <20	57. TOT COLIFORM/MPN <20	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC 730	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Samples from grow out area. Placed there 9.9.01

Grows at lease only

LABORATORY NOTES:

Fe: 22.0 ppm

Ni: 1.32 ppm

EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*

* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.

WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB # 14 213115 29 MAR 902

SAMPLE COLLECTION FORM

Analyzed By: VL/Lee VL/Lee VL/Lee PCE

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
3/28/02 Picked/Rec	3.25.02	4.2.02	TCJ

CONDITION: HOT FROZEN COLD OTHER	SEALED: YES NO	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
		Oysters			G1
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS
DATE OF SHIP	FROM LOT OF	COLLECTED AT: Geib Lease			REASON: 01
		ADDRESS		FOLLOW UP SAMPLE #1: SM 11 SM 13 SM 27	SOURCE: 10
SHIPPER/PACKER & ADDRESS					ANALYSIS: 24(8,13)
COMPLAINT*				ORIGINAL CONTAINER: YES NO	DATE PURCHASED
NAME & LOCATION OF STORE WHERE PURCHASED					PRODUCT USED: YES NO
HOW STORED: FROZEN AMBIENT COLD	IMPORT PRODUCT: YES NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE, TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.

SIGNED FROM: John Miller	SIGNED TO: Phung Tim	DATE: 3-29-02	TIME: 9:30	HOW STORED: in cooler	
AGENT # PRINT NAME: 164 John Miller	PRINT NAME:				
SIGNED FROM: Phung Tim	SIGNED TO: Kerry Patterson	3-29-02	10:00	in Refrigerator	
PRINT NAME: Phung Tim	PRINT NAME: Kerry Patterson				
SIGNED FROM: Kerry Patterson	SIGNED TO: Chuck	4/2/02	10:40	Ref	
PRINT NAME: Kerry Patterson	PRINT NAME: Chuck Ellis				

1. CEREAL %	2. MEXT'PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb <0.20 ppm	36. Cd 0.26 ppm	37. Cu 27.3 ppm	38. Cr <0.37 ppm	39. Zn 926 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN 20	57. TOT COLIFORM/MPN 50	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC 770	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Samples from grow out
Placed there SUMMER 2000
2 WINTERS AT LEASE

LABORATORY NOTES:

LAB 14

Fe: 21.4 ppm
Ni: 1.24 ppm

EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*
* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.

WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB # 13

2003114 29 AM 9:22

SAMPLE COLLECTION FORM

4/5/02 - V&V 4/11/02 PCE

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUB
3/28/02 Final By PCE/TMC	3.29.02	4.2.02	TCJ

CONDITION	SEALED	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
HOT FROZEN COLD OTHER	YES NO	OYSTERS			61
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS

DATE OF SHIP	FROM LOT OF	COLLECTED AT	REASON
		Geib lease	01
		ADDRESS	FOLLOW UP SAMPLE #1 SM 11 SM 13 SM 27 / SOURCE 10

SHIPPER/PACKER & ADDRESS

ANALYSIS 24(8)13

COMPLAINT* | ORIGINAL CONTAINER YES NO | DATE PURCHASED

NAME & LOCATION OF STORE WHERE PURCHASED | PRODUCT USED YES NO

HOW STORED	IMPORT PRODUCT	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING
FROZEN AMBIENT COLD	YES NO				

CHAIN OF CUSTODY - DATE, TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

	SIGNED / PRINT NAME	DATE	TIME	HOW STORED
SIGNED FROM	John Miller			
AGENT # / PRINT NAME	164 John Miller			
SIGNED TO / PRINT NAME	Kerry Patterson	3-29-02	9:30	in cooler
SIGNED FROM	Rhona Tum			
PRINT NAME	Rhona Tum			
SIGNED TO / PRINT NAME	Kerry Patterson	3-29-02	10:00	in Refrigerator
SIGNED FROM	Kerry Patterson			
PRINT NAME	Kerry Patterson			
SIGNED TO / PRINT NAME	Chris Ellis	4/2/02	10:16	Ref

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb 0.28 ppm	36. Cd 0.30 ppm	37. Cu 82.1 ppm	38. Cr 0.38 ppm	39. Zn 533 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN <20	57. TOT COLIFORM/MPN <20	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC 2100	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Sample from a grow out placed there Summer June - Oct. 2001

LABORATORY NOTES: LAB#13 Fe: 28.1 ppm Ni: 1.20 ppm

EVALUATION BY LAB:	ADMINISTRATION FOLLOW-UP:	CONCLUSION:	REVIEWED BY:
1. NOT AN FP OBLIGATION	1. CHECK ESTABLISHMENT	1. SATISFACTORY	
2. FP VIOLATION - NO VIOLATION	2. ADDITIONAL SAMPLES	2. QUESTIONABLE	
3. FP ACTION INDICATED	3. EMBARGO	3. VIOLATES STD	
4. INSUFFICIENT SAMPLE FOR ANALYSIS	4. REVIEW PROCESS	4. UNSATISFACTORY	
5. INSUFFICIENT INFORMATION FOR ANALYSIS	5. NO FURTHER ACTION		
6. IMPROPER CHAIN OF CUSTODY	6. DISPOSE		
7. LAB UNABLE TO PERFORM TESTING (REASON)			

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*

* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.

WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

SAMPLE COLLECTION FORM

LAB #

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
8/4/98 Anal BX 1145	8/13/98	8/17/98	PCE

CONDITION: HOT / COLD / FROZEN / OTHER	SEALED: YES / NO	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
		Oysters			61
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS

DATE OF SHIP	FROM LOT OF	COLLECTED AT	REASON
		Billington Cove Maine	01
		ADDRESS	FOLLOW UP SAMPLE #1 / SOURCE: 10
SHIPPER/PACKER & ADDRESS			ANALYSIS: 13
COMPLAINT*			ORIGINAL CONTAINER YES / NO; DATE PURCHASED
NAME & LOCATION OF STORE WHERE PURCHASED			PRODUCT USED YES / NO

HOW STORED: FROZEN / AMBIENT / COLD	IMPORT PRODUCT: YES / NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE ,TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

SIGNED FROM	SIGNED TO	DATE	TIME	HOW STORED
AGENT / PRINT NAME: 164 John Mullen	PRINT NAME: Molly Nousilva	8/4/98	1:45	ambient
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_3$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb 0.47 ppm	36. Cd 0.45 ppm	37. Cu 127 ppm	38. Cr <0.15 ppm	39. Zn 798 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Moosetone Oyster nursery stock. appx. 36mm

LABORATORY NOTES:

Fe: 133 ppm

EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*

* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.

WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB #

Proposal 19-108

202209 16 PM 1:52

SAMPLE COLLECTION FORM

DATE OF COLLECTION	Rec'd By	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
11/14/00	MCS	11/29/00	11/30/00	PCE

CONDITION HOT FROZEN COLD OTHER	SEALED YES NO	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
		Oysters			61
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS
DATE OF SHIP	FROM LOT OF	COLLECTED AT Point Judith Pond			REASON 04
		ADDRESS Beach Island		FOLLOW UP SAMPLE #1	SOURCE 10
SHIPPER/PACKER & ADDRESS					ANALYSIS 13
COMPLAINT*				ORIGINAL CONTAINER YES NO	DATE PURCHASED
NAME & LOCATION OF STORE WHERE PURCHASED					PRODUCT USED YES NO
HOW STORED FROZEN COLD	AMBIENT	IMPORT PRODUCT YES NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE ,TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

SIGNED FROM John Muller	SIGNED TO Chris Kelly	DATE 11/16/00	TIME 2:00	HOW STORED On ice
AGENT# PRINT NAME 164 John Muller	PRINT NAME Chris Kelly			
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb 0.77 ppm	36. Cd 0.17 ppm	37. Cu 25.4 ppm	38. Cr 0.34 ppm	39. Zn 590 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Samples collected by Bot R Heaulo

LABORATORY NOTES:

Fe: 26.9 ppm

EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*
* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.
WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION FOR OFFICE CONSUMER

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB #

198251 13 AM10:32

SAMPLE COLLECTION FORM

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
7/13/00 Amt Bx MWS	10/27/00	10/27/00	PCE

CONDITION HOT FROZEN COLD OTHER	SEALED YES NO	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
		Oysters			66
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS
DATE OF SHIP	FROM LOT OF	COLLECTED AT Moonstone Oyster			REASON 01
		ADDRESS		FOLLOW UP SAMPLE #1	SOURCE 02
SHIPPER/PACKER & ADDRESS					ANALYSIS 13
COMPLAINT*				ORIGINAL CONTAINER YES NO	DATE PURCHASED
NAME & LOCATION OF STORE WHERE PURCHASED					PRODUCT USED YES NO
HOW STORED: FROZEN COLD / AMBIENT	IMPORT PRODUCT YES NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE ,TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

SIGNED FROM John Miller	SIGNED TO Mary Lou Sabo	DATE 7/13/00	TIME 10:35	HOW STORED Ambient	
AGENT # PRINT NAME 164 John Miller	PRINT NAME MARY LOU SABO, LVT				
SIGNED FROM	SIGNED TO				
PRINT NAME	PRINT NAME				
SIGNED FROM	SIGNED TO				
PRINT NAME	PRINT NAME				

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb <0.54 ppm	36. Cd 0.19 ppm	37. Cu 28.0 ppm	38. Cr <0.48 ppm	39. Zn 830 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Cage #24 oldest oysters on case placed mid-late 98

LABORATORY NOTES:

Fe: 31.4 ppm

RECEIVED OCT 30 2000 RI Department of Health

EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*
* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.
WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB # 198250 13 AM 10:32

SAMPLE COLLECTION FORM

DATE OF COLLECTION	Analyst By	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
7/13/00	MJS	10/27/00	10/27/00	PCE

CONDITION HOT FROZEN COLD OTHER	SEALED YES NO	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
		OYSTERS			66
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS

DATE OF SHIP	FROM LOT OF	COLLECTED AT	REASON
		Moonstone Oyster	01
		ADDRESS	FOLLOW UP SAMPLE #1 / SOURCE
			02
SHIPPER/PACKER & ADDRESS			ANALYSIS
			13
COMPLAINT*			ORIGINAL CONTAINER YES NO / DATE PURCHASED
NAME & LOCATION OF STORE WHERE PURCHASED			PRODUCT USED YES NO

HOW STORED FROZEN COLD	AMBIENT	IMPORT PRODUCT YES NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE ,TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC::

SIGNED FROM	SIGNED TO	DATE	TIME	HOW STORED	
AGENT & PRINT NAME I/C John Miller	PRINT NAME MAKEPEACE/LOA	7/13/00	10:35	Ambient	
SIGNED FROM	SIGNED TO				
PRINT NAME	PRINT NAME				
SIGNED FROM	SIGNED TO				
PRINT NAME	PRINT NAME				

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb <0.54 ppm	36. Cd 0.18 ppm	37. Cu 13.2 ppm	38. Cr <0.48 ppm	39. Zn 578 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:
Cage # 18 July 99

LABORATORY NOTES:
Fe: 34.7

RECEIVED OCT 30 2000 R.I. DEPARTMENT OF HEALTH

EVALUATION BY LAB:	ADMINISTRATION FOLLOW-UP:	CONCLUSION:	REVIEWED BY:
1. NOT AN FP OBLIGATION	1. CHECK ESTABLISHMENT	1. SATISFACTORY	
2. FP VIOLATION - NO VIOLATION	2. ADDITIONAL SAMPLES	2. QUESTIONABLE	
3. FP ACTION INDICATED	3. EMBARGO	3. VIOLATES STD	
4. INSUFFICIENT SAMPLE FOR ANALYSIS	4. REVIEW PROCESS	4. UNSATISFACTORY	
5. INSUFFICIENT INFORMATION FOR ANALYSIS	5. NO FURTHER ACTION		
6. IMPROPER CHAIN OF CUSTODY	6. DISPOSE		
7. LAB UNABLE TO PERFORM TESTING (REASON)			

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD*
* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.
WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER

Proposal 19-108

RHODE ISLAND DEPARTMENT OF HEALTH - OFFICE OF FOOD PROTECTION,
RM 203, CANNON BLDG., 3 CAPITOL HILL, PROVIDENCE, RI 02908

LAB # 198249 13 AM 10:32

SAMPLE COLLECTION FORM

DATE OF COLLECTION	Rec'd By	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
7/13/00	mvs	10/27/00	10/27/00	PCE

CONDITION HOT FROZEN COLD OTHER	SEALED YES NO	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
		Oysters			01
EXP DATE	SIZE	BRAND NAME	TEMP ESTAB	TEMP REC °C	TEMP ANALYSIS

DATE OF SHIP	FROM LOT OF	COLLECTED AT	REASON
		Moonstone Oyster	01

ADDRESS	FOLLOW UP SAMPLE #1	SOURCE
		02

SHIPPER/PACKER & ADDRESS		ANALYSIS
		13

COMPLAINT*	ORIGINAL CONTAINER YES NO	DATE PURCHASED

NAME & LOCATION OF STORE WHERE PURCHASED		PRODUCT USED YES NO

HOW STORED FROZEN COLD	AMBIENT	IMPORT PRODUCT YES NO	INTERVIEWED BY	DATE	TIME	AMOUNT REMAINING

CHAIN OF CUSTODY - DATE ,TIME OF TRANSACTION OF COMPLAINT FROM FIELD TO LAB, ETC.:

SIGNED FROM John Muller	SIGNED TO Mary Lou Silva	DATE 7/13/00	TIME 10:35	HOW STORED ambient
AGENT PRINT NAME 164 John Muller	PRINT NAME MARY LOU SILVA			
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			
SIGNED FROM	SIGNED TO			
PRINT NAME	PRINT NAME			

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY FL %	5. NFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug mal/g	11. FFA %	12. WT/VOL	13. $NaNO_2$ ppm	14. EL'TROPHO	15. TOTAL SOLIDS %	16. HISTAMINE mg/100g
17. NaCl %	18. NH_3 mg/100g	19. SULFITE ppm	20. Hg ppm	21. TVB mg N/100g	22. DOMOIC ACID ug/ml	23. TMA mgN/100g	24. VITAMIN A IU/Qt
25. pH	26. BRIX %	27. INSECT	28. RODENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Qt
33. PKG INT	34. AFLATOXIN ppb	35. Pb <0.54 ppm	36. Cd 0.19 ppm	37. Cu 60.9 ppm	38. Cr <0.48 ppm	39. Zn 711 ppm	40. A_w
41. Na mg/Serving	42. TSP	43. Ca mg/100g	44. P mg/100g	45. Mg mg/100g	46. GLUTAMIC/MSG %	47. ACETIC ACID mg/100g	48. GLUCOSE %
47. FRUCTOSE %	48. SUCROSE %	49. LACTOSE %	50. MALTOSE %	51. YEAST	52. MOLD	53 PSP	54. OTHER
56. FECAL/MPN	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:
Cage #238
Sept. 99

LABORATORY NOTES:
Fe: 38.3 ppm

OCT 30 2000

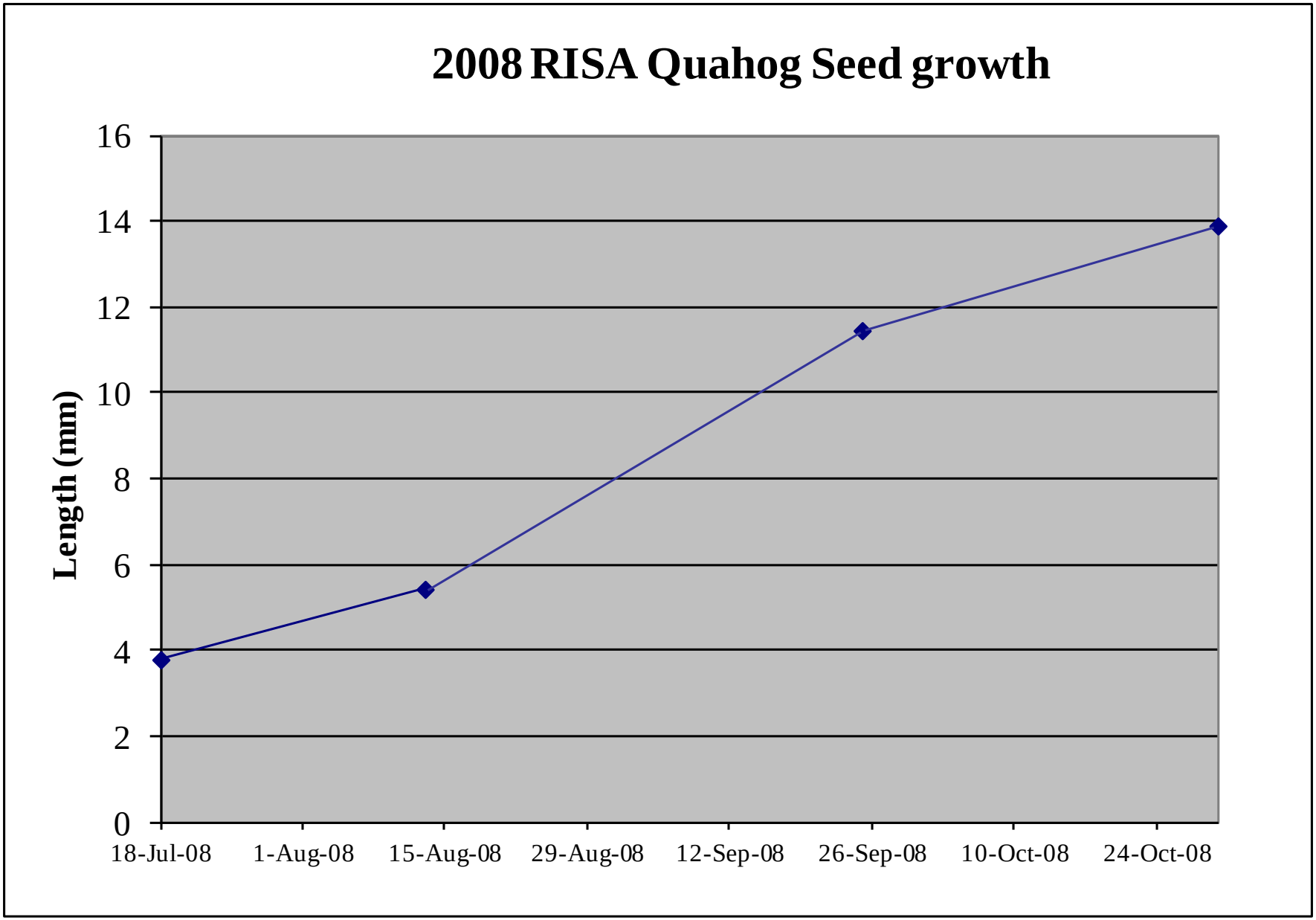
EVALUATION BY LAB:
1. NOT AN FP OBLIGATION
2. FP VIOLATION - NO VIOLATION
3. FP ACTION INDICATED
4. INSUFFICIENT SAMPLE FOR ANALYSIS
5. INSUFFICIENT INFORMATION FOR ANALYSIS
6. IMPROPER CHAIN OF CUSTODY
7. LAB UNABLE TO PERFORM TESTING (REASON)

ADMINISTRATION FOLLOW-UP:
1. CHECK ESTABLISHMENT
2. ADDITIONAL SAMPLES
3. EMBARGO
4. REVIEW PROCESS
5. NO FURTHER ACTION
6. DISPOSE

CONCLUSION:
1. SATISFACTORY
2. QUESTIONABLE
3. VIOLATES STD
4. UNSATISFACTORY

REVIEWED BY:

FOOD QUALITY (SAFETY SHELF-LIFE & CONSUMER ACCEPTANCE) INVOLVES THE ENTIRE CHAIN OF PRODUCTION PROCESSING & DISTRIBUTION OF FOOD"
* SAMPLE WILL BE MAINTAINED FOR THIRTY (30) DAYS AFTER ANALYSIS AND THEN DISPOSED OF BY THE LABORATORY.
WHITE - DATA ENTRY YELLOW - LAB PINK - FOOD PROTECTION GOLDENROD - CONSUMER


2008 RISA Quahog Seed growth
Length (mm)
16
14
12
10
8
6
4
2
0
18-Jul-08
1-Aug-08
15-Aug-08
29-Aug-08
12-Sep-08
26-Sep-08
10-Oct-08
24-Oct-08

Warwick Cove Upweller

Quahog Seed 30-Oct-08 Note: µg/kg = ppb

Group (n=15)	avg Length (mm)	stdev	avg Live Weight (g)	stdev	avg Soft Tissue Wet Weight (g)	stdev			avg Hg/Soft Tissue Wet Weight (µg/kg)	stdev	avg Cr/Soft Tissue Wet Weight (µg/kg)	stdev	avg Fe/Soft Tissue Wet Weight* (µg/kg)	stdev	avg Ni/Soft Tissue Wet Weight (µg/kg)	stdev	avg Cu/Soft Tissue Wet Weight (µg/kg)	stdev
1	15.1	2.9	0.967	0.730	0.267	0.238			7.81		0.20		35.57		0.20		8.18	
2	12.6	1.6	0.545	0.202	0.139	0.056			9.41		0.28		34.00		0.22		11.80	
3	13.9	1.2	0.685	0.201	0.182	0.058			8.24		0.26		33.33		0.20		9.30	
Total	13.9	2.2	0.732	0.476	0.196	0.152			8.49	0.83	0.25	0.04	34.30	1.15	0.21	0.01	9.76	1.85
Group (n=15)					avg Soft Tissue Dry Weight (g)	stdev	avg % Dry Weight	stdev	avg Hg/Soft Tissue Dry Weight (µg/kg)	stdev	avg Cr/Soft Tissue Dry Weight (µg/kg)	stdev	avg Fe/Soft Tissue Dry Weight* (µg/kg)	stdev	avg Ni/Soft Tissue Dry Weight (µg/kg)	stdev	avg Cu/Soft Tissue Dry Weight (µg/kg)	stdev
1					0.041	0.041	14.8%	2.4%	52.75		1.38		240.70		1.37		55.33	
2					0.022	0.008	16.1%	1.5%	58.45		1.73		210.60		1.35		73.09	
3					0.027	0.008	15.2%	1.7%	54.22		1.70		219.20		1.31		61.16	
Total					0.030	0.025	15.4%	2.0%	55.14	2.96	1.60	0.19	223.50	15.50	1.34	0.03	63.19	9.05

Group (n=15)	avg Zn/Soft Tissue Wet Weight* (µg/kg)	stdev	avg As/Soft Tissue Wet Weight (µg/kg)	stdev	avg Se/Soft Tissue Wet Weight (µg/kg)	stdev	avg Sr/Soft Tissue Wet Weight (µg/kg)	stdev	avg Ag/Soft Tissue Wet Weight (µg/kg)	stdev	avg Cd/Soft Tissue Wet Weight (µg/kg)	stdev	avg Pb/Soft Tissue Wet Weight (µg/kg)	stdev
1	55.10		1.77		0.87		15.28		0.03		0.06		0.31	
2	66.07		1.99		1.29		14.07		0.03		0.05		0.28	
3	55.07		1.55		0.53		11.94		0.03		0.11		0.26	
Total	58.75	6.34	1.77	0.22	0.89	0.38	13.76	1.69	0.03	0.00	0.07	0.03	0.28	0.03
Group (n=15)	avg Zn/Soft Tissue Dry Weight* (µg/kg)	stdev	avg As/Soft Tissue Dry Weight (µg/kg)	stdev	avg Se/Soft Tissue Dry Weight (µg/kg)	stdev	avg Sr/Soft Tissue Dry Weight (µg/kg)	stdev	avg Ag/Soft Tissue Dry Weight (µg/kg)	stdev	avg Cd/Soft Tissue Dry Weight (µg/kg)	stdev	avg Pb/Soft Tissue Dry Weight (µg/kg)	stdev
1	372.90		12.01		5.89		103.40		0.22		0.43		2.09	
2	409.30		12.32		7.97		87.14		0.16		0.29		1.76	
3	362.20		10.17		3.47		78.55		0.22		0.69		1.68	
Total	381.47	24.69	11.50	1.16	5.78	2.26	89.70	12.62	0.20	0.04	0.47	0.20	1.84	0.22

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Gina Olson
Affiliation	Washington Dept of Health
Email	Gina.olson.wa.gov
Proposal Subject	Laboratory Method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* Enumeration and Detection Through MPN and Real-Time PCR
Specific NSSP Guide Reference	Section IV Guidance Documents Chapter II Growing Areas .14 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	1. Approved Methods fir Vibrio Enumeration

	Vibrio Type:	**Application : PHP Sample Type:**	**Application : Reopening**
		X	
EIA[1]	***Vibrio vulnificus (V.v.)***	X	
MPN[2]	***Vibrio vulnificus (V.v.)***	X	
SYBR Green 1 QPCR-MPN[5]	***Vibrio vulnificus (V.v.)***	X	
MPN[3]	***Vibrio parahaemolyticus (V.p.)***	X	
PCR[4]	***Vibrio parahaemolyticus (V.p.)***	X	
MPN-Real Time PCR[6]	***tdh+ and trh+ Vibrio parahaemolyticus (V.p.)***	X X	X
MPN-Real Time PCR[7]	***Vibrio parahaemolyticus (V.p.)***	x	X
MPN-Real Time PCR[9]	***Vibrio parahaemolyticus (V.p.)* and *Vibrio vulnificus (V.v.)***		X
Direct Plating Method[8]	***Vibrio parahaemolyticus (V.p.)***		X

Footnotes:

[1] EIA procedure of Tamplin, et al, as described in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, 1992.

MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or by the DNA - alkaline phosphatase gene probe for vvhA as described by Wright et al., or a method that a State can demonstrate is equivalent.

[2] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, followed by confirmation using biochemical analyses or the DNA-alkaline phosphatase gene probe for tlh as described by McCarthy et al., or a method that a State can demonstrate is equivalent.

[3] MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, and as described in the "Direct Plating Procedure for the Enumeration of Total and Pathogenic *Vibrio parahaemolyticus* in Oyster Meats" developed by FDA, Gulf Coast Seafood Laboratory, or a method that a State can demonstrate is equivalent.

[5] *Vibrio vulnificus*, ISSC Summary of Actions 2009. Proposal 09-113, Page 123.

[6] MPN-Real Time PCR Method for the tdh and trh Genes for Total *V. parahaemolyticus* as described in Kinsey et al., 2015. ISSC 2015 Summary of Actions Proposal 15-111, Page 397.

[7] MPN-Real Time PCR Method for the *tlh* gene for total *V. parahaemolyticus* as described in Kinsey et al., 2015. ISSC 2015 Summary of Actions Proposal 15- 113, Page 418

[8] Direct Plating Procedure in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, and as described in the 'Direct Plating Procedure for the Enumeration of Total and Pathogenic *Vibrio parahaemolyticus* in Oyster Meats' developed by FDA, Gulf Coast Seafood Laboratory.

[9] MPN-Real Time PCR Method for *Vibrio parahaemolyticus* and *Vibrio vulnificus*. Washington State Department of Health, Food and Shellfish Bacteriology Laboratory.

Public Health Significance	The purpose of this method is to provide laboratories supporting the NSSP the ability to rapidly quantify *Vibrio parahaemolyticus (Vp)* and *Vibrio vulnificus (Vv)* from oysters using a high throughput real-time PCR assay. Rapid and early detection of these pathogens, complying with the required quantitative detection guidelines suggested by the ISSC, will help the shellfish industry market oysters for consumption that are within regulatory limits for these pathogens. This method once approved would add a testing method of MPN Real-Time PCR for *Vibrio vulnificus* and it would be an alternative to the *Vibrio parahaemolyticus* MPN Real-Time PCR methods already approved in the 2017 Model Ordinance.
Cost Information	The cost for this method is approx. $155 per sample. This estimate is based on recurring costs of consumables, reagents, and supplies needed for routine testing. It does not include indirect materials considered to be standard microbiology equipment such as analytical balance, PCR workstation, DNA purification system, refrigerator, pipettes, etc.
Action by 2019 Laboratory Committee	Recommended referral of Proposal 19-128 to an appropriate committee as determined by the Conference Chair
Action by 2019 Task Force I	Recommended the adoption of Laboratory Committee recommendation on Proposal 19-128
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 19-128.
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 19-128.
Action by 2023 Laboratory Committee	Recommended referral of Proposal 19-128 to an appropriate committee as determined by the Conference Chair
Action by 2023 Task Force I	Recommended the adoption of Laboratory Committee recommendation on Proposal 19-128
Action by 2023 General Assembly	Adopted recommendation of Task Force I on Proposal 19-128.
Action by FDA July 7,2023	Concurred with Conference action on Proposal 19-128.
Action by 2025 Executive Board	Adopted the recommendation of 2025 Laboratory Committee Committee. Interim Approval on October 15,2025

Validation Data for MPN Real-time PCR for Total and Pathogenic *Vibrio parahaemolyticus*

Name of Method Submitter: Gina Olson, Washington State Department of Health

Specific purpose or intent of the method for use in the NSSP:

Requesting adoption of this method as an approved method for Vibrio enumeration, both *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in oysters. The method may be used in the following applications: PHP validation and verification of product and in management of growing areas through environmental testing and surveillance in order to re-open closed growing areas. This method once approved would provide a high-throughput alternative to the current approved MPN real-time PCR method. In addition, this method would be the only approved MPN real-time PCR method to test for total Vp, pathogenic Vp, and Vv in a single assay.

Validation Criteria Data:

All oyster samples used in this validation were collected from different harvest locations and/or different harvest dates in Washington State. All samples were processed using APHA guidelines for the cleaning and shucking of shellstock. Samples were confirmed negative for the target organism of Vp through the FDA BAM culture-based method and through pcr prior to spiking. Spiking levels were determined by spread plating dilutions onto PCA w/2% NaCl in duplicate and averaging the counts.

Vp strain WA4647 was used to spike all samples for all validation criteria. This strain is positive for *tlh*, *tdh*, and *trh*. All data generated for all three targets was identical and has been presented in a single MPN in all validation criteria tables and data calculations.

The validation data for Vp and Vv is presented separately for clarity and ease in reviewing the data, but this is a single assay and all elements were present during the validation of all organisms.

<u>Assay Design</u>

DNA Isolation: Roche MagnaPure 96 using Roche DNA/Viral Nucleic Acid Small Volume Kit

Real-time PCR Instrument: Applied Biosystems QuantStudio Dx (384-well format)

Mastermix: Life Technologies TaqMan Environmental Master Mix 2.0

Real-Time PCR targets: 2 multiplex reactions

- Multiplex 1: Total Vp (*tlh)*, Vv (*vvhA)*, internal control (IC)
- Multiplex 2 (Vp pathogenicity markers): *tdh*, *trh*, *orf8*

Real-Time PCR parameters:

Denaturation: 95°C for 10 mins
Annealing: 95°C for 15 secs
Extension: 59°C for 1 min
Cycles: 40

1. Accuracy/Trueness & Measurement Uncertainty

Accuracy/Trueness

Purpose/Method

Accuracy/Trueness measures the closeness of agreement between the test results (MPNs) and the accepted reference values (CFUs). This was done by analyzing twenty oyster samples over a range of concentrations (low to high) to determine the MPN. The MPN and CFU data set was converted into logs. The average MPN in logs was divided by the average plate count in logs. This provides an estimate (in percent) of the accuracy/trueness of the method.

Results

The average of the plate count CFUs was 2.88 log. The average of MPNs was 3.17 log. Accuracy/Trueness was found to be 109.94%. Results can be found below in Table 1.

Measurement Uncertainty

Purpose/Method

Measurement uncertainty expresses the range of values around the measured result within which the true value is expected to lie. To determine this parameter, twenty oyster samples spiked with a range of concentrations were analyzed. The MPN and CFU data set was converted into logs and the MPN result was subtracted from the CFU result for each sample. A 95% confidence interval was calculated from the difference. This confidence interval represents the measurement uncertainty of the methods.

Results

The measurement uncertainty was determined via 95% CI (0.23, 0.50), resulting in a measurement uncertainty of 0.27. Results can be found below in Table 1.

Table 1. Data for determination of Accuracy/Trueness and Measurement Uncertainty

Sample	Plate Count, log(CFUs)	MPN, log(MPN/g)
1	0.89	0.62
2	0.77	1.36
3	0.85	0.96
4	2.85	2.62
5	2.32	3.36
6	2.32	3.17
7	4.18	5.04
8	4.18	4.66
9	0.36	0.63

10	4.36	4.66
11	2.04	2.62
12	2.45	2.36
13	3.52	3.36
14	4.69	5.04
15	4.69	5.04
16	1.66	1.62
17	2.57	2.96
18	3.60	3.59
19	4.69	5.04
20	4.69	4.66

2. Ruggedness

Purpose/Method

The amount of analyte recovered should be consistent between different lots of media/reagents. Ruggedness tests the impact of different lots used to process samples on the final result. This was done by testing ten oyster samples spiked at a range of concentrations in duplicate. The first replicate was performed using “Lot 1” media/reagents and the second was performed using “Lot 2.” To determine if the method was sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between Lot 1 and Lot 2 samples.

Results

Using data from Table 2, there was no significant difference (p=0.64) between different lots of media and reagents.

Table 2. Data for Determination of Ruggedness

Sample	Replicate 1, log(MPN/g)	Replicate 2, log(MPN/g)
1	0.62	0.96
2	1.36	1.36
3	0.96	0.96
4	2.96	2.62

5	3.36	3.63
6	2.96	3.18
7	5.04	5.34
8	5.34	4.66
9	0.62	1.62
10	5.04	4.66

3. Precision & Recovery

Precision

Purpose/Method

The difference between the methods results (MPNs) and the reference values (CFUs) should be consistent between different samples and also when detecting varying concentrations of measurand. The precision of the method tests the consistency of the difference between the CFU's found on plates and the MPN values. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and CFU data sets were converted into log values. Each MPN was compared to its associate CFU value. A nested ANOVA was then performed, with variance components being the sample, and concentrations within the samples (low, medium, and high), and then the error. The ANOVA component of interest was that comparing the concentrations within the samples to the determinations (or error).

Results

The difference between CFUs and MPNs can be found below in Table 3. The data shows that there are no significant differences between the concentrations in samples and the determinations within concentrations via a nested ANOVA (p=0.08). Additionally, the variance of the method does not exceed the known variance of a 3-tube MPN (p=0.21).

Table 3: Data for determining the Precision

Sample	Concentration	Difference	log(CFUs)	log(MPNs)
1	low	-0.72	-1.17	-0.44
1	low	-1.13	-1.17	-0.04
1	med	-1.40	1.96	3.36
1	med	-1.00	1.96	2.96
1	high	0.13	4.69	4.56

1	high	-0.65	4.69	5.34
2	low	-0.68	-0.51	0.18
2	low	0.24	-0.51	-0.75
2	med	0.08	2.45	2.36
2	med	-0.42	2.45	2.87
2	high	-0.35	4.69	5.04
2	high	0.03	4.69	4.66
3	low	-0.18	-0.92	-0.74
3	low	-0.18	-0.92	-0.74
3	med	-0.45	2.52	2.96
3	med	-0.11	2.52	2.62
3	high	-0.35	4.69	5.04
3	high	-0.35	4.69	5.04
4	low	-0.08	-0.21	-0.13
4	low	-0.57	-0.21	0.36
4	med	-0.13	2.49	2.62
4	med	-0.13	2.49	2.62
4	high	-0.35	4.69	5.04
4	high	0.03	4.69	4.66
5	low	0.07	-0.15	-0.21
5	low	0.30	-0.15	-0.44
5	med	0.13	2.49	2.36
5	med	0.13	2.49	2.36
5	high	-0.35	4.69	5.04
5	high	0.03	4.69	4.66
6	low	-0.24	-0.28	-0.04

6	low	0.17	-0.28	-0.44
6	med	-0.30	2.66	2.96
6	med	0.30	2.66	2.36
6	high	-0.35	4.69	5.04
6	high	-0.35	4.69	5.04
7	low	0.34	-0.41	-0.74
7	low	0.34	-0.41	-0.74
7	med	-0.40	2.57	2.96
7	med	-0.40	2.57	2.96
7	high	0.03	4.69	4.66
7	high	-0.65	4.69	5.34
8	low	-0.12	-0.57	-0.44
8	low	-0.44	-0.57	-0.13
8	med	-0.02	2.60	2.62
8	med	-0.02	2.60	2.62
8	high	0.03	4.69	4.66
8	high	0.03	4.69	4.66
9	low	0.12	-0.33	-0.44
9	low	-0.20	-0.33	-0.13
9	med	-0.12	2.52	2.62
9	med	-0.45	2.52	2.96
9	high	-0.35	4.69	5.04
9	high	-0.35	4.69	5.04
10	low	0.03	-0.48	-0.51
10	low	0.03	-0.48	-0.51
10	med	-0.29	2.67	2.96

10	med	-0.96	2.67	3.63
10	high	0.03	4.69	4.66
10	high	0.03	4.69	4.66

Recovery

Purpose/Method

The amount of analyte recovered should be consistent both between different samples and also when detecting varying concentrations of measurand. The recovery of the method tests the consistency of the analyte recovered via MPNs as compared to the CFUs found on plates. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and CFU data set was converted to logs. Each duplicated MPN was averaged and then compared to its associate CFU, in logs. A single-factor ANOVA was then used to compare the recovery at the three concentrations.

Results

The difference between CFUs and MPNs can be found below in Table 4. The recovery across all samples and concentrations was found to be 109.71%. There was not found to be significant differences in the recovery at the various concentrations (p=0.56).

Table 4: Data for the determination of Recovery

Sample	Concentration	Ave log(CFUs) per Conc.	Ave log(MPN) per Conc.
1	low	-1.17	-0.24
	med	1.96	3.16
	high	4.69	4.95
2	low	-0.51	-0.28
	med	2.45	2.62
	high	4.69	4.85
3	low	-0.92	-0.75
	med	2.52	2.79
	high	4.69	5.04
4	low	-0.21	0.12
	med	2.49	2.62
	high	4.69	4.85
5	low	-0.15	-0.33
	med	2.49	2.36
	high	4.69	4.85
6	low	-0.28	-0.24
	med	2.66	2.66
	high	4.69	5.04

7	low	-0.41	-0.75
	med	2.57	2.96
	high	4.69	5.00
8	low	-0.57	-0.29
	med	2.6	2.62
	high	4.69	4.66
9	low	-0.33	-0.29
	med	2.52	2.79
	high	4.69	5.04
10	low	-0.48	-0.51
	med	2.67	3.3
	high	4.69	4.66

4. Specificity

Purpose/Method

The method should only detect the analyte of interest, even in the presence of interfering organisms. Specificity refers to the ability of the method to measure only the target organism. One matrix sample was divided into three aliquots. One aliquot was spiked with a low but determinable level of *Vibrio parahaemolyticus* (*Vp*). The other two aliquots were spiked with the same level of *Vp* as the first, but were also spiked with a high level of potential interfering organisms. One aliquot received a high level of *Vibrio vulnificus (Vv)* and the other received *Vibrio alginolyticus* (*Va*). Five replicates were performed. Each of the replicates was analyzed by taking the average log MPN and calculating the Specificity Index (SI). A paired t-test was used to determine if the average specificity index obtained from the five replicates differed from 1 (significance level = 0.05).

Results

Using the data from Table 5, the average specificity index was 0.88 when in the presence of *Vv* and 0.98 in the presence of *Va*. These values are not significantly different than 1 *(p*=0.12 for *Vv, p*=0.69 for *Va*).

Table 5. Data for Determination of Specificity

Replicate	***Vp* only, log(MPN/g)**	***Vp* + *Vv*, log(MPN/g)**	***Vp* + *Va*, log(MPN/g)**
1	1.96	2.36	2.36
2	1.62	1.58	2.36
3	1.62	1.96	1.96
4	2.36	2.36	1.96
5	2.17	2.96	1.62

5. Linear Range, Limit of Detection & Limit of Quantification/Sensitivity

Linear Range

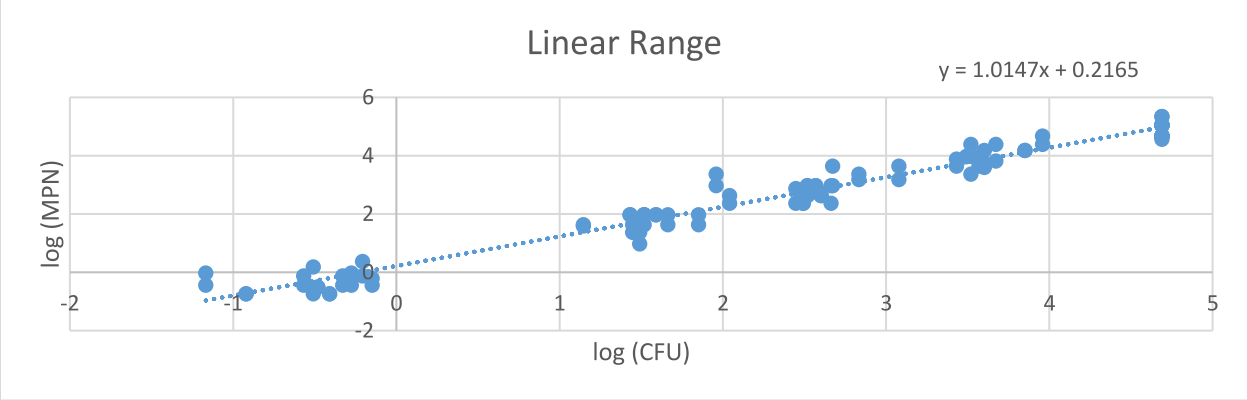
Purpose/Method

The MPN value found should directly correlate to the concentration of analyte within the sample, within the working range of the method. Thus, as the concentration increases, the MPN value should also increase in a linear fashion. Ten Oyster samples were tested at 5 concentration levels, in duplicate. Each MPN was compared to its associate CFU, found by plate count. The relationship between the log(MPN) and log(CFU) was then found by obtaining the correlation coefficient by performing a linear regression with log(CFU) as the independent variable and log(MPN) as the dependent variable.

Results

The relationship between the MPNs and CFUs can be seen in Figure 1 below. The relationship between MPNs and CFUs was found to be linear, with a Pearson's *r* of 0.99. The working range used was of concentrations ranging from 10^{-1} to 10^{4} cells/gram.

Figure 1: Graph representing the relationship between the log values of CFUs and MPNS



Limit of Detection

Purpose/Method

The method should be capable of detecting as little as 1 cell/gram of sample, or 0 cells/gram, in log form. Therefore, it must be determined whether the method can detect one cell per gram of sample. The log(MPN) was compared to the log(CFU) of ten oyster samples, spiked at five varying concentrations, in duplicate. This was done by performing a regression analysis on the data and calculating the Limit of Detection by taking the antilog of the intercept. The independent variable was set as log(CFU) and the dependent variable was set as log(MPN).

Results

The Limit of Detection was found to be 1.65 cells. The overall regression standard error, the

95.0% confidence interval was found to be 0.67, which encompasses the Limit of Detection. The 99.99% confidence interval of the intercept was found to be 0.23. These both contain the intercept of 0.22 within the interval.

Table 7: Data for determination of the Limit of Detection

Sample	Concentration	Log(CFU)	Log(MPN)
Sample 1, Rep 1	**10^-1**	-1.17	-0.45
	10^1	2.05	2.63
	10^2	2.96	3.36
	10^3	3.96	4.66
	10^4	4.69	4.66
Sample 1, Rep 2	**10^-1**	-1.17	-0.04
	10^1	2.05	2.36
	10^2	2.96	2.96
	10^3	3.96	4.38
	10^4	4.69	5.34
Sample 2, Rep 1	**10^-1**	-0.51	0.17
	10^1	1.44	1.36
	10^2	2.44	2.36
	10^3	2.83	3.17
	10^4	4.69	5.04

Sample 2, Rep 2	**10^-1**	-0.51	-0.75
	10^1	1.44	1.63
	10^2	2.44	2.87
	10^3	2.83	3.36
	10^4	4.69	4.66
Sample 3, Rep 1	**10^-1**	-0.93	-0.75
	10^1	1.13	1.58
	10^2	2.51	2.96
	10^3	3.51	3.36
	10^4	4.69	5.04
Sample 3, Rep 2	**10^-1**	-0.93	-0.75
	10^1	1.13	1.63
	10^2	2.51	2.63
	10^3	3.51	4.38
	10^4	4.69	5.04
Sample 4, Rep 1	**10^-1**	-0.21	-0.13
	10^1	1.48	0.96
	10^2	2.48	2.63

	10^3	3.07	3.63
	10^4	4.69	5.04
Sample 4, Rep 2	**10^-1**	-0.21	0.36
	10^1	1.48	1.36
	10^2	2.48	2.63
	10^3	3.07	3.17
	10^4	4.69	4.66
Sample 5, Rep 1	**10^-1**	-0.15	-0.21
	10^1	1.85	1.96
	10^2	2.49	2.36
	10^3	3.49	3.97
	10^4	4.69	5.04
Sample 5, Rep 2	**10^-1**	-0.15	-0.45
	10^1	1.85	1.63
	10^2	2.49	2.36
	10^3	3.49	3.97
	10^4	4.69	4.66
Sample 6, Rep 1	**10^-1**	-0.28	-0.04

	10^1	1.66	1.63
	10^2	2.66	2.96
	10^3	3.85	4.17
	10^4	4.69	5.04
Sample 6, Rep 2	**10^-1**	-0.28	-0.45
	10^1	1.66	1.96
	10^2	2.66	2.36
	10^3	3.85	4.17
	10^4	4.69	5.04
Sample 7, Rep 1	**10^-1**	-0.41	-0.75
	10^1	1.59	1.96
	10^2	2.57	2.96
	10^3	3.57	3.97
	10^4	4.69	4.66
Sample 7, Rep 2	**10^-1**	-0.41	-0.75
	10^1	1.59	1.96
	10^2	2.57	2.96
	10^3	3.57	3.63

	10^4	4.69	5.34
Sample 8, Rep 1	**10^-1**	-0.57	-0.45
	10^1	1.43	1.96
	10^2	2.60	2.63
	10^3	3.60	3.59
	10^4	4.69	4.66
Sample 8, Rep 2	**10^-1**	-0.57	-0.13
	10^1	1.43	1.96
	10^2	2.60	2.63
	10^3	3.60	4.17
	10^4	4.69	4.66
Sample 9, Rep 1	**10^-1**	-0.33	-0.45
	10^1	1.51	1.96
	10^2	2.51	2.63
	10^3	3.43	3.63
	10^4	4.69	5.04
Sample 9, Rep 2	**10^-1**	-0.33	-0.13
	10^1	1.51	1.63

	10^2	2.51	2.96
	10^3	3.43	3.87
	10^4	4.69	5.04
Sample 10, Rep 1	**10^-1**	-0.49	-0.52
	10^1	1.51	1.96
	10^2	2.67	2.96
	10^3	3.67	4.38
	10^4	4.69	4.66
Sample 10, Rep 2	**10^-1**	-0.49	-0.52
	10^1	1.51	1.96
	10^2	2.67	3.63
	10^3	3.67	3.80
	10^4	4.69	4.66

Limit of Quantification/Sensitivity

Purpose/Method

The quantifiable limit of the method is bounded by the values defined by a 3-tube MPN. In the case that the Limit of Detection is not significantly different than 1 cell/gram, than the Limit of Quantification can be extrapolated using the FDA BAM MPN Calculator.

Results

As the method starts with a low dilution of 1 gram of sample per tube, use of a 3-tube MPN and corresponding dilution ratios will result in the Limit of Quantification/Sensitivity for the method being 0.36 MPN/gram.

Inclusivity

Purpose

To assess the ability of the method to detect a wide range of target strains in various oyster tissues.

Method

Vibrio parahaemolyticus (*Vp*) strains were grown in APW for 18-24 hours. DNA was extracted using the MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. The strains that make up the inclusivity panel were obtained from the Center for Disease Control and Prevention (CDC), National Oceanic and Atmospheric Administration (NOAA), American Type Culture Collection (ATCC), or Washington State Public Health Laboratory (WAPHL). The primers and probes utilized in this method have been tested against DNA extracts from the isolates listed in Table 1 below.

Confirmation was performed through a secondary assay. Since *tlh* is a species specific marker, a positive *Vp* identification through biochemicals or by ATCC paperwork was used as confirmation. For *tdh* some isolates were confirmed by NOAA and others were confirmed using *tdh* primers from Nordstrom et al. 2007. The *trh* marker was more challenging due to 2 variations of the *trh* gene. All isolates were confirmed using the FDA BAM *trh* primer set and anything with discrepant results between our assay and the FDA assay we confirmed with a biochemical urease test. The ability of *Vp* to hydrolyze urea has been shown to be indicative of the presence of the *trh* gene (Lida et al paper 1997). The *ORF8* pandemic marker was confirmed using the primer set from Myers et al. 2003.

Results

Primer / Probe Sensitivity
Sensitivity= (# of true positives/ (# of true positives + # of false negatives))
tlh sensitivity = 73/73 = **100% *tlh* sensitivity**
tdh sensitivity =33/33 = **100% *tdh* sensitivity**
trh sensitivity = 33/33 = **100% *trh* sensitivity**
ORF8 sensitivity = 24/24 = **100% *ORF8* sensitivity**

The primers and probes utilized in this method for their respective target demonstrates 100% inclusivity. See Table 1 (Inclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

Table 1. Inclusivity panel with results by target. Targets not tested or confirmed are blocked out.

Strain	Source	tlh	tdh	trh	orf8	vvha
F5828	CDC	+	+		+	
F5835	CDC	+	+		+	
F5847	CDC	+	+		+	
F6820	CDC	+	+		+	
F7630	CDC	+	+		+	
F7635	CDC	+	+		+	
F7636	CDC	+	+		+	
F7680	CDC	+	+		+	
F8701	CDC	+	+		+	
F8949	CDC	+	+		+	
F9083	CDC	+	+		+	
K0071	CDC	+	+		+	
K0456	CDC	+	-	+	-	
17803	ATCC	+	-	+		
27519	ATCC	+	-			
27969	ATCC	+				
33844	ATCC	+	+			
33845	ATCC	+	+			
33846	ATCC	+	+			
33847	ATCC	+	+			
35117	ATCC	+	-			
35118	ATCC	+	+			
43996	ATCC	+	+			
49398	ATCC	+	-			
BAA-238	ATCC	+	+		+	
BAA-239	ATCC	+	+		+	
BAA-240	ATCC	+	+		+	
BAA-241	ATCC	+	+		+	
BAA-242	ATCC	+	+		+	
NWF 261	NOAA - NWFSC	+	-	-	-	
NWF 512	NOAA - NWFSC	+	-	-	-	
NWF 586	NOAA - NWFSC	+	+	-	+	
NWF 605	NOAA - NWFSC	+	+	-	+	
NWF 609	NOAA - NWFSC	+	+	-	+	
NWF 735	NOAA - NWFSC	+	+		+	
NWF 782	NOAA - NWFSC	+	+	-	+	

NWF 797	NOAA - NWFSC	+	-	-		
NWF 800	NOAA - NWFSC	+	-			
NWF 805	NOAA - NWFSC	+	-			
NWF 843	NOAA - NWFSC	+	-			
NWF 846	NOAA - NWFSC	+	+	+		
NWF 864	NOAA - NWFSC	+	+		+	
NWF 930	NOAA - NWFSC	+	+	-	+	
5412	WA PHL	+		+		
5419	WA PHL	+		+		
5423	WA PHL	+	+	+		
5424	WA PHL	+		+		
5425	WA PHL	+	+	+		
5426	WA PHL	+		+		
5429	WA PHL	+		+		
5430	WA PHL	+		+		
5434	WA PHL	+		+		
5436	WA PHL	+		+		
5437	WA PHL	+		+		
5442	WA PHL	+		+		
5444	WA PHL	+		+		
5454	WA PHL	+		+		
5456	WA PHL	+		+		
5463	WA PHL	+		+		
5468	WA PHL	+		+		
5469	WA PHL	+		+		
5470	WA PHL	+		+		
5471	WA PHL	+		+		
5473	WA PHL	+		+		
5474	WA PHL	+		+		
5475	WA PHL	+		+		
5487	WA PHL	+		+		
5488	WA PHL	+		+		
5492	WA PHL	+		+		
5501	WA PHL	+		+		
5508	WA PHL	+		+		
5518	WA PHL	+		+		
5519	WA PHL	+		+		
06-2410	06-2410 (CDC)					+

06-2450	06-2450 (CDC)					+
07-2405	07-2405 (CDC)					+
08-2468	08-2468 (CDC)					+
08-2470	08-2470 (CDC)					+
08-2472	08-2472 (CDC)					+
08-2485	08-2485 (CDC)					+
1831-81	1831-81 (CDC)					+
2009V-1002	2009V-1002 (CDC)					+
2009V-1055	2009V-1055 (CDC)					+
2010V-1021	2010V-1021 (CDC)					+
209V-1035	209V-1035 (CDC)					+
2431-04	2431-04 (CDC)					+
2473-85	2473-85 (CDC)					+
2492-88	2492-88 (CDC)					+
2809-78	2809-78 (CDC)					+
430-79	430-79 (CDC)					+
AM38622	AM38622 (CDC)					+
AM38623	AM38623 (CDC)					+
27562	27562					+
29307	29307					+
Total Confirmed Isolated		73	33	33	24	21

Exclusivity

Purpose

To demonstrate the ability of the method to distinguish the targeted analyte from other potentially cross-reactive non-target strains that could possibly contaminate shellfish.

Method

All organisms were inoculated into APW and incubated for 18-24 hours. DNA was extracted using the MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. All strains were obtained from the Center for Disease Control and Prevention (CDC) and American Type Culture Collection (ATCC).

Results

Primer / Probe Specificity

Specificity = (# of true negative/ (# of true negatives + # of false positives))

tlh specificity= 49/49 = **100% *tlh* Specificity**
tdh specificity = 49/49 = **100% *tdh* Specificity**
trh specificity = 49/50 = **98% *trh* Specificity**
ORF8 specificity = 49/49 = **100% *ORF8* Specificity**

The tlh, tdh, and ORF8 primers and probes utilized in this method demonstrate 100% exclusivity. The trh primers and probe demonstrate a 98% specificity (see Known Limitations below). See Table 2 (Exclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

Known limitations and interferences

Vibrio alginolyticus possesses a *trh* gene with 98% homology to the *trh* gene in *Vibrio parahaemolyticus*. Most probable number (MPN) values for *trh* should be reported only if *tlh (V. parahaemolyticus* specific gene) is present in the corresponding tube. Tubes only positive for *trh* should not be accounted for when generating the MPN value. This assay utilizes the Taqman Environmental Mastermix 2.0, which is specifically formulated to detect bacterial pathogens with greater specificity and sensitivity. There are no additional known limitations when using the Taqman Environmental Mastermix 2.0.

Table 2. Exclusivity panel with results by target. Targets not tested or confirmed are blocked out.

Organism	Strain	*tlh*	*tdh*	*trh*	*orf8*	*vvha*
E. coli	ATCC 25922	-	-	-	-	-
G. hollisae	ATCC 33564	-	-	-	-	-
K. pneumoniae	ATCC 33495	-	-	-	-	-
P. aeruginosa	ATCC 33495	-	-	-	-	-
S. aureus	ATCC 10145	-	-	-	-	-
S. sonnei	ATCC 25925	-	-	-	-	-
S. typhimurium	ATCC 9290	-	-	-	-	-
V. aestuarians	ATCC 35048	-	-	-	-	-
V. alginolyticus	ATCC 17749	-	-	-	-	-
V. alginolyticus	S14-048 (Environmental- WA PHL)	-	-	+	-	-
V. algosus	ATCC 14390	-	-	-	-	-
V. campbellii	ATCC 25920	-	-	-	-	-
V. cholerae	ATCC 39050	-	-	-	-	-
V. cinncinatiensis	ATCC 35912	-	-	-	-	-
V. furnissii	ATCC 33813	-	-	-	-	-
V. marinagilis	ATCC 14398	-	-	-	-	-

V. marinofulvus	ATCC 14395	-	-	-	-	-
V. marinovulgaris	ATCC 14394	-	-	-	-	-
V. metschnikovii	ATCC 700040	-	-	-	-	-
V. mimicus	ATCC 33653	-	-	-	-	-
V. natriegens	ATCC 14048	-	-	-	-	-
V. nereis	ATCC 25917	-	-	-	-	-
V. nigripulchritudo	ATCC 27043	-	-	-	-	-
V. ponticus	ATCC 14391	-	-	-	-	-
V. proteolyticus	ATCC 15338	-	-	-	-	-
V. splendidus	ATCC 33789	-	-	-	-	-
V. tubiashii	ATCC 19106	-	-	-	-	-
V. vulnificus	06-2410 (CDC)	-	-	-	-	
V. vulnificus	06-2450 (CDC)	-	-	-	-	
V. vulnificus	07-2405 (CDC)	-	-	-	-	
V. vulnificus	08-2468 (CDC)	-	-	-	-	
V. vulnificus	08-2470 (CDC)	-	-	-	-	
V. vulnificus	08-2472 (CDC)	-	-	-	-	
V. vulnificus	08-2485 (CDC)	-	-	-	-	
V. vulnificus	1831-81 (CDC)	-	-	-	-	
V. vulnificus	2009V-1002 (CDC)	-	-	-	-	
V. vulnificus	2009V-1055 (CDC)	-	-	-	-	
V. vulnificus	2010V-1021 (CDC)	-	-	-	-	
V. vulnificus	209V-1035 (CDC)	-	-	-	-	
V. vulnificus	2431-04 (CDC)	-	-	-	-	
V. vulnificus	2473-85 (CDC)	-	-	-	-	
V. vulnificus	2492-88 (CDC)	-	-	-	-	
V. vulnificus	2809-78 (CDC)	-	-	-	-	
V. vulnificus	430-79 (CDC)	-	-	-	-	
V. vulnificus	AM38622 (CDC)	-	-	-	-	
V. vulnificus	AM38623 (CDC)	-	-	-	-	
V. vulnificus	27562	-	-	-	-	
V. vulnificus	29307	-	-	-	-	
A. trota	2013V-1197 (CDC)	-	-	-	-	-
A. veronii	N/A (CDC)	-	-	-	-	-
Total Strains	49					

Validation Data for MPN Real-time PCR for *Vibrio vulnificus*

Name of Method Submitter: Gina Olson, Washington State Department of Health

Specific purpose or intent of the method for use in the NSSP:

Requesting adoption of this method as an approved method for Vibrio enumeration, both *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in oysters. The method may be used in the following applications: PHP validation and verification of product and in management of growing areas through environmental testing and surveillance in order to re-open closed growing areas. This method once approved would provide a high-throughput alternative to the current approved MPN real-time PCR method. In addition, this method would be the only approved MPN real-time PCR method to test for total Vp, pathogenic Vp, and Vv in a single assay.

Validation Criteria Data:

All oyster samples used in this validation were collected from different harvest locations and/or different harvest dates in Washington State. All samples were processed using APHA guidelines for the cleaning and shucking of shellstock. Samples were confirmed negative for the target organism of Vv through the FDA BAM culture-based method and through pcr prior to spiking. Spiking levels were determined using a 5-tube MPN dilution series in APW in duplicate (averaging the 2 values).

Vv strain ATCC 29307 was used to spike all samples for all validation criteria. This strain is positive for *vvhA*.

The validation data for Vp and Vv is presented separately for clarity and ease in reviewing the data, but this is a single assay and all elements were present during the validation of all organisms.

<u>Assay Design</u>

DNA Isolation: Roche MagnaPure 96 using Roche DNA/Viral Nucleic Acid Small Volume Kit

Real-time PCR Instrument: Applied Biosystems QuantStudio Dx (384-well format)

Mastermix: Life Technologies TaqMan Environmental Master Mix 2.0

Real-Time PCR targets: 2 multiplex reactions

- Multiplex 1: Total Vp (*tlh)*, Vv (*vvhA)*, internal control (IC)
- Multiplex 2 (Vp pathogenicity markers): *tdh*, *trh*, *orf8*

Real-Time PCR parameters:

Denaturation: 95°C for 10 mins
Annealing: 95°C for 15 secs
Extension: 59°C for 1 min
Cycles: 40

1. Accuracy/Trueness & Measurement Uncertainty

Accuracy/Trueness

Purpose/Method

Accuracy/Trueness measures the closeness of agreement between the test results (MPNs) and the reference results (spiked MPNs without matrix). This was done by analyzing twenty oyster samples over a range of concentrations (low to high) to determine the MPN. The MPN and reference data set was converted into logs. The average MPN in logs was divided by the average reference value in logs. This provides an estimate in percent of the accuracy/trueness of the method.

Results

The average of the reference values was 2.20 log. The average of MPNs was 2.15 log. Accuracy/Trueness was found to be 97.69%. Results can be found below in Table 1.

Measurement Uncertainty

Purpose/Method

Measurement uncertainty expresses the range of values around the measured result within which the true value is expected to lie. To determine this parameter, twenty oyster samples spiked with a range of concentrations were analyzed. Each MPN and reference value was converted into logs and the MPN result was subtracted from the reference result for each sample. A 95% confidence interval was calculated from the difference. This confidence interval represents the measurement uncertainty of the methods.

Results

The measurement uncertainty was determined via 95% CI (0.16, 0.30), resulting in a measurement uncertainty of 0.14. Results can be found below in Table 1.

Table 1. Data for determination of Accuracy/Trueness and Measurement Uncertainty

Sample	Reference MPN, log(MPN/g)	MPN, log(MPN/g)
1	0.11	-0.44
2	0.11	-0.04
3	0.11	0.36
4	2.11	1.96
5	2.11	2.36
6	2.20	1.87

7	2.20	2.36
8	4.08	3.97
9	4.08	4.38
10	4.08	3.88
11	0.23	-0.04
12	1.18	1.18
13	2.30	1.96
14	3.15	3.36
15	4.30	4.38
16	0.49	0.36
17	1.32	0.96
18	2.08	2.62
19	3.45	3.36
20	4.30	4.18

2. Ruggedness

Purpose/Method

The amount of analyte recovered should be consistent between different lots of media/reagents. Ruggedness tests the impact of different lots used to process samples on the final result. This was done by testing ten oyster samples spiked at a range of concentrations in duplicate. One replicate was performed using “Lot 1” media/reagents and one replicate was performed using “Lot 2.” To determine if the method was sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between Lot 1 and Lot 2 samples.

Results

Using data from Table 2, there was no significant difference (p=0.37) between different lots of media and reagents.

Table 2. Data for Determination of Ruggedness

Sample	Replicate 1, log(MPN/g)	Replicate 2, log(MPN/g)
1	-0.44	0.17
2	-0.04	0.36
3	0.36	0.36
4	1.96	1.96
5	2.36	1.87
6	1.87	1.96
7	2.36	2.36
8	3.97	3.88
9	4.38	4.66
10	3.88	3.97

3. Precision & Recovery

Precision

Purpose/Method

The difference between the methods results (MPNs) and the reference values should be consistent both between different samples and also when detecting varying concentrations of measurand. The precision of the method tests the consistency of the difference between the reference values and the MPN values found in spiked matrix. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and reference data sets were converted into log values. Each MPN was compared to its associate reference value. A nested ANOVA was then performed, with variance components being the sample, and concentrations within the samples (low, medium, and high), and then the error. The ANOVA component of interest was that comparing the concentrations within the samples to the determinations (or error).

Results

The difference between reference values and MPNs can be found below in Table 3. The data shows that there are no significant differences between the concentrations in samples and the determinations within concentrations via a nested ANOVA (p=0.69). Additionally, the variance of the method does not exceed the known variance of a 3-tube MPN (p=0.16).

Table 3: Data for determining the Precision

Sample	Concentration	Difference	log(reference MPNs)	log(MPNs)
1	low	0.27	0.23	-0.04
1	low	-0.40	0.23	0.63
1	med	-0.55	3.41	3.97
1	med	0.45	3.41	2.96
1	high	-0.24	4.41	4.66
1	high	-0.24	4.41	4.66
2	low	-0.19	0.18	0.36
2	low	0.21	0.18	-0.04
2	med	0.06	3.23	3.17
2	med	-0.13	3.23	3.36
2	high	0.26	4.23	3.97
2	high	0.06	4.23	4.17
3	low	0.27	0.13	-0.14
3	low	0.17	0.13	-0.04
3	med	0.21	3.18	2.96
3	med	-0.28	3.18	3.46
3	high	-0.20	4.18	4.38
3	high	0.21	4.18	3.97
4	low	0.23	0.29	0.06
4	low	0.73	0.29	-0.44
4	med	-0.23	3.13	3.36
4	med	0.17	3.13	2.96

4	high	-0.20	4.13	4.33
4	high	-0.53	4.13	4.66
5	low	-0.13	0.18	0.31
5	low	-0.45	0.18	0.63
5	med	-0.07	3.29	3.36
5	med	-0.07	3.29	3.36
5	high	-0.09	4.29	4.38
5	high	0.32	4.29	3.97
6	low	0.13	0.49	0.36
6	low	0.53	0.49	-0.04
6	med	-0.14	3.49	3.63
6	med	0.32	3.49	3.17
6	high	0.00	4.18	4.17
6	high	0.00	4.18	4.17
7	low	-0.05	0.31	0.36
7	low	0.35	0.31	-0.04
7	med	-0.05	3.31	3.36
7	med	0.35	3.31	2.96
7	high	0.34	4.31	3.97
7	high	0.34	4.31	3.97
8	low	-0.28	0.08	0.36
8	low	0.22	0.08	-0.14
8	med	0.12	3.08	2.96
8	med	-0.28	3.08	3.36
8	high	-0.25	4.08	4.33

8	high	-0.09	4.08	4.17
9	low	0.27	0.44	0.17
9	low	0.12	0.44	0.32
9	med	0.08	3.44	3.36
9	med	0.27	3.44	3.17
9	high	0.06	4.44	4.38
9	high	0.27	4.44	4.17
10	low	0.13	0.30	0.17
10	low	0.34	0.30	-0.04
10	med	-0.06	3.30	3.36
10	med	0.67	3.30	2.63
10	high	0.13	4.30	4.17
10	high	0.33	4.30	3.97

Recovery

Purpose/Method

The amount of analyte recovered should be consistent both between different samples and also when detecting varying concentrations of measurand. The recovery of the method tests the consistency of the analyte recovered via MPNs as compared to the reference values. This was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. Each MPN and reference value was converted to logs. Each duplicated MPN was averaged and then compared to its associate reference value, in logs. A single-factor ANOVA was then used to compare the recovery at the three concentrations.

Results

The difference between reference values and MPNs can be found below in Table 4. The recovery across all samples and concentrations was found to be 97.44%. There was not found to be significant differences in the recovery at the various concentrations (p=0.49).

Table 4: Data for the determination of Recovery

Sample	Concentration	Avg log(Reference) per Conc.	Avg log(MPN) per Conc.
1	low	0.23	0.30
	med	3.41	3.47
	high	4.41	4.66
2	low	0.18	0.16
	med	3.23	3.27
	high	4.23	4.07
3	low	0.13	-0.09
	med	3.18	3.21
	high	4.18	4.17
4	low	0.29	-0.19
	med	3.13	3.16
	high	4.13	4.49
5	low	0.18	0.47
	med	3.29	3.36
	high	4.29	4.17
6	low	0.49	0.16
	med	3.49	3.40
	high	4.18	4.17
7	low	0.31	0.16
	med	3.31	3.16
	high	4.31	3.97
8	low	0.08	0.11
	med	3.08	3.16
	high	4.08	4.25
9	low	0.44	0.24
	med	3.44	3.27
	high	4.44	4.28
10	low	0.30	0.07
	med	3.30	3.00
	high	4.30	4.07

4. Specificity

Purpose/Method

The method should only detect the analyte of interest, even in the presence of interfering

organisms. Specificity refers to the ability of the method to measure only the target organism. One matrix sample was divided into two aliquots. One aliquot was spiked with a low but determinable level of *Vibrio vulnificus* (*Vv*). The other aliquot was spiked with the same level of *Vv* as the first, but also spiked with a high level of potential interfering *Vibrio parahaemolyticus (Vp)*. Five replicates were performed. Each of the replicates was analyzed by taking the average log MPN and calculating the Specificity Index (SI). A paired *t*-test was used to determine if the average specificity index obtained from the five replicates differed from 1 (significance level = 0.05).

Results
Using the data from Table 5, the average specificity index was 0.99 when in the presence of *Vp.* These values are not significantly different than 1 (p=0.74).

Table 5. Data for Determination of Specificity

Replicate	*Vv* only, log(MPN/g)	*Vp* + *Vv*, log(MPN/g)
1	1.63	1.96
2	2.32	2.17
3	1.96	1.63
4	1.96	2.36
5	2.17	2.17

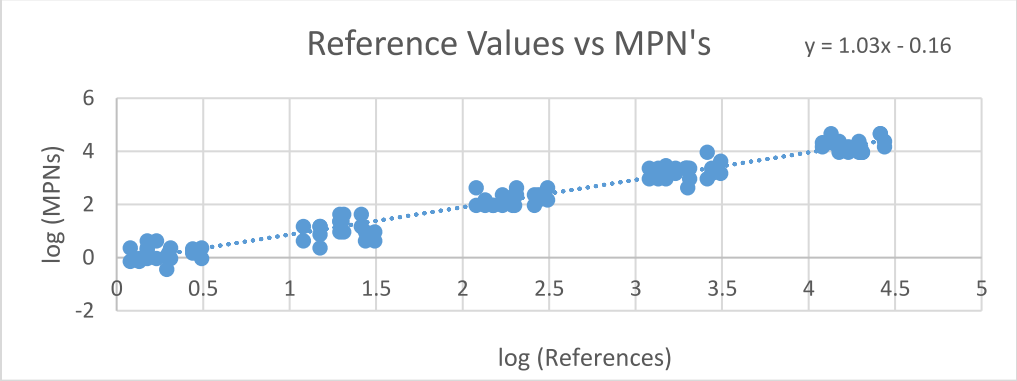
5. Linear Range, Limit of Detection & Limit of Quantification/Sensitivity

Linear Range

Purpose/Method
The MPN value found should directly correlate to the concentration of analyte within the sample, within the working range of the method. Thus, as the concentration increases, the MPN value should also increase in a linear fashion. Ten Oyster samples were tested at 5 concentration levels, in duplicate. Each MPN was compared to its associate reference value. The relationship between the log(MPN) and log(reference) was then found by obtaining the correlation coefficient by performing a linear regression with log(reference) as the independent variable and log(MPN) as the dependent variable.

Results
The relationship between the MPNs and reference values can be seen in Figure 1 below. The relationship between MPNs and reference values was found to be linear, with a Pearson's *r* of 0.98. The working range used was of concentrations ranging from 10^0 to 10^4 cells/gram.

Figure 1: Graph representing the relationship between the log of reference values and MPNS



Limit of Detection

Purpose/Method

The method should be capable of detecting as little as 1 cell/gram of sample, or 0 cells/gram, in log form. Therefore, it must be determined whether the method can detect one cell per gram of sample. The log(MPN) was compared to the log(reference) of ten oyster samples, spiked at five varying concentrations, in duplicate. This was done by performing a regression analysis on the data and calculating the Limit of Detection by taking the antilog of the intercept. The independent variable was set as log(reference) and the dependent variable was set as log(MPN).

Results

The Limit of Detection was found to be 0.68 cells. The overall regression 95.0% confidence interval was found to be 0.58, which encompasses the Limit of Detection. The 99.99% confidence interval of the intercept was found to be 0.23. These both contain the intercept value of -0.16 within the interval.

Table 7: Data for determination of the Limit of Detection

Sample	Concentration	Log(reference)	Log(MPN)
Sample 1, Rep 1	**10^0**	0.23	-0.04
	10^1	1.41	1.17
	10^2	2.41	1.96

	10^3	3.41	3.97
	10^4	4.41	4.66
Sample 1, Rep 2	**10^0**	0.23	0.63
	10^1	1.41	1.63
	10^2	2.41	2.36
	10^3	3.41	2.96
	10^4	4.41	4.66
Sample 2, Rep 1	**10^0**	0.18	0.36
	10^1	1.18	1.17
	10^2	2.23	1.96
	10^3	3.23	3.17
	10^4	4.23	3.97
Sample 2, Rep 2	**10^0**	0.18	-0.04
	10^1	1.18	0.87
	10^2	2.23	2.36
	10^3	3.23	3.36
	10^4	4.23	4.17

Sample 3, Rep 1	**10^0**	0.13	-0.14
	10^1	1.29	0.96
	10^2	2.29	1.96
	10^3	3.18	2.96
	10^4	4.18	4.38
Sample 3, Rep 2	**10^0**	0.13	-0.04
	10^1	1.29	1.36
	10^2	2.29	2.17
	10^3	3.18	3.46
	10^4	4.18	3.97
Sample 4, Rep 1	**10^0**	0.29	0.06
	10^1	1.29	1.36
	10^2	2.13	2.17
	10^3	3.13	3.36
	10^4	4.13	4.33
Sample 4, Rep 2	**10^0**	0.29	-0.44
	10^1	1.29	1.63

	10^2	2.13	1.96
	10^3	3.13	2.96
	10^4	4.13	4.66
Sample 5, Rep 1	**10^0**	0.18	0.31
	10^1	1.18	0.36
	10^2	2.18	1.96
	10^3	3.29	3.36
	10^4	4.29	4.38
Sample 5, Rep 2	**10^0**	0.18	0.63
	10^1	1.18	1.17
	10^2	2.18	1.96
	10^3	3.29	3.36
	10^4	4.29	3.97
Sample 6, Rep 1	**10^0**	0.49	0.36
	10^0	1.49	0.63
	10^2	2.49	2.63
	10^3	3.49	3.63

	10^4	4.18	4.17
Sample 6, Rep 2	**10^0**	0.49	-0.04
	10^1	1.49	0.96
	10^2	2.49	2.17
	10^3	3.49	3.17
	10^4	4.18	4.17
Sample 7, Rep 1	**10^0**	0.31	0.36
	10^1	1.31	0.96
	10^2	2.31	2.36
	10^3	3.31	3.36
	10^4	4.31	3.97
Sample 7, Rep 2	**10^0**	0.31	-0.04
	10^1	1.31	1.63
	10^2	2.31	2.63
	10^3	3.31	2.96
	10^4	4.31	3.97
Sample 8, Rep 1	**10^0**	0.08	0.36

	10^1	1.08	1.17
	10^2	2.08	2.63
	10^3	3.08	2.96
	10^4	4.08	4.33
Sample 8, Rep 2	**10^0**	0.08	-0.14
	10^1	1.08	0.63
	10^2	2.08	1.96
	10^3	3.08	3.36
	10^4	4.08	4.17
Sample 9, Rep 1	**10^0**	0.44	0.17
	10^1	1.44	0.96
	10^2	2.44	2.17
	10^3	3.44	3.36
	10^4	4.44	4.38
Sample 9, Rep 2	**10^0**	0.44	0.32
	10^1	1.44	0.63
	10^2	2.44	2.36

	10^3	3.44	3.17
	10^4	4.44	4.17
Sample 10, Rep 1	**10^0**	0.30	0.17
	10^1	1.30	1.36
	10^2	2.30	1.96
	10^3	3.30	3.36
	10^4	4.30	4.17
Sample 10, Rep 2	**10^0**	0.30	-0.04
	10^1	1.30	1.36
	10^2	2.30	2.36
	10^3	3.30	2.63
	10^4	4.30	3.97

Limit of Quantification/Sensitivity

Purpose/Method

The quantifiable limit of the method is bounded by the values defined by a 3-tube MPN. In the case that the Limit of Detection is not significantly different than 1 cell/gram, than the Limit of Quantification can be extrapolated using the FDA BAM MPN Calculator.

Results

As the method starts with a low dilution of 1 gram of sample per tube, use of a 3-tube MPN and corresponding dilution ratios will result in the Limit of Quantification/Sensitivity for the method being 0.36 MPN/gram.

Inclusivity

Purpose

To assess the ability of the method to detect a wide range of target strains in various oyster tissues.

Method

Vibrio vulnificus (*Vv*) strains were grown in APW for 18-24 hours. DNA was extracted using the MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. The strains that make up the inclusivity panel were obtained from the Center for Disease Control and Prevention (CDC). The primers and probes utilized in this method have been tested against DNA extracts from the isolates listed in Table 1 below. The *vvhA* marker was either confirmed by the CDC or ATCC paperwork.

Results

Primer / Probe Sensitivity
Sensitivity= (# of true positives/ (# of true positives + # of false negatives))
vvhA sensitivity = 21/21 = **100% *vvhA* sensitivity**

The primers and probes utilized in this method demonstrates 100% inclusivity. See Table 1 (Inclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

Table 1. Inclusivity panel with results by target. Targets not tested or confirmed are blocked out.

Strain	Source	tlh	tdh	trh	orf8	vvha
06-2410	06-2410 (CDC)					+
06-2450	06-2450 (CDC)					+
07-2405	07-2405 (CDC)					+
08-2468	08-2468 (CDC)					+
08-2470	08-2470 (CDC)					+
08-2472	08-2472 (CDC)					+
08-2485	08-2485 (CDC)					+
1831-81	1831-81 (CDC)					+
2009V-1002	2009V-1002 (CDC)					+
2009V-1055	2009V-1055 (CDC)					+
2010V-1021	2010V-1021 (CDC)					+
209V-1035	209V-1035 (CDC)					+
2431-04	2431-04 (CDC)					+
2473-85	2473-85 (CDC)					+
2492-88	2492-88 (CDC)					+

2809-78	2809-78 (CDC)					+
430-79	430-79 (CDC)					+
AM38622	AM38622 (CDC)					+
AM38623	AM38623 (CDC)					+
27562	27562					+
29307	29307					+
Total Confirmed Isolated		0	0	0	0	21

Exclusivity

Purpose

To demonstrate the ability of the method to distinguish the targeted analyte from other potentially cross-reactive non-target strains that could possibly contaminate shellfish.

Method

All organisms were inoculated into APW and incubated for 18-24 hours. DNA was extracted using the MagNAPure 96 instrument and PCR performed using the QuantStudio Dx Real-Time PCR System. All strains were obtained from the Center for Disease Control and Prevention (CDC) and American Type Culture Collection (ATCC).

Results

Primer / Probe Specificity

Specificity = (# of true negative/ (# of true negatives + # of false positives))

vvhA specificity = 28/28 = **100% *vvhA* Specificity**

The vvhA primers and probes utilized in this method demonstrate 100% exclusivity. None of the exclusivity panel had detection of *vvhA*. See Table 2 (Exclusivity Panel) for the breakdown of strains, source, targets tested, and its corresponding result.

Table 2. Exclusivity panel with results by target. Targets not tested or confirmed are blocked out.

Organism	ATCC #	*tlh*	*tdh*	*trh*	*orf8*	*vvha*
E. coli	25922	-	-	-	-	-
G. hollisae	33564	-	-	-	-	-
K. pneumoniae	33495	-	-	-	-	-
P. aeruginosa	33495	-	-	-	-	-
S. aureus	10145	-	-	-	-	-
S. sonnei	25925	-	-	-	-	-

S. typhimurium	9290	-	-	-	-	-
V. aestuarians	35048	-	-	-	-	-
V. alginolyticus	17749	-	-	-	-	-
V. algosus	14390	-	-	-	-	-
V. campbellii	25920	-	-	-	-	-
V. cholerae	39050	-	-	-	-	-
V. cinncinatiensis	35912	-	-	-	-	-
V. furnissii	33813	-	-	-	-	-
V. marinagilis	14398	-	-	-	-	-
V. marinofulvus	14395	-	-	-	-	-
V. marinovulgaris	14394	-	-	-	-	-
V. metschnikovii	700040	-	-	-	-	-
V. mimicus	33653	-	-	-	-	-
V. natriegens	14048	-	-	-	-	-
V. nereis	25917	-	-	-	-	-
V. nigripulchritudo	27043	-	-	-	-	-
V. ponticus	14391	-	-	-	-	-
V. proteolyticus	15338	-	-	-	-	-
V. spledidus	33789	-	-	-	-	-
V. tubiashii	19106	-	-	-	-	-
A. trota	2013V-1197 (CDC)	-	-	-	-	-
A. veronii	N/A (CDC)	-	-	-	-	-
Total Strains	28					

Supplemental Validation Data for MPN Real-Time PCR for *Vibrio parahaemolyticus*

Name of Method Submitter: Anna Pickett, Washington State Department of Health

Specific purpose or intent of the method for use in the NSSP:

Requesting adoption of this method as an approved method for *Vibrio* enumeration, both *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in oysters. The method may be used in the following applications: PHP validation and verification of product and in management of growing areas and to re-open closed growing areas. This method, once approved, would provide a high-throughput alternative to the current approved MPN real-time PCR method. Additionally, this method would be the only approved MPN real-time PCR method to test for total Vp, pathogenic Vp, and Vv in a single assay.

Supplemental Validation Criteria Data:

All oyster samples used in the supplemental validation were collected from different harvest locations and/or harvest dates in Washington state. All samples were processed using APHA guidelines for the cleaning and shucking of shellstock. Samples were confirmed negative for the target organisms of both Vp and Vv through the FDA BAM culture-based method and through qPCR prior to spiking. Spiking levels were determined by spread plating dilutions onto PCA w/2% NaCl in duplicate and averaging the counts.

Vp strain WA4647 was used to spike all samples for all validation criteria. This strain is positive for tlh, tdh, and trh. All data generated for all three targets was identical and has been presented in a single MPN in all validation criteria tables and data calculations.

The validation data for Vp and Vv is presented separately for clarity and ease in reviewing the data, but this is a single assay and all elements were present during the validation of all organisms.

All supplemental testing performed as a response to specific comments issued by ISSC Committee in 2019.

1. Assay Design

See amended SOP for mastermix formulation appendix.

2. Comparability

Purpose/Method

To assess performance of the method as compared to the established qPCR method in the NSSP, 35 oyster samples from ten different growing areas were collected over multiple weeks. Each oyster sample was assessed both culturally and through PCR to verify the absence of detectable *V. parahaemolyticus* and *V. vulnificus*. Oyster homogenate was then frozen until testing. Testing occurred on 30 *Vp* spiked samples with a range of 10^0 through 10^5, five replicates at each spiking level and 5 unspiked blank samples. After processing the MPNs and enriching overnight, each replicate was extracted via WAPHL method (MagNAPure 96) and through the NSSP boil extraction. Both the MP96 and boil extraction were frozen until PCR could be performed. Boil preps were shipped to FDA Dauphin Island on dry ice. WAPHL performed the proposed real-time PCR method at the same time as the FDA performed the NSSP method. To compare the performance of the

proposed method with the established NSSP method, a two sided t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between the two data sets.

Additionally, to ascertain if freezing matrix had any impact on performance of the assay or the effects of spiking, a subset (six) of fresh samples were spiked at the same levels as the frozen matrix and run alongside testing. A two sided t-test was also performed on the fresh vs. frozen log-transformed data with a significance level (α) of 0.05. There should be no significant difference between the two data sets.

Results

Using results from Table 1. Data for Comparability of *Vp,* there was no significant difference (p=0.96) between the NSSP approved method and the proposed method when testing spiked tissue. Both assays showed no detection of *Vp* in the five blank samples. When assessing fresh vs. frozen matrix using the data from Table 2, there was no significant difference between the matrices (p=0.94).

Table 1. Data for Comparability of *Vp*

Sample	Spike Level	WAPHL Method log(MPN/g)	NSSP Method log(MPN/g)
1	10^5	5.04	5.04
2	10^5	5.04	5.04
3	10^5	5.04	5.04
4	10^5	5.04	5.04
5	10^5	5.04	5.04
6	10^4	5.04	5.04
7	10^4	4.66	4.66
8	10^4	5.04	5.04
9	10^4	4.66	4.66
10	10^4	5.04	5.04
11	10^3	4.66	4.66
12	10^3	3.63	4.17
13	10^3	3.89	3.89
14	10^3	4.38	4.38
15	10^3	4.38	4.38
16	10^2	2.96	2.96
17	10^2	2.62	2.62
18	10^2	2.96	2.96
19	10^2	2.62	2.62
20	10^2	2.62	2.62
21	10^1	1.62	1.62
22	10^1	1.36	1.36
23	10^1	2.18	2.18
24	10^1	2.36	2.36
25	10^1	1.96	1.96
26	10^0	0.62	0.62
27	10^0	0.87	0.87
28	10^0	1.36	1.36
29	10^0	1.62	1.62
30	10^0	0.87	0.87

Table 2. Fresh vs. Frozen Matrix (*Vp)*

Sample	Spike Level	Fresh Matrix log(MPN/g)	Frozen Matrix log(MPN/g)
1	10^5	5.04	5.04
2	10^4	5.04	5.04
3	10^3	4.66	3.98
4	10^2	2.98	3.18
5	10^1	1.63	1.96
6	10^0	0.62	1.18

3. Ruggedness

Purpose/Method

During the initial assessment of ruggedness, samples were tested on different days, by different analysts, utilizing different lots of all material components, including APW, PBS, MP96 kits, mastermix components, etc. to demonstrate variability in conditions garnered no impact on results. After initial submission, the committee requested variation in testing conditions also be analyzed. Ruggedness tests the impacts of several differences in process steps on the final result. To assess performance over a myriad of conditions, ten oyster samples were spiked at a varied levels across the detection limits and run under several potential testing circumstances. First, the samples were tested in accordance with SOP guidance as a baseline. Samples were then tested after the APW enrichment MPN tubes were held at room temperature after four hours, and again after 24 hours of refrigeration. Additionally, extracted DNA was analyzed on qPCR again after the extracted DNA had been held under refrigeration for both 24-hour and 48-hour periods. Finally, extracted DNA was tested with prepared mastermix held for four hours under refrigeration and 24 hours under refrigeration prior to template addition. All ten oyster samples were tested under these seven conditions to determine if change in conditions impacted Vp MPN determination. To determine if the method was sufficiently rugged under suggested conditions, a ANOVA was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between any of the samples.

Results

Using the data from Table 3. Expanded *Vp* Ruggedness below, there was no significant difference between different testing conditions (p=1). Please also see ANOVA below.

Table 3. Expanded *Vp* Ruggedness

Sample	SOP Conditions log(MPN/g)	APW 4hrs @ Room T log(MPN/g)	APW 24hrs Refrig. log(MPN/g)	Extract 24hrs Refrig. log(MPN/g)	Extract 48 hrs. Refrig log(MPN/g)	MM 4hrs. Refrig log(MPN/g)	MM 24hrs. Refrig. log(MPN/g)
1	0.96	0.96	0.96	0.96	0.96	0.96	0.96
2	0.87	0.87	0.87	0.87	0.87	0.87	0.87
3	1.34	1.34	1.34	1.34	1.34	1.34	1.34
4	1.87	1.87	1.87	1.87	1.87	1.87	1.87
5	2.96	2.96	2.96	2.96	2.96	2.96	2.96
6	2.96	2.96	2.96	2.96	2.96	2.96	2.96
7	3.63	3.63	3.63	3.63	3.63	3.63	3.63
8	4.66	4.66	4.66	4.66	4.66	4.66	4.66

9	5.04	5.04	5.04	5.04	5.04	5.04	5.04
10	4.38	4.38	4.38	4.38	4.38	4.38	4.38

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	28.70938497	2.870938497	2.413697005
Column 2	10	28.70938497	2.870938497	2.413697005
Column 3	10	28.70938497	2.870938497	2.413697005
Column 4	10	28.70938497	2.870938497	2.413697005
Column 5	10	28.70938497	2.870938497	2.413697005
Column 6	10	28.70938497	2.870938497	2.413697005
Column 7	10	28.70938497	2.870938497	2.413697005

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	8.52651E-14	6	1.42109E-14	5.88759E-15	1	2.246408
Within Groups	152.0629113	63	2.413697005			
Total	152.0629113	69				

4. Precision & Recovery

The difference between the methods results (MPNs) and the reference values should be consistent both between different samples and also when detecting varying concentrations of measurand. The precision of the method tests the consistency of the difference between the reference values and the MPN values found in spiked matrix. In the original submission, this was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and reference data sets were converted into log values. Each MPN was compared to its associate reference value. A nested ANOVA was then performed, with variance components being the sample, and concentrations within the samples (low, medium, and high), and then the error. The ANOVA component of interest was that comparing the concentrations within the samples to the determinations (or error). Please see the requested ANOVA below.

Results

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	-1.46549	-0.14655	0.117716
Column 2	10	-2.96761	-0.29676	0.147948
Column 3	10	-2.05591	-0.20559	0.021358

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.114538	2	0.057269	0.598586	0.55671888	3.354131
Within Groups	2.583191	27	0.095674			
Total	2.697729	29				

Supplemental Validation Data for MPN Real-Time PCR for *Vibrio vulnificus*

Name of Method Submitter: Anna Pickett, Washington State Department of Health

Specific purpose or intent of the method for use in the NSSP:

Requesting adoption of this method as an approved method for *Vibrio* enumeration, both *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in oysters. The method may be used in the following applications: PHP balidation and verification of product and in management of growing areas through environmental testing and surveillance, to re-open closed growing areas. This method, once approved, would provide a high-throughput alternative to the current approved MPN real-time PCR method. Additionally, this method would be the only approved MPN real-time PCR method to test for total Vp, pathogenic Vp, and Vv in a single assay.

Supplemental Validation Criteria Data:

All oyster samples used in the supplemental validation were collected from different harvest locations and/or harvest dates in Washington state. All samples were processed using APHA guidelines for the cleaning and shucking of shellstock. Samples were confirmed negative for the target organisms of both Vp and Vv through the FDA BAM culture-based method and through qPCR prior to spiking. Spiking levels were determined using a 5-tube MPN dilution series in APW in duplicate, averaging the two values.

Vv strain ATCC 29307 was used to spike all samples for all validation criteria. This strain is positive for gene target *vvhA.*

The validation data for Vp and Vv is presented separately for clarity and ease in reviewing the data, but this is a single assay and all elements were present during validation of all organisms.

All supplemental testing performed as a response to recommendations issued by ISSC Committee in 2019.

1. Assay Design

See amended SOP for mastermix formulation appendix.

2. Comparability

Purpose/Method

To assess performance of the method as compared to the established qPCR method in the NSSP, 35 oyster samples from ten different growing areas were collected over multiple weeks. Each oyster sample was assessed both culturally and through PCR to determine the absence of detectable *V. parahaemolyticus* and *V. vulnificus*. Oyster samples were then frozen until testing. Testing occurred on 30 *Vv* spiked samples with a range of 10^0 through 10^5, five replicates at each spiking level and 5 unspiked blank samples. After processing the MPNs and enriching overnight, each replicate was extracted via WAPHL method (MagNAPure 96) and through the NSSP boil extraction. Both the MP96 and boil extraction were frozen until PCR could be performed. Boil preps were shipped to FDA Dauphin Island. WAPHL performed the proposed real-time PCR method at the same time as the FDA performed the NSSP method. To compare the performance of the proposed method with the

established NSSP method, a paired t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between the two data sets.

Additionally, to ascertain if freezing matrix had any impact on performance of the assay or the effects of spiking, a subset (six) of fresh samples were spiked at the same levels as the frozen matrix and run alongside testing. A two sided t-test was also performed on the fresh vs. frozen log-transformed data with a significance level (α) of 0.05. There should be no significant difference between the two data sets.

NOTE: Several results generated by FDA Dauphin Island were determined to be statistically improbable when put into the FDA BAM MPN Calculator due to drop-off of detection in the upper ends of the assay. In other words, the PCR resulted negative in the higher dilutions (A, B, C series) but would show positive through the lower dilutions (D, E, F series). If such instances occurred in-house, the results would be considered improbable and the sample would be re-run on PCR. Given that these results occurred at a different institution, WA DOH consulted with FDA who stated that this can happen with higher level dilutions in the NSSP *Vv* assay. As the samples could not be re-run, for all testing analytes where this occurred, the negatives in the higher dilutions were treated as positives for the purpose of determining MPN. The samples for which such drop-off occurred are noted with an asterisk. Since this phenomenon was not observed with samples at WA DOH, the MPNs, as determined with the proposed method, utilized the FDA BAM calculator unchanged.

Results

Using results from Table 1. Data for Comparability of *Vv,* there was no significant difference (p=0.58) between the NSSP approved method and the proposed method when testing spiked tissue. Both assays showed no detection of *Vv* in the five blank samples. When assessing fresh vs. frozen matrix using the data from Table 2, there was no significant difference between the matrices (p=0.81).

Table 1. Data for Comparability of *Vv*

Sample	Spike Level	WAPHL Method log(MPN/g)	NSSP Method log(MPN/g)
1	10^5	5.04	5.04*
2	10^5	5.04	5.04
3	10^5	5.04	5.04
4	10^5	5.04	5.04
5	10^5	5.04	5.04
6	10^4	4.66	5.04
7	10^4	4.66	5.04*
8	10^4	4.66	4.66
9	10^4	4.66	4.66
10	10^4	4.38	4.38
11	10^3	3.18	5.04*
12	10^3	3.36	4.36*
13	10^3	3.63	3.63
14	10^3	3.63	3.63
15	10^3	3.63	3.63
16	10^2	2.96	3.63*
17	10^2	1.96	3.36*
18	10^2	2.36	2.62

19	10^2	2.32	2.45
20	10^2	2.96	2.96
21	10^1	1.36	3.63*
22	10^1	1.36	1.36
23	10^1	1.96	1.96
24	10^1	1.96	1.96
25	10^1	0.96	0.96
26	10^0	-0.04	-0.04
27	10^0	0.18	0.18
28	10^0	0.15	0.15
29	10^0	-0.04	-0.04
30	10^0	1.36	1.36

Table 2. Fresh vs. Frozen Matrix (*Vv)*

Sample	Spike Level	Fresh Matrix log(MPN/g)	Frozen Matrix log(MPN/g)
1	10^5	5.04	5.04
2	10^4	5.04	5.04
3	10^3	3.63	3.36
4	10^2	2.36	2.18
5	10^1	1.96	1.18

3. Ruggedness

Purpose/Method

During the initial assessment of ruggedness, samples were tested on different days, by different analysts, utilizing different lots of all material components, including APW, PBS, MP96 kits, mastermix components, etc. to demonstrate variability in conditions garnered no impact on results. After initial submission, the committee requested variation in testing conditions also be assessed, in addition to varied lots of media reagents. Ruggedness tests the impacts of several differences in process steps on the final result. To assess performance over a myriad of conditions, ten oyster samples were spiked at a varied levels across the detection limits and run under several potential testing circumstances. First, the samples were tested in accordance with SOP guidance as a baseline. Samples were then tested after the APW enrichment MPN tubes were held at room temperature after four hours, and again after 24 hours of refrigeration. Additionally, extracted DNA was analyzed on qPCR again after the extracted DNA had been held under refrigeration for both 24-hour and 48-hour periods. Finally, extracted DNA was tested with prepared mastermix held for four hours under refrigeration and 24 hours under refrigeration prior to template addition. All ten oyster samples were tested under these seven conditions to determine if change in conditions impacted Vv MPN determination. To determine if the method was sufficiently rugged under suggested conditions, an ANOVA was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between any of the samples.

Results

Using the data from Table 3. Expanded *Vv* Ruggedness below, there was no significant difference between different testing conditions (p=1). Please also see ANOVA below.

Table 3. Expanded *Vv* Ruggedness

Sample	SOP Conditions log(MPN/g)	APW 4hrs @ Room T log(MPN/g)	APW 24hrs Refrig. log(MPN/g)	Extract 24hrs Refrig. log(MPN/g)	Extract 48 hrs. Refrig log(MPN/g)	MM 4hrs. Refrig log(MPN/g)	MM 24hrs. Refrig. log(MPN/g)
1	0.18	0.18	0.18	0.18	0.18	0.18	0.18
2	0.62	0.62	0.62	0.62	0.62	0.62	0.62
3	0.96	0.96	0.96	0.96	0.96	0.96	0.96
4	1.62	1.62	1.62	1.62	1.62	1.62	1.62
5	1.62	1.62	1.62	1.62	1.62	1.62	1.62
6	1.96	1.96	1.96	1.96	1.96	1.96	1.96
7	3.36	3.36	3.36	3.36	3.36	3.36	3.36
8	2.62	2.62	2.62	2.62	2.62	2.62	2.62
9	4.18	4.18	4.18	4.18	4.18	4.18	4.18
10	4.18	4.18	4.18	4.18	4.18	4.18	4.18

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	21.31057443	2.131057443	2.013638108
Column 2	10	21.31057443	2.131057443	2.013638108
Column 3	10	21.31057443	2.131057443	2.013638108
Column 4	10	21.31057443	2.131057443	2.013638108
Column 5	10	21.31057443	2.131057443	2.013638108
Column 6	10	21.31057443	2.131057443	2.013638108
Column 7	10	21.31057443	2.131057443	2.013638108

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.26326E-14	6	7.10543E-15	3.52865E-15	1	2.246408
Within Groups	126.8592008	63	2.013638108			
Total	126.8592008	69				

4. Precision & Recovery

The difference between the methods results (MPNs) and the reference values should be consistent both between different samples and also when detecting varying concentrations of measurand. The precision of the method tests the consistency of the difference between the reference values and the MPN values found in spiked matrix. In the original submission, this was done by testing ten oyster samples at low, medium, and high concentrations in duplicate to determine the MPN. The MPN and reference data sets were converted into log values. Each MPN was compared to its associate reference value. A nested ANOVA was then performed, with variance components being the sample, and concentrations within the samples (low, medium, and high), and then the error. The

ANOVA component of interest was that comparing the concentrations within the samples to the determinations (or error). Please see the requested ANOVA below.

Results

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	1.282785	0.128278	0.050537
Column 3	10	0.273956	0.027396	0.049981

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.058282	2	0.029141	0.741478	0.485862	3.354130829
Total	1.119422	29				

5. Specificity

Purpose/Method

After ISSC feedback on the initial proposal, the interactions between *V. vulnificus, V. alginolyticus,* and *V. parahaemolyticus* were assessed in greater depth, particularly, how lower levels of competitor organisms may impact *vvha* specificity. To perform this assessment in addition to data already presented, five oyster samples were divided into three aliquots. All three aliquots were spiked with 10^1 levels of *Vv*. Five of those aliquots were additionally spiked with 10^3 levels of potentially interfering *V. alginolyticus* and five were spiked with 10^3 levels of potentially interfering *V. parahaemolyticus*. Replicates were tested on different days to ensure reproducibility of results. Each of the replicates was analyzed by taking the average log MPN and calculating the Specificity Index (SI). A paired *t*-test was used to determine if the average specificity index obtained from the five replicates differed from 1 (significance level (α) of 0.05).

Results

Using the data from Table 4. Supplementary Data for Determination of Specificity, the average specificity index was 1.26 when in the presence of *Vp* and 1.02 in the presence of *Va.* These values are not significantly different than 1 (*p*=0.11 for *Vp, p*=0.71 for *Va).*

Table 4. Supplementary Data for Determination of Specificity

Replicate	*Vv* only, log(MPN/g)	*Vv* + *Vp,* log(MPN/g)	*Vv* + *Va,* log (MPN/g)
1	1.62	0.96	1.62
2	1.36	1.36	1.18
3	1.36	0.96	0.87

4	1.18	0.96	1.36
5	0.96	0.96	1.96

6. Inclusivity

Purpose/Method

To assess the ability of the method to detect a wide range of target strains, the committee requested additional information on environmental isolate inclusivity. WAPHL subsequently tested three environmental *V. vulnificus* isolates cultured from Washington shell stock and confirmed to be *Vibrio vulnificus* biochemically. WAPHL then coordinated with FDA Dauphin Island to receive additional environmental isolates. Following up on the initial 21 *Vv* isolates run in 2018, WAPHL ran 12 environmental *Vv* on the assay to ensure robust inclusivity.

Results

Sensitivity = (True Positives/(True positives + false negatives))
Supplemental *vvhA* sensitivity = 12/(12+0) = 100%
Total *vvhA* sensitivity = 33/(33+0) = 100%

The primers and probes utilized in this method demonstrate 100% inclusivity. See Table 6. Supplementary Inclusivity Panel for strain information and result.

Table 6. Supplementary Inclusivity Panel

Strain	Source	vvha
S21-123A	Washington DOH	+
S21-137A	Washington DOH	+
S21-145B	Washington DOH	+
R101-A9	FDA Dauphin Island	+
R30-C10	FDA Dauphin Island	+
R42-D10	FDA Dauphin Island	+
R84-F4	FDA Dauphin Island	+
R74-C3	FDA Dauphin Island	+
R99-A10	FDA Dauphin Island	+
R595-A3	FDA Dauphin Island	+
R80-G3	FDA Dauphin Island	+
R56-B5	FDA Dauphin Island	+

Supplemental Validation Data for MPN Real-Time PCR for *Vibrio parahaemolyticus* – Naturally Incurred Samples

Name of Method Submitter: Anna Pickett, Washington State Department of Health

Specific purpose or intent of the method for use in the NSSP:

Requesting adoption of this method as an approved method for *Vibrio* enumeration, both *Vibrio parahaemolyticus* (Vp) and *Vibrio vulnificus* (Vv) in oysters. The method may be used in the following applications: PHP validation and verification of product and in management of growing areas and to re-open closed growing areas. This method, once approved, would provide a high-throughput alternative to the current approved MPN real-time PCR method. Additionally, this method would be the only approved MPN real-time PCR method to test for total Vp, pathogenic Vp, and Vv in a single assay.

Supplemental Naturally Incurred Validation Criteria Data:

All oyster samples used in the supplemental validation were collected from different harvest locations and/or harvest dates in Washington state. All samples were processed using APHA guidelines for the cleaning and shucking of shellstock. Samples were analyzed in accordance with submitted method and also using the NSSP PCR method. All samples incurred *V. parahaemolyticus* naturally.

All supplemental testing performed as a response to specific comments issued by ISSC Committee in 2022.

1. **Non-Detect Samples**

   Naturally incurred non-detect samples were previously run and submitted to Dauphin Island for testing in 2022, but the data was not captured in the previous supplemental submission. Data is presented below.

   To assess performance of the method as compared to the established qPCR method in the NSSP, five oyster samples from five different growing areas were collected over multiple weeks. Each oyster was cleaned and processed in accordance with APHA guidelines. After processing the MPNs and enriching overnight, each replicate was extracted via WAPHL method (MagNAPure 96) and through the NSSP boil extraction. Boil preps were shipped to FDA Dauphin Island on dry ice where PCR was performed. To compare the performance of the proposed method with the established NSSP method, a two sided t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between the two data sets.

   For both NSSP and WAPHL method, no targets were detected for any samples. (*tlh, tdh, trh,* or *vvha).* Given the limits of the assay non-detects are reported as <0.36 CFUs/g. For this report, CFUs were treated as 0.36 for ease of data handling.

   Results
   Using results from Table 1. Data for Comparability of *Vp,* there was no significant difference (p=1) between the NSSP approved method and the proposed method when testing naturally incurred non-detect samples for *tlh, tdh, trh,* or *vvha.*

Table 1. Data for Comparability of *Vp*

Sample	WAPHL Method *tlh* log(MPN/g)	WAPHL Method *trh* log(MPN/g)	WAPHL Method *tdh* log(MPN/g)	WAPHL Method *vvha* log(MPN/g)	NSSP Method *tlh* log(MPN/g)	NSSP Method *tdh* log(MPN/g)	NSSP Method *trh* log(MPN/g)	NSSP Method *vvha* log(MPN/g)
1	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44
2	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44
3	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44
4	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44
5	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44	-0.44

2. Naturally Incurred *Vp* levels

Purpose/Method

To assess performance of the method as compared to the established qPCR method in the NSSP, five oyster samples from five different growing areas were collected over multiple weeks as a part of routine screening in the summer months. Each oyster was cleaned and processed in accordance with APHA guidelines. After processing the MPNs and enriching overnight, each replicate was extracted via WAPHL method (MagNAPure 96) and through the NSSP boil extraction. Boil preps were shipped to FDA Dauphin Island on dry ice where PCR was performed. To compare the performance of the proposed method with the established NSSP method, a two sided t-test was utilized on log-transformed data with a significance level (α) of 0.05. There should be no significant difference between the two data sets.

Results

Using results from Table 1. Data for Comparability of *Vp,* there was no significant difference between the NSSP approved method and the proposed method when testing naturally incurred samples (*tlh:* p= 0.89, *tdh:* p=1, trh: p=1).

Table 1. Data for Comparability of *Vp*

Sample	WAPHL Method *tlh* log(MPN/g)	NSSP Method *tlh* log(MPN/g)	WAPHL Method *tdh* log(MPN/g)	NSSP Method *tdh* log(MPN/g)	WAPHL Method *trh* log(MPN/g)	NSSP Method *trh* log(MPN/g)
1	1.62	1.62	0.36	0.36	0.36	0.36
2	2.96	2.96	1.18	1.18	1.18	1.18
3	3.36	3.36	0.96	0.96	1.36	1.36
4	4.66	4.18	1.58	1.58	2.18	2.18
5	4.18	4.18	1.36	1.36	1.36	1.36

1. Purpose/Principle

The purpose of this test is to rapidly quantify *Vibrio parahaemolyticus* (*Vp*) and *Vibrio vulnificus* (*Vv*) from oysters using a high throughput MPN based real-time PCR protocol. Culture based assays for the enumeration of *Vp* and *Vv* require a minimum of four days and require the Kanagawa test (media based) to detect pathogenicity. This assay was designed to provide quantitative results for total *Vibrio parahaemolyticus* (*Vp tlh*+), known pathogenic markers of *Vibrio parahaemolyticus* (*Vp tdh*+ and *Vp trh*+), as well as a strain of potential pandemic *Vibrio* (*Vp* ORF8+). *Vp* ORF+ is part of the assay and used for surveillance purpose and was not used in the validation of this method. Additionally, the assay provides quantitative results for total *Vibrio vulnificus* (*vvhA*) and utilizes an exogenous internal control (WA IC).

This test utilizes Taqman® probe real-time polymerase chain reactions to amplify 4 target genes from the *Vibrio parahaemolyticus (Vp)* genome as well as 1 target from the *Vibrio vulnificus* (*Vv*) genome.

Vp

- Thermolabile hemolysin, *tlh* gene
- Thermostable direct hemolysin, *tdh* gene
- Thermostable direct related hemolysin, *trh* gene
- Filamentous phage (f237) Orf8, gene

Vv

- Cytolysin-hemolysin, *vvhA* gene

2. Reagents / Media

- Master Mix: TaqMan™ Environmental Master Mix 2.0; Thermo Fisher Cat. #4396838
- Molecular PCR grade water
- TE buffer
- Primers (See appendix for sequences)
- Probes (See appendix for sequences)
- Internal Control Plasmid (See appendix for sequences)
- MagNAPure 96 DNA and Viral NA Small Volume kit; Roche, Cat. # 06543588001
- Alkaline Peptone Water (APW)
- Phosphate Buffer Saline (PBS)
- Quant Studio 384 well Calibration plates: VIC®, FAM™, NED™ and ROX™ dye Spectral Calibration plates Life Technologies Cat # 4432278, 4432271, 4432302, 4432284. ROI and Background plate Cat # 4432320. FAM™/ROX™ and VIC®/ROX™ dye normalization plates Cat # 4432308.

Lyophilized primers are diluted to 100µM stock concentrations with TE Buffer and stored at -15°C (or below) until use. Store in low light transmitting tubes. Original stock solutions are good for 2 years unless otherwise stated by manufacturers. Working concentrations of primers and probes should also be stored in low light transmitting tubes and are good for 6 months in freezer (-15°C or below). Thawed working concentrations are good for 2 weeks refrigerated (2-8°C).

The exogenous internal control plasmid (1:100 concentration) is stored at -15°C or below. Prepare working stock by adding 990µL molecular grade H_2O to single IC tube (containing 10µL). The working stock can be stored at 2-8°C. On day of use, dilute working stock an additional 1:100. The final concentration of the final product is 1:100,000. See Appendix for Internal Control Plasmid information.

3. Supplies / Materials

- Isopropanol, 70%
- RNAse AWAY®
- Sterile scrub brushes
- Sterile oyster knives
- Sterile wide mouth containers (1 liter capacity)
- Oyster shucking block
- Chain-mail glove
- Dishwashing gloves
- Serological pipettes (1ml, 10ml, 25ml)
- Filtered pipette tips
- Sterile dilution bottles
- Microcentrifuge tubes (1.7mL)
- 384 well PCR plates; Thermo Fisher Cat. # 4326270
- Optical covers
- MagNAPure 96 Cartridge seals; Roche Cat. # 06241638001
- MagNAPure 96 Processing Cartridge; Roche Cat. # 06241603001
- MagNaPure 96 Output plate; Roche Cat. # 06241611001
- MagNAPure 96 System Fluid; Roche Cat. # 06640729001
- MagNAPure 96 Tips 1000µL; Roche Cat. #06241620001

3.1 Bacterial Cultures

- *Vibrio parahaemolyticus* strain (*tlh*+, *tdh*+ and *Orf8*+)
- *Vibrio vulnificus* (*vvha*+)

4. Equipment

- High Speed blender
- Balance (±0.1 g)
- Sterile blender jars
- Timer
- Vortex mixer

- Incubator (35°C±0.5)
- Refrigerator, 2-8°C
- Freezer, -15°C to -25°C
- Traceable thermometers
- Biological safety cabinets (BSC) or Air Clean PCR stations
- Pipettes P-1000, P-200, P-20
- Multi-channel Pipette (8) 2µL-25µL
- Applied Biosystems® QuantStudio™ Dx™ Real-Time PCR station
- Roche MagNAPure 96 DNA purification system
- PCR plate centrifuge.

5. Specimen Information

Samples are collected, transported, and processed in accordance with Recommended Procedures for the Examination of Sea Water and Shellfish described by the American Public Health Association[iii].

Sample processing at the lab must be initiated no more than 36 hours after collection. Samples received more than 36 hours after collection are considered unsatisfactory.

6. Quality Control

- Instructions
    - Three process controls are included in every extraction run. To prepare the process control material, enrich *V. parahaemolyticus* (*tlh*+, *tdh*+, *trh*+) and *V. vulnificus* (vvha+) overnight in APW. The overnight enrichment is diluted and a $<10^3$ per ml culture is used as a positive control. The two organisms will also serve as negative controls for each other. Include an uninoculated APW blank to serve as the uninoculated control. Process control cultures are to be run concurrent with the samples, and accompany the samples throughout incubation, extraction, and confirmation.
    - To ensure that all MagNA Pure 96 plastics (i.e. processing cartridge, output plate, internal control tubes, 1000µl tips) and external system fluid are free of interfering contaminants, process controls will serve as quality control.
    - Two amplification controls are included in every PCR run. To prepare the positive PCR control, enrich *Vibrio parahaemolyticus* and *Vibrio vulnificus* overnight in APW. DNA is extracted individually, combined in equal parts, and divided into 10µL aliquots. Pre-extracted amplification controls should be stored frozen at -15°C or below. Expiration is one year from the date it was prepared. Sterile molecular grade water will be used for the no-template-control (NTC) and will serve as the negative amplification control.
    - Every time a new lot of working stock primer/probe mix is prepared, twenty runs using the Master mix positive control will be used to determine the acceptable limits for the lot. The standard deviation of each mix will be calculated. In every run, ranges will be checked.
    - Disposable pipettes (i.e. serological) will be tested for sterility.

- Frequency
    - Process controls, exogenous internal control, and amplification controls are included with every PCR run.
    - Quality controls will be run on all media and reagents, mastermix, and primer/probe mixes prior to use or concurrent with testing.

7. Calibration

- **QuantStudio Dx PCR Workstation**

  Calibration kits are used to maintain the Real-Time PCR system with 384-Well Block. For this assay it is required to perform a spectral calibration with FAM™, VIC®, ROX™, NED™ dyes, plates to perform region-of-interest (ROI) calibration and normalization calibration.

  Calibration and verification should be run at least every six months and following a Performance Maintenance. Kits are stored at -15°C to -25°C. The maximum degree of accuracy for each dye of interest in florescence emission wavelength is ±5nm. Follow manufacturer's instruction on performing calibrations. Additional plates (SYBR®,TAMRA™ and RNase P) may be required by the technician after a major repair or if a new instrument is installed.

8. Procedure

8.1 Sample Preparation- Scrubbing

a. The intent of the assay is to determine the concentration of *Vp* and *Vv* in the oyster tissue and liquor. Any material on the outside of the oyster that gets introduced into the interior of the animal during shucking can alter the concentration.
b. The sink must be clean before scrubbing can begin. Wash the sink with water or soap and water.
c. The gloved hands of the analyst are to be washed with soap immediately prior to cleaning the shells of debris. The gloves worn are latex, nitrile and/or stainless-steel mesh to protect analyst's hands from injury.
d. Using sterile scrub brushes, each oyster is cleaned under cold running water. All barnacles, mud, vegetation and debris should be removed.
   Note: The faucet used for rinsing the shellfish should not contain an aerator. Pay close attention to the hinge and shell seam. A sterile brush should only be used for one sample. Do not re-use brushes when scrubbing multiple samples. Any oyster that does not tightly close during handling is likely dead and should be discarded. In addition, any oyster whose shell is broken to expose tissue should be discarded.
e. A representative sample of at least 12 shellfish and no less than 200g is used for analysis.

8.2 Sample Preparation- Shucking

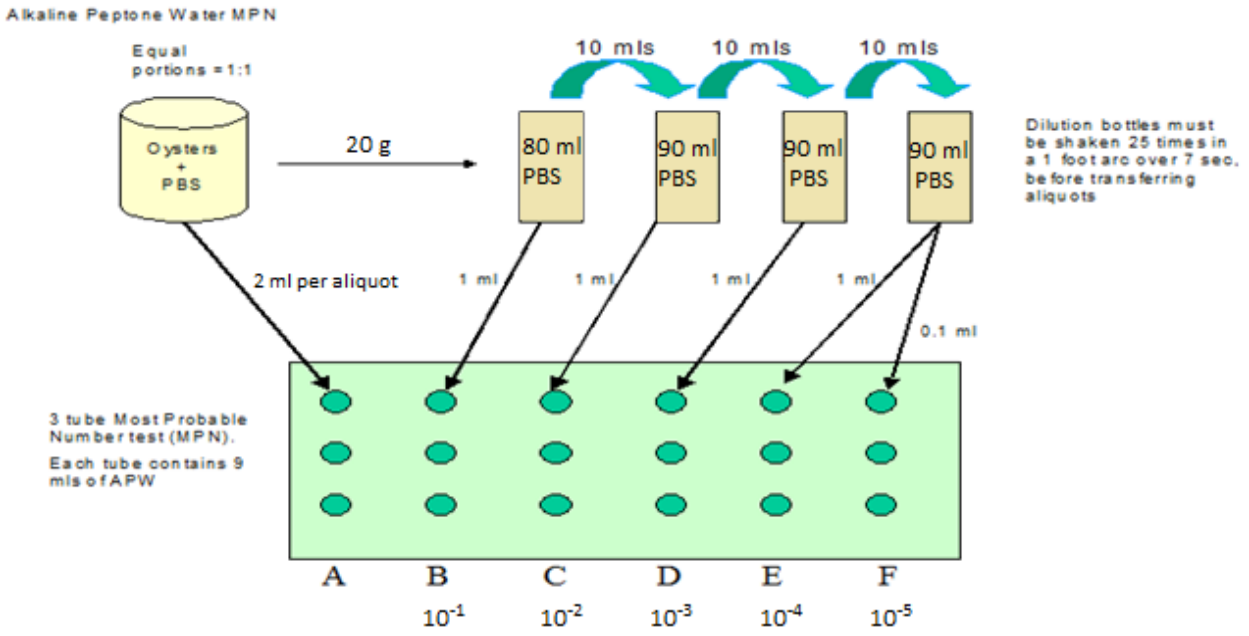
a. In order to accurately quantify *Vp* and *Vv* in oyster tissue it is very important to avoid introduction of bacteria (*Vp* or other) into the oyster tissue.
b. The sink must be disinfected before shucking can begin. Wash the sink with water or soap and water. Completely dry the sink. Wipe the sink down with 70% isopropanol and allow it to air dry.
c. Place a sterile pre-weighed tissue collection container on the sink counter.
d. Disinfect a shucking block by washing with soap and water and wiping down with 70% isopropanol. Place block on the sink counter to air dry.
e. Place the oyster sample to be shucked on the sink counter.
f. Put on clean nitrile gloves.
g. Over one of the gloves put on a chain-mail glove. The chain-mail glove should be on the hand that will not be holding the knife.
h. Put another nitrile glove on over the chain-mail glove. Cover both hands in 70% isopropanol and allow them to air dry.
i. Grab and hold each oyster with the chain-mail hand and use the other hand and a sterile oyster knife to shuck each oyster.
j. A fresh knife, shucking container and gloves must be used for each sample.
k. Use the disinfected shucking block while shucking to minimize knife accidents and to protect the counter surface.
l. Collect all tissue and liquor (fluid) in the sterile pre-weighed container. 10^{-1}
m. The shucking block and counter must be washed and sterilized between samples.

8.3 Sample Processing- Setting up MPN

Enumeration in this assay is achieved by conducting an MPN (most probable number) analysis.

a. Weigh the shellfish tissue collection container. Subtract the original container weight from the total weight to determine the amount of oyster tissue and liquor.
b. Record the weight of tissue on the sample worksheet.
c. Transfer the oyster tissue and liquor to a sterile blender jar.
d. Add an equal weight of diluent (PBS) to the sample container. If needed, PBS can be used to rinse any residual tissue from the container just as long as a 1:1 dilution can be maintained (±0.1g).
e. Transfer the PBS to the blender jar. Record the weight of PBS used on the sample worksheet.
f. Blend the shellfish sample with PBS at high speed for 90 seconds (60 to 120 seconds is acceptable).
g. From this homogenized sample, set up a 3-tube most probable number (MPN) serial dilution series. Use PBS for making dilutions and alkaline peptone water (APW) as the enrichment broth in each of the MPN tubes. See Figure below.

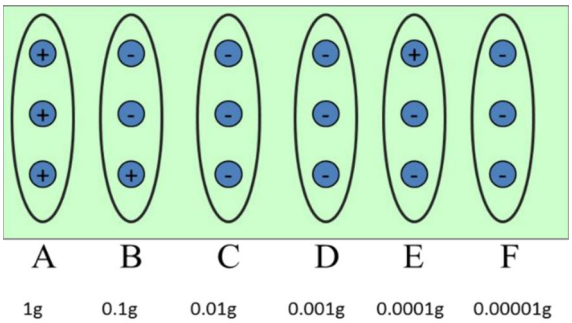
The initial 1:10 dilution is prepared gravimetrically with PBS (20 g of sample homogenate to 80 mL of PBS). Record the weight and volume used on the sample worksheet for the sample worksheet. All successive dilutions are prepared volumetrically.

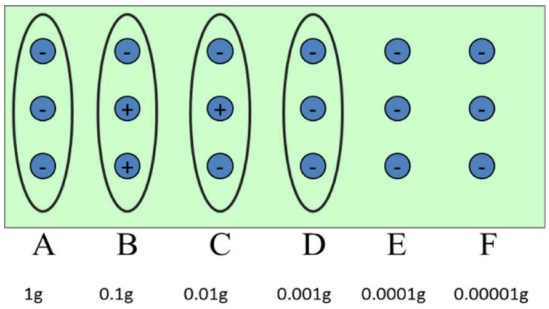


h. Incubate at 35°C± 1 for 18 to 24 hours. .

8.4 Reading MPN

a. Each APW tube must be checked for growth following 18-24 hrs. of incubation. Use the following criteria to select tubes for further testing.
    i. Examine all tubes for turbidity. Examine each tube with a light source shining through the tube.
    ii. Record all positive and negative results on the lab worksheet.
    iii. The following examples illustrate the selection process. Each tube is labeled as +/- for turbidity. The dilutions circled should be selected for further testing.





b. If one tube in a given three tube dilution series is positive for turbidity, all tubes in that series must be tested (you will still record the actual positive or negative values).
c. In addition, test one complete dilution series beyond the last series that contained any growth and all tubes of higher concentration.

8.5 DNA Extraction

DNA extraction must be initiated within the 18–24-hour incubation window.

Once extraction is complete, the material is considered stable and may be stored at 2-8°C for 1-2 days or frozen at -20°C to -80°C for extended periods. NOTE: Multiple freeze thaw cycles should be avoided due to potential DNA degradation.

a. Create a plate map to keep track of your samples and load the MagNA Pure 96 cartridge accordingly. 200µL of each sample should be added to the 96 well cartridge. Include 200µL of *Vp* culture, 200µL of *Vv* culture, and 200µL of uninoculated APW. These will act as process controls for the assay.
b. Once loaded, seal the MagNA Pure cartridge with an adhesive cartridge seal.
c. Confirm that the MagNA Pure 96 instrument and its linked computer are turned on.
d. Ensure that the correct MagNA Pure kit is selected “DNA/Viral SV 2.0”.
e. Select the protocol “Pathogen Universal 200.3.1”
f. Sample volume should be entered as 200µL.
g. Elution volume should be entered as 100µL.
h. Next to the Internal Control section, click the More Options icon. Scan the barcode located on the IC tube. Enter the number of tests. This will determine the amount of IC needed. Since IC is prepped for single use, the auto-filled expiration date is not relevant.
i. Enter in your sample order. Be sure that the correct cartridge wells are highlighted on the screen. Incorrect set up here will lead to a failed extraction.
j. Click the “Stage Set-up” button.
k. Begin adding in the appropriate reagents/plastics in accordance with the outlined requirements on the MagNA Pure load screen (software).
l. Once the reagent trays are completely loaded and the tips are adequately filled, place the remaining trays back into the instrument.
m. Remove the cartridge seal from the processing cartridge and place into the instrument. Discard the seal into an autoclave waste container.

n. Ensure that all plastics, reagents and sample cartridges are in place and accounted for on the computer screen.

o. Close the door and press the “start extraction” button.
p. Note the time that the run will be completed. The final extracted template DNA will be refrigerated on-board the MagNA Pure 96 instrument until it is removed. It is however not advisable to leave the extract uncovered for any length of time.
q. Once completed, open the door, remove the extracted DNA, immediately seal the cartridge with a new cartridge seal, and refrigerate at 2-8°C until ready for PCR (if PCR is to be completed in 1-2 days). If PCR will not be complete in the next two days freeze the DNA at -20°C to -80°C.

8.6 MagNAPure 96 Waste Removal and Decontamination.

Use manufacturer’s suggested cleaning procedure. Run the UV decontamination protocol.

8.7 PCR Mastermix Preparation

Mastermix preparation is performed within an Airclean hood. This includes primer and probe manipulations and mastermix loading onto the PCR plate. Thorough decontamination before and after use of the Airclean hood is advisable.

a. Prepare a PCR platemap . Be sure to include positive and negative process controls, in addition to a positive and negative amplification control.
b. Determine the number of reactions needed. This will help you determine how much of each mastermix component will be required. It is advisable to prepare several reactions more than are needed to account for pipetting variability.
c. Once in the Pre-PCR room, follow mastermix recipe and MagNAPure plate map to prepare Multiplex 1 and 2. The mastermix can be prepared in a microcentrifuge tube or sterile disposable reagent reservoir.
d. Briefly vortex (swirl, pipette up and down, or equivalent) to completely mix the components.
e. Using a pipette (multichannel advisable), add 18µL of mastermix to each appropriate well (384 well plate) according to the PCR platemap.

8.8 Template Addition

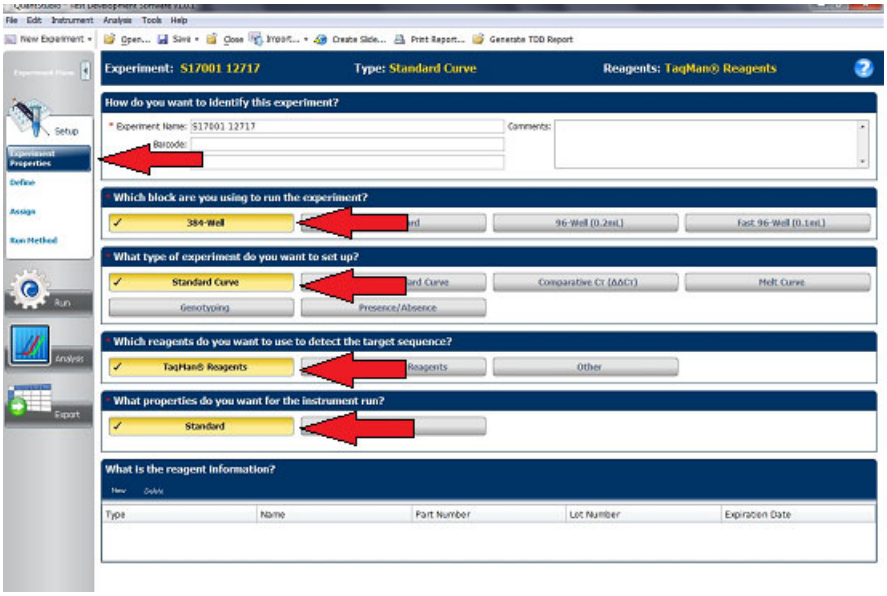
a. Add the extracted DNA template to the appropriate wells according to your PCR platemap. Use 2µL of DNA for a total reaction volume of 20µL.
b. Once all wells are loaded including the positive and negative amplification controls, seal the plate with an optical seal.
   Avoid touching either side of the seal as the adhesive side will come into contact with your reactions (poses a contamination risk) and the outside must be clear of smudges to allow accurate readings. Apply the seal using the plastic applicator supplied with the instrument. Take care to completely seal each well. Any unsealed well will rapidly evaporate during PCR and lead to inaccurate results.
c. Centrifuge the plate briefly to remove bubbles from the wells and ensure that the template is in contact with the reaction mix.

8.9 Setting up Real-Time PCR Station

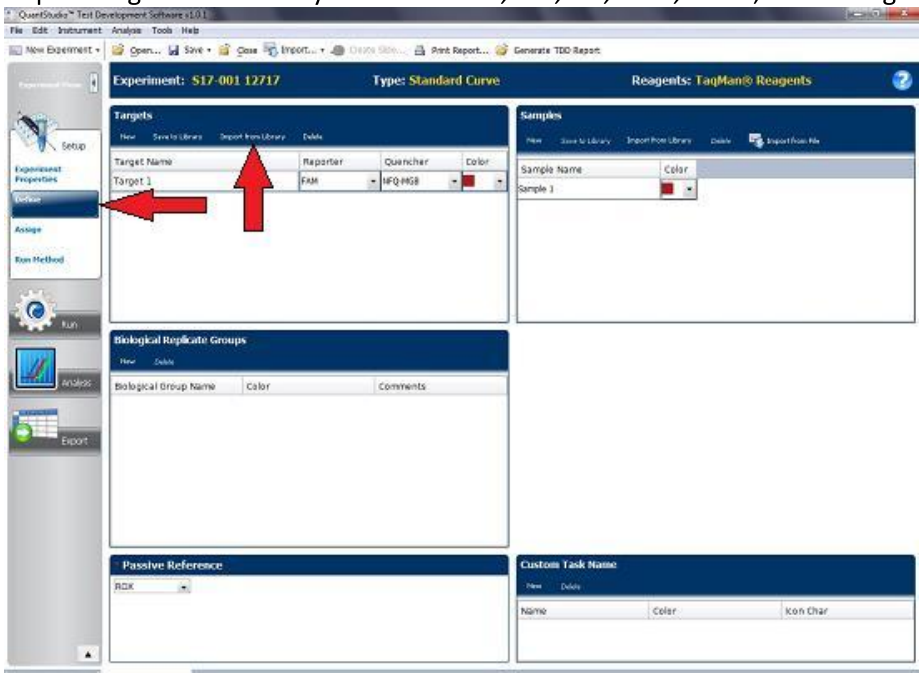
a. Turn on the Applied Biosystems® QuantStudio™ Dx™ instrument and the computer.

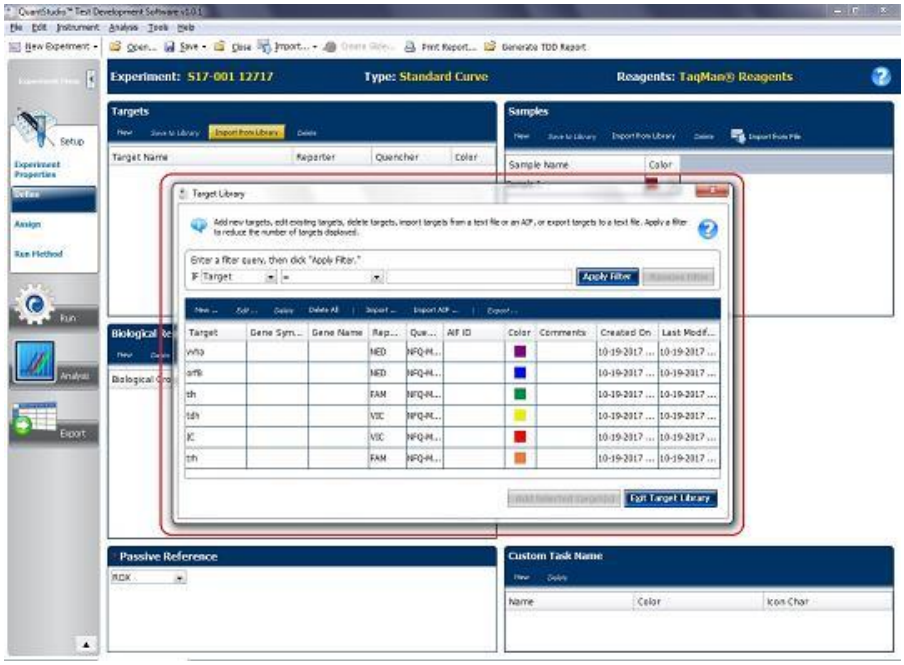
b. Open the Test Development software, under the File menu select “New Experiment”.

c. Rename experiment with the appropriate sample numbers and date of run.
d. Under the tab “Experiment Properties” ensure “384-Well Block”, “Standard Curve”, TaqMan® Reagents”, and “Standard” (for run mode) are selected.

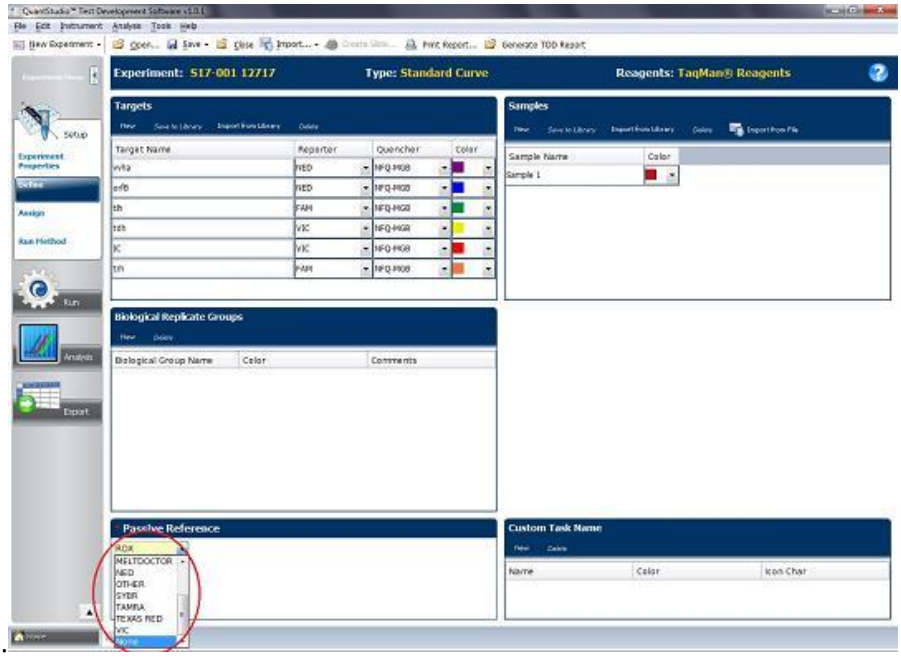


e. The “Define” tab is used to select the targets of detection. The targets are saved to the library. Import targets from library. Select the *tlh, tdh, trh, vvha,* ORF8, and IC targets.

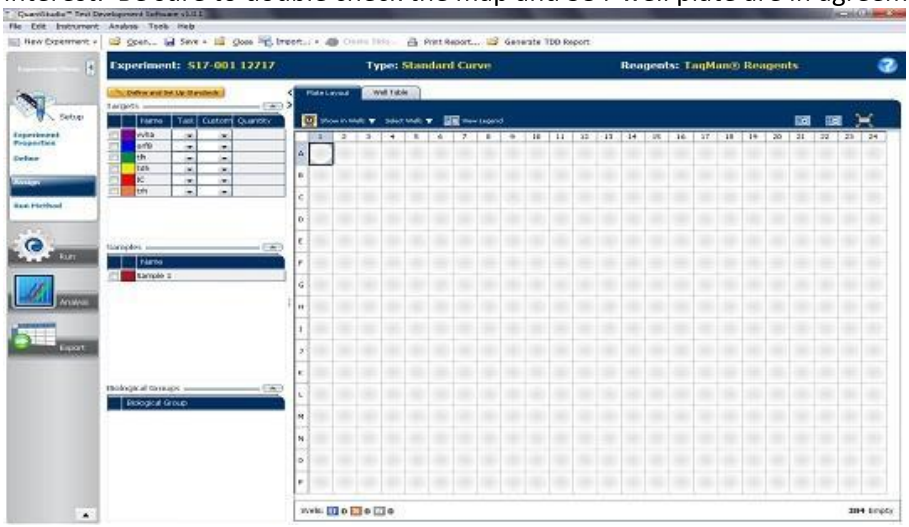




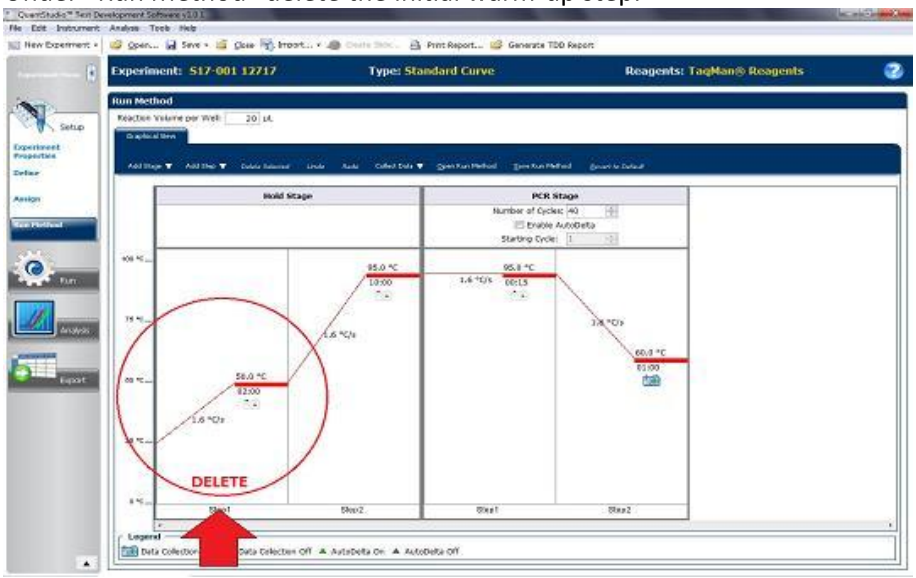
f. At the bottom of the screen, select “ROX” from the drop down menu regarding “Passive Reference”



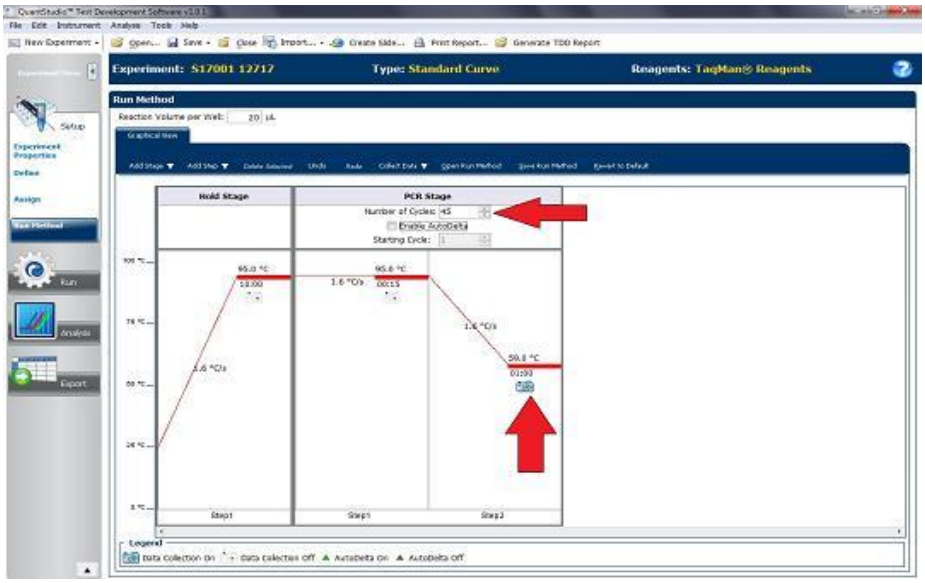
g. Select the “Assign” tab and assign the appropriate wells with the corresponding targets of interest. Be sure to double check the map and 384-well plate are in agreement.



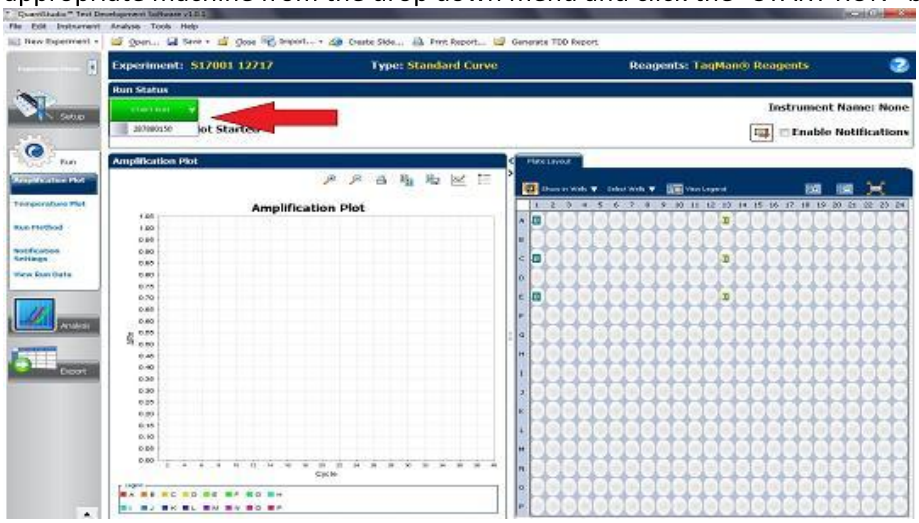
h. Under “Run Method” delete the initial warm-up step.



i. After doing so the parameters are the following:
   95° C for 10 minutes
   40 cycles
   95° C for 15 seconds
   59° C for 60 seconds



j. Turn on the instrument user interface by touching the touchscreen. To open loading tray, touch the eject button.
k. Load the plate and close tray.
l. From the PC, click the "Run" tab and find the green "START RUN" button. Select the appropriate machine from the drop down menu and click the "START RUN" button.



m. Save run file using sample numbers and date.

9. Interpretation

a. Select "Analysis Settings" on the Amplification Plot screen.
b. Change all thresholds and baseline settings to "manual" and set all thresholds, except trh, to 0.04 and leave baseline settings at 5 to 15. Set trh threshold to 0.08.
c. Apply analysis settings and exit to Amplification Plot screen.
d. Record quality control Ct values on the Vibrio parahaemolyticus Real-Time PCR Ct Value QC worksheet. For all targets record Ct value from the positive mastermix control. Record Ct value for the Internal Control (IC) using the negative mastermix control. All Ct values should be recorded with the threshold set at 0.04 and baseline set at 5 to 15.
e. Select all wells in the plate by clicking in the upper left box of the plate layout.

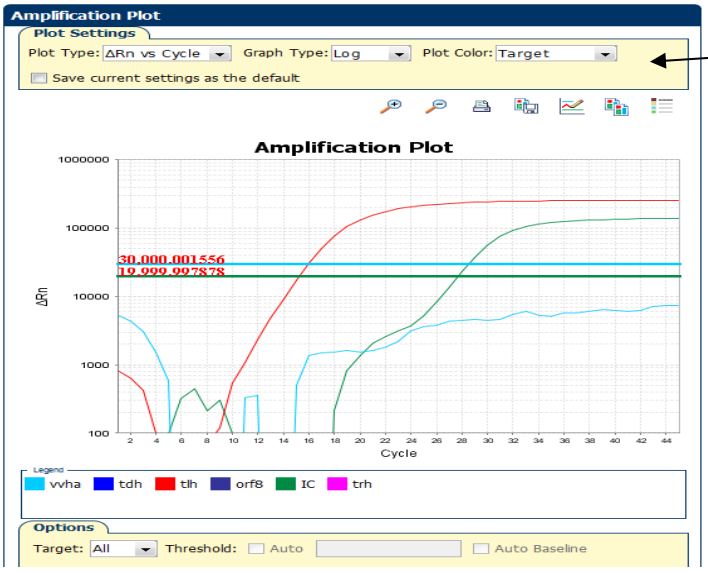
f. View each target individually and make necessary changes to the threshold and baseline. The threshold should be set above background levels. It may be necessary to change baseline settings to lower background levels.

g. If baseline changes are necessary - view individual wells in the Multicomponent Plot screen. Change baseline settings as needed to exclude early background noise.

Exclude early background noise when setting baseline



h. Once threshold and baseline are set at appropriate levels, record results from each well for every target. Targets within the amplification plot may have high background in the early stages of the run (i.e. <10 cycles). Disregard background that crosses the threshold before cycle 10. Change the Plot color to "Target" to help read results.



Plot Color = Target

Positive = amplification above threshold

Negative = No amplification

9.1 Interfering Substances

Vibrio alginolyticus possesses a *trh* gene with 98% homology to the *trh* gene in *Vibrio parahaemolyticus*[i]. Most probable number values for *trh* should be reported only if *tlh* is present in the corresponding tube. Tubes only positive for *trh* should not be accounted for when generating the MPN value.

10. Calculations

Upon determination of positive reactions, record the number of confirmed positive tubes per dilution series onto the *Vibrio* Sample Worksheet, and generate an MPN index. MPN values (concentration) of each target is derived from the FDA Bacteriological Analytical Manual (BAM) MPN Calculator. This Excel document can be located in Appendix 2 of the FDA BAM. To compute an MPN value, follow the instructions as noted in the FDA BAM MPN Calculator, found here: https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions

11. Reference Range

Reportable Range

tlh: <0.36 MPN/g to >110,000 MPN/g
tdh: <0.36 MPN/g to >110,000 MPN/g
trh: <0.36 MPN/g to >110,000 MPN/g
ORF8: <0.36 MPN/g to >110,000 MPN/g
vvhA: <0.36 MPN/g to >110,000 MPN/g

12. References

[i] González-Escalona, Narjol, George M. Blackstone, and Angelo DePaola. Characterization of a Vibrio alginolyticus strain, isolated from Alaskan oysters, carrying a hemolysin gene similar to the thermostable direct hemolysin-related hemolysin gene (trh) of Vibrio parahaemolyticus." Applied and environmental microbiology 72.12 (2006): 7925-7929.

[ii]Dauphin L. A. et. al. Evaluation of Automated and Manual Commercial DNA Extraction Methods for Recovery of *Brucella* DNA from suspensions and spiked swabs. 2009. JCM Vol. 47, No. 12. p. 3920-3926.

[iii]The American Public Health Association, Inc. *Recommended Procedures for the Examination of Sea Water and Shellfish*. 4th ed., 1970.

13. Appendix

Appendix A- Primers and Probes

VIBRIO PARAHAEMOLYTICUS

TRH
Primers
Trh 627F
ATA CCT TTT CCT TCT CCW GGT TC
Trh 731b R
TTG TCC AGT AGT CAT CAA CGA TTG
Trh Glov R
TTG TCC AAT AGT CCT CCA CAA TTG
(Ward) Probe-- Trh P
FAM TAT TTG TYG TTA GAA ATA CAA CAA T **MGBNFQ**

(WA PHL *Vibrio* Internal Control)
Primers
WA IC F
GGC GAA GCG AAT CTG GAA A
WA IC R
GGT GTA GTT GTG CGT GTA ATA TGA GA
Probe-- WA PHL ICP
VIC CGT AAG ACA ATC TGA TAG TAG T **MGBNFQ**

Orf8
Primers
Orf8 F TCA CCT GAG GAC GCA GTT ACG
Orf8 R TTC AAT TGT AGA ACC GCC AGC TA
Orf8 Probe
NED TCC TGC TGT ACT TTT AG **MGBNFQ**

TLH (69 bp amplicon)
Primers
Tlh-F CCG CTG ACA ATC GCT TCT C
Tlh-R TTT GAT CTG GCT GCA TTG CT
Tlh probe
FAM ACC ACA CGA TCT GGA GCA ACG ACG **MGBNFQ**

TDH (94 bp amplicon)
Primers
TDH-F 2013 TAT CCA TGT TGG CTG CAT TC
TDH-R 2013 CGA ACA ACA AAC AAT ATC TCA TCA GA
TDH Probe
VIC TGT CCC TTT TCC TGC CCC CGG MGBNFQ

VIBRIO VULNIFICUS

VVHA (79 bp amplicon)
vvha-F GAT CGT TGT TTG ACC GTA AAC G
vvha-R TGC TAA GTT CGC ACC ACA CTG T
vvha Probe
NED-CAA AAC GCT CAC AGT CG-**MGB probe**

Appendix B- Internal Control Plasmid

The *Vibrio parahaemolyticus* and *Vibrio vulnificus* enumeration and detection through MPN and real-time PCR assay utilizes an exogenous internal control (WA IC). This plasmid is added to the during DNA extraction for the detection of matrix inhibition or other assay failures. The 73bp fragment can be synthesized and clones into a pIDTSMART-AMP plasmid by Intergrated DNA Technologies (IDT), Ref ID: 88772700.

Sequence:
GGCGAAGCGAATCTGGAAAACGTAAGACAATCTGATAGTAGTATATTTCTCATATTACACGCACAACTAC ACC

Additionally, the Invitrogen OneShot Top10 Chemically Competent Cells and QIAGEN Plasmid Midi Kit can be used collectively to manufacture and purify additional plasmid DNA.

Protocol for Transforming Chemically Competent Cells.

This section provides a procedure to transform Invitrogen One Shot TOP10 chemically compentent E.coli via regular Chemical transformation protocol, as described by the manufacturer's instructions.

Step-by-step Procedure:

1. Prepare a plasmid dilution by transferring 1 µl of 40mM Plasmid (stock) into 3µl of molecular water. Briefly place on ice.

2. Thaw, on ice, one 50 µL vial of One Shot® cells for each ligation/transformation.

3. Pipet 1µl of plasmid dilution (from step 1) directly into the vial of competent cells and mix by tapping gently. Do not mix by pipetting up and down. The remaining ligation mixture(s) can be stored at −20°C.

4. Incubate the vial(s) on ice for 30 minutes.

5. Incubate for exactly 30 seconds in the 42°C water bath. Do not mix or shake.

6. Remove vial(s) from the 42°C bath and place them on ice.

7. Add 250 µL of pre-warmed (room temperature) S.O.C medium to each vial. S.O.C is a rich medium; sterile technique must be practiced to avoid contamination.

8. Place the vial(s) in a microcentrifuge rack on its side and secure with tape to avoid loss of the vial(s). Shake the vial(s) at 37°C for exactly 1 hour at 225 rpm in a shaking incubator.

9. Spread 100µL from each transformation vial on separate, labeled LB agar plates. The remaining transformation mix may be stored at 4°C and plated out the next day, if desired.

10. Invert the plate(s) and incubate at 37°C overnight.

11. Select isolated colonies and pick to LB medium with ampicillin.

Protocol for Plasmid DNA Purification using QIAGEN Plasmid Midi Kit

This protocol is designed for preparation of up to 100µg of high or low copy plasmid DNA using the QIAGEN Plasmid Midi Kit. Consult the manufactures instructions for additional information.

Before starting:
Prepare Buffer P1 according to step 5
Prepare Buffer P2
Prepare buffer P3

Step-by-step Procedure:

1. Pick up to 2 colonies per plate from a freshly streaked selective plate and inoculate a starter culture of 5 ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8 hr. at 37°C with vigorous shaking (approx. 300 rpm).

Use a tube or flask with a volume of at least 4 times the volume of the culture.

2. Dilute the starter culture 1/500 to 1/1000 into selective LB medium. For high-copy plasmids, in a 250ml flask inoculate 25 ml medium with 50 µl of starter 8hr culture. Grow at
37°C for 12–16 h with vigorous shaking (approx. 300 rpm). Either change shaking incubator platform or attach flask holder to allow for vigorous shaking.

Use a flask or vessel with a volume of at least 4 times the volume of the culture. The culture should reach a cell density of approximately 3–4 x 109 cells per milliliter, which typically corresponds to a pellet wet weight of approximately 3 g/liter medium.

3. Harvest the bacterial cells by centrifugation at 6000 x *g* for 15 min at 4°C. Transfer to 50ml centrifuge tubes (falcon or equivalent). Decant the supernatant and retain the pellet.
If you wish to stop the protocol and continue later, freeze the cell pellets at –20°C.

4. Resuspend the bacterial pellet in 4 ml Buffer P1. Vortex in Falcon tubes until no clumps are visible.

For efficient lysis, it is important to use a vessel that is large enough to allow complete mixing of the lysis buffers. Ensure that RNase A has been added to Buffer P1.
If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle before use to ensure LyseBlue particles are completely resuspended. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

5. Add 4 ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4–6 times, and incubate at room temperature (15–25°C) for 5 min. Total volume is now 8ml.

Do not vortex, as this will result in shearing of genomic DNA. The lysate should appear viscous. Do not allow the lysis reaction to proceed for more than 5 min. After use, the bottle containing Buffer P2 should be closed immediately to avoid acidification from CO2 in the air.
If LyseBlue has been added to Buffer P1, the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously colored suspension.
If the suspension contains localized colorless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colored suspension is achieved.

6. Add 4 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 15 min. Total volume is now 12ml.

Precipitation is enhanced by using chilled Buffer P3 and incubating on ice. After addition of Buffer P3, a fluffy white material forms and the lysate becomes less viscous. The precipitated material contains genomic DNA, proteins, cell debris, and KDS. The lysate should be mixed

thoroughly to ensure even potassium dodecyl sulfate precipitation. If the mixture still appears viscous, more mixing is required to completely neutralize the solution.
If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colorless. A homogeneous colorless suspension indicates that the SDS has been effectively precipitated.

7. Centrifuge 12 ml volume at ≥20,000 x *g* for 30min at 4°C. Remove supernatant containing plasmid DNA promptly. Use high-speed centrifuge (in BSL3 or equivalent). If BSL3 centrifuge is utilized, all steps there after must take place within the BSL3.

Before loading the centrifuge, the sample should be mixed again. Centrifugation should be performed in non-glass tubes (e.g., polypropylene). After centrifugation the supernatant should be clear.

8. Centrifuge the supernatant again at ≥20,000 x *g* for 15 min at 4°C. Remove supernatant containing plasmid DNA promptly.

This second centrifugation step should be carried out to avoid applying suspended or particulate material to the QIAGEN-tip. Suspended material (causing the sample to appear turbid) can clog the QIAGEN-tip and reduce or eliminate gravity flow.

9. Equilibrate a QIAGEN-tip 100 by applying 4 ml Buffer QBT, and allow the column to empty by gravity flow. Prepare Qiagen-Tip 100 during centrifugation by adding 4ml of Buffer QBT.

Flow of buffer will begin automatically by reduction in surface tension due to the presence of detergent in the equilibration buffer. Allow the QIAGEN-tip to drain completely. QIAGEN-tips can be left unattended, since the flow of buffer will stop when the meniscus reaches the upper frit in the column.

10. Apply the supernatant from step 8 to the QIAGEN-tip immediately after centrifugation and allow it to enter the resin by gravity flow.

The supernatant should be loaded onto the QIAGEN-tip promptly. If it is left too long and becomes cloudy due to further precipitation of protein, it must be centrifuged again or filtered before loading to prevent clogging of the QIAGEN-tip.

11. Wash the QIAGEN-tip twice with 10 ml Buffer QC.

Allow Buffer QC to move through the QIAGEN-tip by gravity flow. The first wash is sufficient to remove contaminants in the majority of plasmid DNA preparations. The second wash is especially necessary when large culture volumes or bacterial strains producing large amounts of carbohydrates are used.

12. Elute DNA with 5 ml Buffer QF into a centrifuge tube.

Collect the eluate in a 15 ml or 50 ml tube (not supplied). Use of polycarbonate centrifuge tubes is not recommended as polycarbonate is not resistant to the alcohol used in subsequent steps. For constructs larger than 45–50 kb, prewarming the elution buffer to 65°C may help to increase yield.

Optional: If you wish to stop the protocol and continue later, store the eluate at 4°C. Storage periods longer than overnight are not recommended.

13. Precipitate DNA by adding 3.5 ml (0.7 volumes) room-temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at ≥15,000 x *g* for 30 min at 4°C. Carefully decant the supernatant.

All solutions should be at room temperature to minimize salt precipitation, although centrifugation is carried out at 4°C to prevent overheating of the sample. Alternatively, disposable conical bottom centrifuge tubes can be used for centrifugation at 5000 x g for 60 min at 4°C. Isopropanol pellets have a glassy appearance and may be more difficult to see than the fluffy, salt-containing pellets that result from ethanol precipitation. Marking the outside of the tube before centrifugation allows the pellet to be more easily located. Isopropanol pellets are also more loosely attached to the side of the tube, and care should be taken when removing the supernatant.

14. Wash DNA pellet with 2 ml of room-temperature 70% ethanol, and centrifuge at ≥15,000 x *g* for 10 min. Carefully decant the supernatant without disturbing the pellet.

Alternatively, disposable conical-bottom centrifuge tubes can be used for centrifugation at 5000 x g for 60 min at 4°C. The 70% ethanol removes precipitated salt and replaces isopropanol with the more volatile ethanol, making the DNA easier to redissolve.

15. Air-dry the pellet for 5–10min, and redissolve the DNA in a suitable volume of buffer (e.g., TE buffer, pH 8.0, or 10 mM Tris·Cl, pH 8.5). Add approx. 100µl of TE Buffer to dissolve the plasmid DNA.

Redissolve the DNA pellet by rinsing the walls to recover the DNA, especially if glass tubes have been used. Pipetting the DNA up and down to promote resuspension may cause shearing and should be avoided. Overdrying the pellet will make the DNA difficult to redissolve. DNA dissolves best under slightly alkaline conditions; it does not easily dissolve in acidic buffers.

16. Plate the Plasmid onto a Blood Agar Plate and incubate for 72hrs. Plate may be checked every 24hrs for growth. If no growth is observed after 72hrs, the plasmid can be removed from the BSL3.

Quantification

1. Determine the concentration of plasmid DNA recovered using the Thermo Scientific NanoDrop instrument (or other spectrophotometer). Further dilutions can be made with TE Buffer to achieve target concentration of 40mM.

2. Additional dilutions should be made according to the *Vibrio parahaemolyticus* and *Vibrio vulnificus* enumeration and detection through MPN and real-time PCR assay protocol.

3. Plasmid dilutions must be r run on a PCR Detection System to verify concentration, and ensure the amplification falls within its expected Ct range.

4. 10µl aliquots of 1:100 dilution will serve as freezer stock and be stored at -15°C or below. Working stocks can be prepared by adding 990µl molecular grade water to the freezer stock, and should be stored at 2-8°C. On day of use, dilute working stock an additional 1:100. The final concentration of the final product is 1:100,000.

Appendix C- Environmental Mastermix

According to the manufacturer's product information, the The TaqMan® Environmental Master Mix 2.0 is supplied in a 2✕ concentration and contains: AmpliTaq Gold® DNA Polymerase, UP (Ultra Pure), dNTPs with dUTP, ROX™ Passive Reference and Optimized buffer components.

Appendix D – Primer/Probe Mix Worksheets

MultiPlex 1 Primer/Probe Mixes

Calculations for individual aliquots are based on 100rxn increments. When making in increments other than multiples of 100 aliquot and H2O calculations will not be correct.

	Date Made:			Analyst:		
# of Rxn	**tlh mix**	**Volume (µL of 100µM stock)**	**n**	**[FINAL]**	**Total**	**Lot #**
5000			5000			
	tlh F	0.020	5000	20000	**100**	
	tlh R	0.020	5000	20000	**100**	
	tlh Probe	0.015	5000	15000	**75**	
	TE Buffer	0.045	5000		**225**	
	Total Volume				**500**	
	Individual Aliquots	20uL			**25**	Number of Aliquots
	Total Molecular H2O to be added before use				**380uL**	Tubes should be labeled as needing this amount of H2O
	IC mix	**Volume (µL of 100µM stock)**	**n**	**[FINAL]**	**Total**	**Lot #**
			5000			
	IC F	0.020	5000	20000	**100**	
	IC R	0.020	5000	20000	**100**	
	IC Probe	0.020	5000	20000	**100**	
	TE Buffer	0.040	5000		**200**	
	Total Volume				**500**	
	Individual Aliquots	20uL			**25**	Number of Aliquots
	Total Molecular H2O to be added before use				**380uL**	Tubes should be labeled as needing this amount of H2O
	vvha mix	**Volume (µL of 100µM stock)**	**n**	**[FINAL]**	**Total**	**Lot #**
			5000			
	vvha F	0.080	5000	40000	**400**	
	vvha R	0.080	5000	40000	**400**	
	vvha probe	0.040	5000	20000	**200**	
	Total Volume				**1000**	
	Individual Aliquots	40uL			**25**	Number of Aliquots
					360uL	

Total Molecular H2O to be added before use					Tubes should be labeled as needing this amount of H2O

MultiPlex 2 Primer/Probe Mixes

Calculations for individual aliquots are based on 100rxn increments. When making in increments other than multiples of 100 aliquot and H2O calculations will not be correct.

	Date Made:			Analyst:		
# of Rxn	**trh mix**	**Volume (µL of 100µM stock)**	**n**	**[FINAL]**	**Total**	**Lot #**
5000			5000			
	trh F	0.100	5000	25000	**500**	
	trh GlovR	0.160	5000	40000	**800**	
	trh 731bR	0.080	5000	20000	**400**	
	trh Probe	0.050	5000	12500	**250**	
	TE Buffer	0.010	5000		**50**	
	Total Volume				**2000**	
	Individual Aliquots	80uL			**25**	Number of Aliquots
	Total Molecular H2O to be added before use				**320uL**	Tubes should be labeled as needing this amount of H2O
	tdh mix	**Volume (µL of 100µM stock)**	**n**	**[FINAL]**	**Total**	**Lot #**
			5000			
	tdh F	0.080	5000	40000	**400**	
	tdh R	0.080	5000	40000	**400**	
	tdh Probe	0.030	5000	15000	**150**	
	TE Buffer	0.010	5000		**50**	
	Total Volume				**1000**	
	Individual Aliquots	40uL			**25**	Number of Aliquots
	Total Molecular H2O to be added before use				**360uL**	Tubes should be labeled as needing this amount of H2O
	orf8 mix	**Volume (µL of 100µM stock)**	**n**	**[FINAL]**	**Total**	**Lot #**
			5000			
	orf8 F	0.140	5000	40000	**700**	
	orf8 R	0.140	5000	40000	**700**	
	orf8 probe	0.050	5000	16667	**250**	

TE Buffer	0.010	5000		**50**	
Total Volume				**1700**	
Individual Aliquots	60uL			**28.333**	Number of Aliquots
Total Molecular H2O to be added before use				**340uL**	Tubes should be labeled as needing this amount of H2O

Appendix D- Mastermix preparation for 20 reactions:

Multiplex 1	**Vol. (µl)**	**n**	**[FINAL]**	**Total**
		20		
tlh primer/probe mix	2	20		**40**
IC primer/probe mix	2	20		**40**
vvha primer/probe mix	2	20		**40**
Molecular H_2O	2	20		**40**
Env MM	10	20	1x	**200**
Mastermix Volume	**18µl**			**360**
Template	**2.0 µl**			
Multiplex 2	**Vol. (µl)**	**n**	**[FINAL]**	**Total**
		20		
tdh primer/probe mix	2	20		**40**
trh primer/probe mix	2	20		**40**
orf8 primer/probe mix	2	20		**40**
Molecular H_2O	2	20		**40**
Env MM	10	20	1x	**200**
Mastermix Volume	**18µl**			**360**
Template	**2.0 µl**			
18µl Master Mix 2.0ul template				

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CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* detection in Oysters [PART IV] QuantStudio DX

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15-minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11**	**Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ 0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22	All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.

K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3**	**The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14	Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.

C	**2**		**1.6.16**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18**	**The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19	Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation		
K	13, 14		1.7.1	Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2	Media components are properly stored in a cool dry place.
O	6		1.7.3	Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**6**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**6**		**1.7.6**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (<0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8	The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9**	**Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4	Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5	Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6	All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II – SAMPLES AND PROCESSING				
		2.1 Sample Collection, Transportation and Receipt		
C	**2, 6**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**

K	5		2.1.2	Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3	Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 6		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2	Blades of shucking knives are not corroded.
K	5		2.2.3	The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6	Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7	Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9**	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10**	**A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11**	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14**	**Samples are blended for 60 to 120 seconds until homogenous.**
		2.3 APW Enrichment		
K	5		2.3.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**2.3.2**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**2.3.3**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution_____**

C	**2, 15, 23**		**2.3.4 For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **An uninoculated APW blank will serve as the uninoculated process control.** **The process control cultures accompany the samples throughout the method. Records are maintained.**
C	**13**		**2.3.5 Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**2.3.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
PART III- PCR METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN OYSTERS SMARTCYCLER II AND AB 7500 FAST			
		3.1 PCR Reagents	
C	**14, 15**		**3.1.1 Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.1.2 Fluorescent probes are stored in light occluding tubes or containers.
C	**14, 15, 18, 19**		**3.1.3 The PCR forward and reverse primers and probes are appropriate for the platform.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE/TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.1.4 Lyophilized forward and reverse primers, and probes, are reconstituted with TE buffer to produce a 0.1 mM stock solution.**
C	**14, 18**		**3.1.5 Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.1.6 Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**

C	**21, 22**		**3.1.7**	**Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.1.8**	**PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.2 DNA Extraction		
C	**14, 18**		**3.2.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.2.2**	**Pipet tips have aerosol barriers.**
K	14, 18		3.2.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.2.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.2.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.2.6**	**One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.2.7**	**Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.2.8	A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.2.9**	**After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**
K	14, 18		3.2.10	Frozen extracts are analyzed within 6 months of frozen storage.
		3.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**3.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.3.2**	**For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.3.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.3.4**	**Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.3.5**	**Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.4 PCR		
C	**14, 19**		**3.4.1**	**If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.4.2**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.4.3	Two (2) µL of APW blank extracted from the uninoculated process control is added to a reaction tube or plate well containing 23µL of Master Mix.
K	14, 19		3.4.4	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
K	14, 19		3.4.5	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
C	**14, 19**		**3.4.6**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix as a no template control. A no template control is included with each PCR run.**

C	**14, 19**		**3.4.7**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control. A positive PCR control is included with each PCR run.**
K	14, 19		3.4.8	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.4.9**	**After centrifugation, tubes or plates are inserted into the instrument.**
		3.5 PCR Amplification		
C	**14, 19**		**3.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		3.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.5.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.6 Computation of Results		
K	14, 19		3.6.1	All runs in which the positive PCR control generates a Ct value for the target(s) of interest and the no template control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**3.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**3.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control is considered invalid and should be re-tested.**
C	**13**		**3.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from either the calculator or MPN table in conjunction with dilution selection tool in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.6.8	For APW enrichment, results are reported as MPN/g of sample.

PART IV- PCR METHOD FOR *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* DETECTION IN OYSTERS QUANTSTUDIO DX			
		4.1 PCR Reagents	
C	**14, 15**	**4.1.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15	4.1.2	Fluorescent probes are stored in light occluding tubes or containers.
C	**26**	**4.1.3**	**The PCR forward and reverse primers, and probes target.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3' Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3' Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3' WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3' WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3' Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3' Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3' Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3' Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3' TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3' TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3' Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ **<u>For Vv Real-time PCR Method</u>** vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3' vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3' vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGBNFQ
C	**14, 18**	**4.1.4**	**Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**26**	**4.1.5**	**Storage of thawed working stocks of primers and probes are stored between 2-8°C, not to exceed 2 weeks.**
C	**26**	**4.1.6**	**Storage of aliquoted working stocks of primers and probes stored in -20°C manual defrost freezer does not exceed 1 year.**
C	**26**	**4.1.7**	**Taqman Environmental Mastermix 2.0 is stored in -20°C manual defrost freezer until first use. After first use, it is stored between 2-8°C.**
C	**26**	**4.1.8**	**Internal control (IC) is stored in -20°C manual defrost freezer until first use. After first use, they are stored between 2-8°C.**
		4.2 DNA Extraction	
C	**14, 18**	**4.2.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**	**4.2.2**	**Pipet tips have aerosol barriers.**
K	14, 18	4.2.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18	4.2.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**	**4.2.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**

C	**26**		**4.2.6**	**Two-hundred (200) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**26**		**4.2.7**	**For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.**
K	26		4.2.8	Extracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen extracts are analyzed within 1 month of frozen storage.
C	**26**		**4.2.9**	**A tlh+ trh+ tdh+ *V. parahaemolyticus* (WA4647 or equivalent), a tlh+ tdh+ Orf8+ *V. parahaemolyticus* (BAA-240 or equivalent), and vvha+ *V. vulnificus* (ATCC 27562 or equivalent) cultures are extracted and combined to serve as the positive PCR (amplification) control.**
		4.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**4.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**26**		**4.3.2**	**For each reaction, add the specified amount of water, appropriate primer and probe concentrations, and Taqman Environmental Master Mix.**
K	14, 16, 18		4.3.3	The Master Mix is vortexed to mix constituents and then briefly spun immediately prior to dispensing aliquots to reaction tubes or plates.
C	**14, 16, 18, 26**		**4.3.4**	**Eighteen (18) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**4.3.5**	**Master Mix must be used on the day of preparation or stored at –20°C until time of use.**
		4.4 PCR		
C	**14, 19, 26**		**4.4.1**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 18µL of Master Mix for a total PCR reaction volume of 20µL.**
K	14, 19, 26		4.4.2	Two (2) µL of extracted blank APW from the uninoculated process control is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.3	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.4	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
C	**14, 19, 26**		**4.4.5**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as a no template control.**
C	**14, 19, 26**		**4.4.6**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as the positive PCR (amplification) control.**
K	14, 19, 26		4.4.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 30 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**16**		**4.4.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		4.5 PCR Amplification		
C	**14, 19**		**4.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		4.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**4.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		4.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**4.5.5**	**The analysis settings are adjusted as specified in the protocol.**

		4.6 Computation of Results		
K	14, 19		4.6.1	All runs in which the NTC has no amplification; positive control generates a Ct value for the target(s) of interest; and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	2		**4.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**4.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**4.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		4.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**4.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control (except NTC) is considered invalid and should be re-tested.**
C	**13**		**4.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		4.6.8	For APW enrichment, results are reported as MPN/g of sample.

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PCR MICROBIOLOGY COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the PCR Microbiology Component:

1. **Does Not Conform Status**: The PCR Microbiology component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥4 or**
   b. The total # of Key nonconformities is **≥13 or**
   c. The total # of Critical, Key and Other is **≥18**
2. **Provisionally Conforms Status**: The PCR Microbiology component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *6 – PCR Microbiology Checklist*, Rev. December 2025

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Thomas Howell
Affiliation	Spinney Creek Shellfish Inc.
Email	tlhowell@spinneycreek.com
Proposal Subject	Guidance for Assessing the Viral Impact from Waste Water Treatment Plant Outfall on Adjacent Growing Areas using the Male-specific Coliphage Method on Effluent Samples.
Specific NSSP Guide Reference	Section IV Guidance Documents - Chapter II. Growing Areas - .19 Classification of the Shellfish Growing Waters Adjacent to Waste Water Treatment Plants
Text of Proposal/ Requested Action	The requested action is that an ISSC committee be formed to draft guidance language describing how to best use MSC effluent sampling techniques to assess the viral impact on adjacent growing areas. This proposed action is the result of recent collaborative work funded by New Hampshire Sea Grant. The PI's and project participants on this project included University of New Hampshire Sea Grant, Connecticut Sea Grant, Spinney Creek Shellfish, Connecticut Department of Agriculture, New Hampshire Department of Environmental Services, US Food and Drug Administration Center for Food Safety and Applied Nutrition, and US Food and Drug Administration Gulf Coast Seafood Laboratory. An optimized method to determine MSC in effluent samples, both pre-treatment (disinfection) and final effluent has been submitted to the Lab Committee for approval. Two years of field studies were recently completed which looked closely at 2 plants in CT and 4 plants in NH. Results of these field studies were reported at the 2019 NESSA meeting in Plymouth MA. By taking effluent samples from WTP's two to three times per week over an extended period, a database can be assembled including Geomean and P95 values in a strategy consistent with NSSP practices. Plotting the effluent time-series data can be used to identify times when plant performance is degraded by predictable, challenging, conditions whether they are operational or environmental. By informing dye study work with WWTF effluent analysis, much more informed decisions can be made with respect to classification of adjacent growing waters. Simply multiplying the P95 results from final effluent statistical analysis by the dilution line in question, an upper level of MSC concentration MSC in the growing waters can be estimated. An interpretation matrix for final effluent MSC time- series analysis to interpret results in a relative way is proposed.
Public Health Significance	The Public Health Significance of this proposal is substantial. Dye studies alone are protective of public health using the 1000:1 dilution line for classification purposes. However, MSC assessment of effluent samples gives a much more informed picture of how appropriate the 1000:1 line is in a particular situation. If an under-designed, problematic WWTP is not adequately deactivating viruses, a higher dilution may be required. This is an important consideration when

	dealing with a WWTP that does not perform to typical standards of secondary treatment with effective disinfection. However, the study has shown that many modern and advanced WWTPs can be reliably operated at sufficient performance levels to justify the 300:1 dilution line for the establishment of a prohibited classification around the WWTP outfall. As time continues and WWTPs are upgraded, this method and technique may permit increased utility of the growing area between the 300:1 and 1000:1 dilution line. In conclusion, public health can be informed and optimized while maximum commercial utilization of growing areas can be achieved.
Cost Information	The MSC method for WWTP effluent samples is inexpensive and easy to perform. Costs become more significant when one considers the personnel and travel time needed to sample the WWTP's. The state control agency can optimize this work by focusing field work during the winter months when the WWTP are likely more challenged and personnel resources are more available.
Action by 2019 Task force I	Recommended referral of Proposal 19-144 to an appropriate committee as determined by the Conference Chairman.
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 19-144.
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 19-144.
Action by 2023 Male Specific Coliphage Committee	Recommended referral of Proposal 19-144 to an appropriate committee as determined by the Conference Chairman.
Action by 2023 Task Force I	Recommended adoption of the Male Specific Coliphage Committee recommendation for Proposal 19-144.
Action by 2023 General Assembly	Adopted recommendation of Task Force I on Proposal 19-144.
Action by FDA July 7,2023	Concurred with Conference action on Proposal 19-144.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Guidance on Cleansing Studies
Specific NSSP Guide Reference	NSSP Section IV Chapter II .19 VI B.
Text of Proposal/ Requested Action	A. Guidance for a Conditional Area Management Plan The management plan for a growing area in the conditionally approved or conditionally restricted classification must meet certain minimum requirements to ensure that the safety of the shellfish for human consumption is maintained. The use and success of the conditional classification depends upon a thorough and accurate management plan. Therefore, it is important that all aspects of the management plan be fully considered and implemented. The minimum requirements to be addressed are: (1) An understanding of and an agreement to the conditions of the management plan by the one (1) or more Authorities involved, other local, State and Federal agencies which may be involved, the affected shellfish industry, and the persons responsible for the operation of any treatment plants or other discharges that may be involved; (2) A written management plan for the growing area being placed in the conditional classification, which includes a general description of the growing area with a map showing the area's boundaries, and which addresses all items in C. through H. (3) A sanitary survey that shows the growing area will be in the open status of its conditional classification for reasonable periods of time. The survey must provide a description of the factors determining the growing area's suitability for being classified conditionally approved or conditionally restricted, and the supporting information and data. (4) A description of the predictable pollution event or events that are being managed and the performance standards established for each pollution source contributing to the pollution event including: (a) For a wastewater treatment facility, the performance standard should be

based on:
(i) Peak effluent flow
(ii) Bacteriological quality of the effluent
(iii) Physical and chemical quality of the effluent
(iv) Bypasses from the treatment plant or its collection system
(v) Design, construction, and maintenance to minimize mechanical failure or overloading (i.e., the reliability of the treatment system and collection system components)

(vi) Provisions for verifying and monitoring efficiency of the wastewater treatment plant and the feedback system for addressing inadequate treatment.
(vii) Identification of conditions that lead to WWTP failure, a lapse in WWTP treatment leading to untreated or partially treated sewage discharge, and closure of the conditionally approved area.

(b) For meteorological or hydrological events, the performance standard should be based on:
(i) Identification of the specific meteorological and/or hydrologic event that will cause the growing area to be placed in the closed status;
(ii) Discussion and data analyses concluding that effects on water quality from these specific meteorological and/or hydrologic events are predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for the growing area placed in the conditional classification; and
(iii) The predicted number of times, based on historical findings, that the pollution event will occur within one (1) year.

(c) For seasonal events, such as marina operation, seasonal rainfall, and waterfowl migration, the performance

	standard should be based on: (i) Identification of the seasonal event that will cause the growing area to be placed in the closed status, including its estimated duration; and (ii) Discussion and data concluding that the seasonal event is predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for a growing area placed in the conditional classification; (5) A description of the plan for monitoring water quality including numbers and frequency; (6) A description of how the closed status for the conditional classification will be implemented, which must include: (a) A clear statement that when the performance standards are not met, the growing area will immediately be placed in the closed status; (b) A requirement to notify the Authority or Authorities that the management plan performance standards have not been met, including: (i) The name of the agency or other party responsible for notifying the Authority; (ii) The anticipated response time between the performance standards not being met and notification of the Authority; and (iii) The procedures for prompt notification including contingencies such as night, weekend and absences of key personnel; (c) A description of the implementation and enforcement, including: (a) The response time between the notification to the Authority of the failure to meet performance standards and activation of the legal closure of the growing area by the Authority; (b) The procedures and methods to be used to notify the shellfish industry; and

(c) The procedures and methods to be used to notify the patrol agency (enforcement agency) including:
- The name of the responsible patrol agency;
- The anticipated response time between the Authority's legal closure of the growing area and notification of closure to the patrol agency; and
- A description of the patrol agencies anticipated activities to enforce the closed status.

(7) A description of the criteria that must be met prior to reopening a growing area in the closed status, including the need to determine that:
(a) The performance standards established in the management plan are again fully met;
(b) The flushing time for pollution dissipation is adequate;
(c) A time interval has elapsed which is sufficient to permit reduction of human pathogens as measured by the coliform indicator group in the shellstock; . Studies shall be conducted to document the time interval necessary for the reduction of coliform levels in the shellstock to pre- closure levels. The Authority shall develop and implement a study design that includes:
(i) The utilization of NSSP-conforming laboratories and NSSP-approved methods to analyze coliform in shellstock and water.
(ii) Establishing a pre-closure coliform baseline in shellstock for each species under consideration in the conditional area management plan.
(iii) If re-opening is to be based on coliform levels in the water, identify and describe an association between coliform levels in shellstock for each species under consideration in the conditional area management plan and coliform levels in growing area water.

(iv) Defining conditions under the conditional area management plan which considers various factors including water temperature, salinity, seasonality,and other environmental conditions that may affect the pumping

activity of each species of shellstock under consideration.

(i)(v) A study design and data analysis approach providing statistical reliability. At a minimum, this should include consideration of:

- variability of measurements of indicator levels in replicate samples
- the likelihood or probability that a significant difference in indicator levels will be identified based on the sample outcomes if a substantial difference exists between the populations being sampled.

Irrespective of the type of study design, these considerations apply and should be used to ensure that the number of samples collected is adequate. The number of samples needed increases with increasing variability of the measurements. When there is a substantial difference between indicator levels in the populations being sampled, the study should have at least an 80% probability of identifying this as such.

(ii)(vi) Determining the time interval for post- closure coliform levels in shellstock and water to return to the pre-closure established baseline.

(d) When utilizing MSC in shellstock in growing areas subjected to suspected human sewage to reopen a closed growing area, studies (utilizing the same format as (c) above) establishing sufficient elapsed time shall document the interval necessary for reduction of viral levels in the shellstock. The utilization of NSSP- conforming laboratories and NSSP-approved methods to analyze MSC in shellstock. Analytical shellstock sample results shall not exceed a level of 50 MSC per 100 grams or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions. These studies may establish criteria for reopening based on viral levels in the shellfish meats or the area must be in the closed status until the event is over and twenty-one (21) days have passed;

(d)(e) Where necessary, the bacteriological quality of the water must be verified; and

(e)(f) Shellstock feeding activity is sufficient to achieve reduction of pathogens to levels present prior to the pollution event.

(8) A commitment to a reevaluation of the management plan at least annually using, at a minimum, the reevaluation requirements in the NSSP Model Ordinance.

Public Health Significance	This language will provide state shellfish Authorities with guidance regarding establishing the elapsed time to reopen closed conditional management areas and assure that shellstock are not adulterated. The public health significance of the proposed guidance for statistical reliability of studies used to establish an elapsed time to reopen is evident by considering an example of the effect of application of these criteria. While several different types of study designs are suitable to identify a minimum elapsed time for pathogen reduction, a common approach is to compare mean log concentrations of fecal indicators in a group of samples collected pre-closure, and representative of baseline, to that in a group of samples collected at the candidate elapsed time post-closure. For this type of study, a two-sample one-sided t-test is typically applied to test the null hypothesis that mean log concentrations are equal. If the test statistic is statistically significant (i.e., $p<0.05$), the null hypothesis is rejected; otherwise, mean concentrations are considered equivalent and the candidate elapsed time sufficient for pathogen reduction. To satisfy the proposed criteria of statistical reliability the sample size of the study will need to be large enough to achieve, based on expected variability of sample measurements about mean levels, an 80% probability of rejecting the null hypothesis when a minimally consequential difference in means exists. This determination of the sample size is made based on what is called the power function of the test statistic. Explicit formula and/or software to calculate sample sizes based on power functions are widely available for most commonly used hypothesis tests and test statistics. Using such calculations, it can be determined that, when the expected standard deviation of log sample measurements about mean levels is 0.5 logs, the example study design requires 13 samples per group to achieve 80% power (probability) to reject the null hypothesis when a true difference in means of 0.5 logs exists. Consequently, when a difference in means of 0.5 logs is considered consequential, a study of this type with fewer than 13 samples per group would not be considered sufficiently reliable. With an expected standard deviation of 0.5 logs, a sample size of 3 per group would have only a 27% probability of rejecting the null hypothesis when a consequential difference in means of 0.5 logs exists and an 80% probability of rejecting the null hypothesis would be achieved only when the true difference in means is equal to or greater than 1.25 logs
Cost Information	No additional cost. This is simply providing guidance for a requirement already in place
Action by 2019 Task Force I	Recommended referral of Proposal 19-145 to an appropriate committee as determined by the Conference Chairperson with the following instructions to develop guidance for cleansing studies and to assess scenarios where water quality sampling could be used in place of cleansing studies
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 19-145.
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 19-145.

Action by 2023 Cleansing Study Committee	**Recommended:** 1) The committee recommended adoption of Proposal 19-145 as substituted. Guidance on Studies Used in the Reopening of an Area Temporarily Placed in the Closed Status Due to an Emergency Condition, a Discharge of Raw Sewage, or when Conditional Area Management Plan (CAMP) Performance Standards are not Met *Note: Similar contaminant reduction studies associated with shellstock relaying and validation studies associated with the depuration process are not covered in this g uidance document. Instead, each has their own specific requirements which are covered in Chapter V. and Chapter XV., as well as Guidance Documents Chapter II .10 and .19, respectively.* A. When Are Studies Required? Per Chapter IV. @.03 A.(5)(d) and C.(2)(c), studies are required for reopening a closed area to establish the environmental conditions and time required for pathogens (as measured by microbiological indicators) in shellstock and water to return to acceptable levels following the impact from an emergency condition, discharge of raw sewage, or when conditional area management plan (CAMP) performance standards are not met. Listed below is a summary of scenarios for reopening options: *1) Scenarios where studies are required to reopen once the emergency situation or condition has returned to normal, or CAMP performance standards are fully met, and sufficient time has elapsed to allow the shellstock to reduce pathogens and for the growing area water quality to return to acceptable levels:* (a) Chapter IV. @.03A.(5)(d): • Reopening due to closures resulting from an emergency condition or situation when pathogens are of concern (other than raw untreated sewage discharged from a sewage collection system or WWSD), studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. Such coliform studies may establish criteria for reopening based on coliform levels in the water. • Reopening due to emergency closures caused by the occurrence of raw untreated sewage discharged from a sewage collection system or WWSD, when the closure duration is less than 21 days or when analytical shellstock samples are utilized for comparison to the levels established in the Chapter IV. @.02 E. (4). The authority may use studies to establish pre-determined male-specific coliphage (MSC) levels in shellfish samples that are conducted no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area

potentially impacted.

(b) Chapter IV. @.03 C.(2)(c)(iii):
For management plans based on WWSD function or pollution sources other than WWSD criteria that reliably predict when an area that was placed in the closed status because of failure to comply with its conditional management plan can be returned to the open status.

- Reopening due to closures impacted by pathogens (other than raw untreated sewage discharged from a sewage collection system or WWSD) from a failure to comply with its conditional management plan, studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. These studies may establish criteria for reopening based on coliform levels in the water.

- Reopening due to temporary closures impacted by sewage from a failure to comply with the conditional management plan based on the WWSD performance standards, studies may be conducted to establish sufficient elapsed time and shall document the interval necessary for reduction of viral levels in the shellstock. These studies may establish pre-determined levels based on regional species under regional conditions. These studies may establish criteria for reopening based on viral levels in shellfish meats.

2) Scenarios where sampling is required to reopen when a study is not conducted, include:

(a) Chapter IV. @.03A.(5)(d):

- Reopening due to emergency closures of harvest areas caused by the occurrence of raw untreated sewage discharged from a sewage collection system or WWSD, when the closure duration is intended to be less than 21 days, the analytical sample results shall not exceed the levels established in Chapter IV. @.02 E. (4).

- Reopening due to emergency closures of harvest areas when poisonous or deleterious substances are the concern, sampling shall establish that poisonous or deleterious substances in shellstock do not exceed FDA action levels, tolerances, guidance levels, and levels that are deemed unsafe through risk evaluation.

(b) Chapter IV. @.03C.(2)(c)(iii):

- Reopening due to temporary closures impacted by sewage from a failure to comply with the conditional management

	plan based on the WWSD performance standards, analytical sample results shall not exceed the MSC level established in Chapter IV. @.02 E. (4). • Water quality sampling can be used to reopen an area following temporary closures resulting from a failure to comply with conditional management plan performance standards based on the effects of non- point sources of pollution such as rain events and/or stormwater runoff. *3) Scenarios where no studies or sampling are required to reopen, include:* (a) Chapter IV. @.03A.(5)(d)(ii) and C.(2)(c)(iii): • Reopening due to the temporary closure from a discharge of raw untreated sewage or exceedance of management plan performance standards relating to WWTP function. If no studies or analytical samples are collected and compared to the levels established in Chapter IV. @.02 E. (4), the area must be in the closed status until the event is over and twenty-one (21) days have passed. 2) proposal be referred back to an appropriate committee as determined by the conference chair to allow for further development of additional sections of the Guidance Document. 3) expanding the charge of the committee to include reviewing Model Ordinance language relating to cleansing studies for reopening.
Action by 2023 Task Force I	Recommended adoption of the Cleansing Study Committee recommendation for Proposal 19-145
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 19-145.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 19-145

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Brooke Roman
Affiliation	Neogen Corporation
Email	broman@neogen.com
Proposal Subject	Neogen's 'Reveal 2.0 for PSP' for detection of PSP
Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter II. Growing Areas, .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	The intention is for this method to be an Approved Limited Use Method for Biotoxin testing for PSP toxins under the NSSP (for mussels and oysters) and that it should appear in Section IV (Guidance Documents), Table 4 (Approved Limited Use Methods for Biotoxin Testing). Full SLV validation data is provided for mussels and oysters.
Public Health Significance	PSP is a serious intoxication which still occurs in the USA and elsewhere. The USFDA and the European Union (EU) have established action levels for PSP toxins at 800 ppb (800 µg/kg) STX equivalents in shellfish. PCOX, has been accepted as a quantitative reference method in the USA and some other countries, although Pre- COX is also accepted by regulatory agencies in other areas of the world such as the UK, various EU countries, AU and NZ. Shellfish need to be more easily screened for toxins that cause paralytic shellfish poisoning (PSP), and they need to be screened closer to growing/harvesting areas to better protect public health. A reliable and simple screening tool for end product testing (EPT) by industry, for community- based and remote surveillance, and for screening out negative samples from the regulatory sample stream. Implementation of these approaches would broaden the food safety net and reduce outbreaks of PSP intoxication. Neogen is the only antibody-based test to detect both the STX and NEO parts of the PSP family of toxins at similar levels. No other antibody-based rapid test for PSP can detect NEO to any significant degree. Other ISSC approved "rapid" methods for PSP screening are largely limited to laboratory settings because of complexity which limits their use in EPT and community-based and remote surveillance of shellfish resources. The only ISSC-approved LFA rapid method, the Scotia LFI, has had many issues with reliability that have limited its applicability in screening for PSP, and concerns about the stability of the method have also been published [1,2,3,4,5]. The Neogen Reveal 2.0 for PSP is an

	excellent candidate for rapid screening of shellfish for PSP toxins in both laboratory and field situations, and is an extension of a platform used by Neogen for many reliable rapid tests in the meat, dairy and food sectors, many of which are approved for use by FDA, USFDA and/or EPA. The test has undergone SLV and ILV evaluations [5,6]and has been shown to be an accurate and reliable candidate for approval for use in the NSSP. [1] Cefas 2006 [2] Turner et al. 2015 [3] Harrison et al. 2016 [4] Dorantes-Aranda et al.2017a [5] Jawaid et al. 2015 [6] Dorantes-Aranda et al. 2017b
Cost Information	Approximately $20 per test. Reader based assay – approximate cost of reader is $2,700.00 USD
Action by 2019 Laboratory Committee	Recommended referral of Proposal 19-150 to an appropriate committee as determined by the Conference Chair.
Action by 2019 Task Force	Recommended adoption of Laboratory Committee recommendation on Proposal 19-150.
Action by 2019 General Assembly	Adopted recommendation of Task Force I on Proposal 19-150.
Action by FDA February 21, 2020	Concurred with Conference action on Proposal 19-150.
Action by 2023 Laboratory Committee	Recommended referral of Proposal 19-150 to an appropriate committee as determined by the Conference Chairperson
Action by 2023 Task Force I	Recommended adoption of the Laboratory Committee recommendation for Proposal 19-150.
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 19- 150
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 19-150.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Robert Rheault
Affiliation	ECSGA
Email	Bob@ecsga.org
Proposal Subject	Data evaluation when the nonpoint sources impacting a growing area are not from a human sewage source
Specific NSSP Guide Reference	Section II. Model Ordinance; Chapter IV Growing Areas; Section @.02 Microbiological Standards F.1.
Text of Proposal/ Requested Action	F. Standard for the Approved Classification of Growing Areas when Evaluated for Nonpoint Sources. (1) Exception. (a) If the tidal stage increases the fecal coliform concentration, the authority shall use sample results collected during that tidal stage to classify the area. (b) If the Authority has documentation supporting that the nonpoint sources impacting the growing area are not from a human sewage origin they may exclude up to two outlier datapoints from the dataset being evaluated. (2) Pollution Sources. Growing areas shall be impacted only by randomly occurring, intermittent events. (3) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section E. (2) or Section F. (4). (4) Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median (or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed fourteen (14) per 100 ml and the estimated 90th percentile shall not exceed an MPN or MF (mTEC) of: (a) 43 MPN per 100 ml for a five-tube decimal dilution test; (b) 49 MPN per 100 ml for a three-tube decimal dilution test; or (c) 31 CFU per 100 ml for a MF (mTEC) test. (5) Estimated 90th Percentile. The estimated 90th percentile shall be calculated by: (a) Calculating the arithmetic mean and standard deviation of the sample result logarithms (base 10); (b) Multiplying the standard deviation in (a) by 1.28; (c) Adding the product from (b) to the arithmetic mean; (d) Taking the antilog (base 10) of the results in (c) to get the estimated 90th percentile; and (e) The MPN values that signify the upper or lower range of sensitivity of the MPN tests in the 90th percentile calculation shall be increased or decreased by one significant number. (6) Required Sample Collection (a) Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity

	and frequency as described in Section E.(3) for application of the standard under Section E. (2). (b) Systematic Random Sampling Standard. The requirement for systematic random sample collection shall be met when: (i) Sample station locations are adequate to produce the data to effectively evaluate all nonpoint sources of pollution; (ii)Sample collection is scheduled sufficiently far in advance to support random collection with respect to environmental conditions. Compliance requires that, prior to implementation, the schedule for random sampling shall be documented in the master file for the growing area, and if conditions at the time of scheduled sample collection are believed to be hazardous to the safety of the individuals assigned to collect samples, sample collection shall be rescheduled at a later date as soon as practical; (iii) A minimum of six (6) random samples shall be collected annually from each sample station in the growing area; (iv) A minimum of two (2) random samples shall be collected annually from each sample station in the growing area while in the inactive status. The sample collection frequency of six (6) random samples per station per year specified under @.02 F. (6)(b) (iii) must resume at least six (6) months before an area is reactivated; and (v) A minimum of the thirty (30) most recent randomly collected samples from each sample station shall be used to calculate the median or geometric mean and 90th percentile to determine compliance with this standard. (c) Transition from Adverse Pollution Condition Standard to Systematic Random Sampling Standard. If the Authority: (i) Does not have thirty (30) recent randomly collected sample results from each station, then the previous fifteen (15) samples collected under adverse pollution conditions may be used with the most recent random samples to meet the minimum thirty (30) sample requirement for a transition period not to exceed three (3) years; and (ii) Uses the transition period described in (i), as additional random samples are collected; the random samples shall replace chronologically the samples collected under adverse pollution conditions (e.g. sample 31 replaces sample 1).

Public Health Significance	It is recognized that on occasion water quality may be impacted by non-human sources such as birds. Scientific literature also indicates that the presence of human enteric pathogens in wild birds is overestimated with the use of the coliform indicator (Smith et al. 2021) https://doi.org/10.1111%2Fbrv.12581 If a few aberrant samples can be reliably attributed to birds it is likely that the closure of the harvest area is an unwarranted response
Cost Information	
Research Needs Information (Optional) a. Proposed specific research need/ problem to be addressed	At this time we do not have an estimate of the correlation of human enteric pathogens with coliforms in wild bird waste. Our growing area classification has been entirely built on the correlation between pathogens and coliforms in wastewater. Using the coliform standard to close harvest areas impacted by birds assumes the relationship is similar, when scientific literature indicates that the risk is being overestimated. Research is needed to describe the persistence of bird-sourced pathogens in the marine environment, and how long these pathogens persist in the shellfish if they are taken up by filter feeding bivalves
b. Explain the relationship between proposed research need and program change recommended in the proposal	Research to elucidate the relationship between human enteric pathogens and coliforms will help define the risk of illness associated with consumption of shellfish that may have been impacted by birds. Studies evaluating how these pathogens survive in the marine environment will further inform this relationship. Studies evaluating the purge rates of these pathogens will help growers devise management approaches to ensure potentially impacted product is held away for contaminated sites and is safe for consumption
Action by 2023 Task Force I	Recommended referral of Proposal 23-107 as amended to an appropriate committee as determined by the Conference Chair F. Standard for the Approved Classification of Growing Areas when Evaluated for Nonpoint Sources. (7) Exception. (a)If the tidal stage increases the fecal coliform concentration, the authority shall use sample results collected during that tidal stage to classify the area. (b) ~~If the Authority has documentation supporting that the nonpoint sources impacting the growing area are not from a human sewage origin they may exclude up to two outlier datapoints from the dataset being evaluated.~~ (8)Pollution Sources. Growing areas shall be impacted only by randomly occurring, intermittent events. (9)Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section E. (2) or Section F. (4). (10) Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median (or geometric mean MPN or MF

	(mTEC) of the water sample results shall not exceed fourteen (14) per 100 ml and the estimated 90th percentile shall not exceed an MPN or MF (mTEC) of: (a) 43 MPN per 100 ml for a five-tube decimal dilution test; (b) 49 MPN per 100 ml for a three-tube decimal dilution test; or (c) 31 CFU per 100 ml for a MF (mTEC) test. (11) Estimated 90th Percentile. The estimated 90th percentile shall be calculated by: (a) Calculating the arithmetic mean and standard deviation of the sample result logarithms (base 10); (b) Multiplying the standard deviation in (a) by 1.28; (c) Adding the product from (b) to the arithmetic mean; (d) Taking the antilog (base 10) of the results in (c) to get the estimated 90th percentile; and (e) The MPN values that signify the upper or lower range of sensitivity of the MPN tests in the 90th percentile calculation shall be increased or decreased by one significant number. Required Sample Collection. (a) Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity and frequency as described in Section E. (3) for application of the standard under Section E. (2). Systematic Random Sampling Standard. The requirement for systematic random sample collection shall be met when: (i) Sample station locations are adequate to produce the data to effectively evaluate all nonpoint sources of pollution; (ii) Sample collection is scheduled sufficiently far in advance to support random collection with respect to environmental conditions. Compliance requires that, prior to implementation, the schedule for random sampling shall be documented in the master file for the growing area, and if conditions at the time of scheduled sample collection are believed to be hazardous to the safety of the individuals assigned to collect samples, sample collection shall be rescheduled at a later date as soon as practical; (iii) A minimum of six (6) random samples shall be collected annually from each sample station in the growing area; (iv) A minimum of two (2) random samples shall be collected annually from each sample station in the growing area while in the inactive status. The sample collection frequency of six (6) random samples per station per year specified under @.02 F. (6)(b) (iii) must resume at least six (6) months before an area is reactivated; and (v) A minimum of the thirty (30) most recent randomly collected samples from each sample station shall be used to calculate the median or geometric mean and 90th percentile to determine compliance with this standard.

	(d) Transition from Adverse Pollution Condition Standard to Systematic Random Sampling Standard. If the Authority: (i) Does not have thirty (30) recent randomly collected sample results from each station, then the previous fifteen (15) samples collected under adverse pollution conditions may be used with the most recent random samples to meet the minimum thirty (30) sample requirement for a transition period not to exceed three (3) years; and (ii) Uses the transition period described in (i), as additional random samples are collected; the random samples shall replace chronologically the samples collected under adverse pollution conditions (e.g. sample 31 replaces sample 1)
Action by 2023 General Assembly	Adopted the recommendations of Task Force I on Proposal 23-107.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 23-107

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Adam Wood
Affiliation	Virginia Department of Health
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Proposal Subject	Relay Timeframe
Specific NSSP Guide Reference	Section II Model Ordinance, Ch. V Shellshock Relaying @.02 Contaminant Reduction C(3)
Text of Proposal/ Requested Action	(1) The Authority may waive the requirements for a contaminant reduction study if: (1) Only microbial contaminants need to be reduced; and (2) The shellstock are relayed from a conditionally approved, restricted, or conditionally restricted area meeting the bacteriological water quality for restricted areas used for shellstock depuration per Chapter IV. @.02 G. and Chapter IV. @.02 H.; and (3) The treatment period exceeds ~~sixty~~fourteen (~~60~~14) days. D. The time period shall be at least fourteen (14) consecutive days when environmental conditions are suitable for shellfish feeding and cleansing unless shorter time periods are demonstrated to be adequa
Public Health Significance	The change to 14 days is consistent with the literature available and already cited in the NSSP. The Guidance documents already have established 14 days as the ideal acceptable time for elimination of microbial contaminants. 60 days is not in any literature nor in any other already voted on sections of the NSSP for relaying. 21 days is the agreed upon value for harvesting waters adulterated with raw sewage, which is likely the worst-case scenario, relay from areas only impacted by microbial contamination should surely be less than those contaminated by raw sewage
Cost Information	N/A
Action by 2023 Task Force I	Recommended referral of Proposal 23-111 to an appropriate committee as determined by the Conference Chairperson.
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 23-111.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 23-111.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Jackie Knue
Affiliation	State of Alaska Environmental Health Laboratory
Email	Jacqueline.knue@alaska.gov
Proposal Subject	Domoic Acid (Amnesic Shellfish Poisoning) HPLC Method Laboratory Evaluation Checklist
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklist
Text of Proposal/ Requested Action	The requested action is to edit the text of the attached checklist for the HPLC method for detecting domoic acid and to append the checklist to the list of NSSP Laboratory Evaluation Checklists at the end of .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Public Health Significance	The attached checklist provides the quality assurance and method requirements that laboratory evaluation officers will use to evaluate laboratories implementing the HPLC method for domoic acid to support the NSSP. The checklist documents the number of critical, key or other nonconformities and how overall laboratory status for the method is determined
Cost Information	None
Action by 2023 Laboratory Committee	Recommended referral of Proposal 23-114 to an appropriate committee as determined by the Conference Chairperson.
Action by 2023 Task Force I	Recommended adoption of the Laboratory Committee recommendation on Proposal 23- 114
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 23-114.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 23-114

PUBLIC HEALTH SERVICE
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SHELLFISH LABORATORY EVALUATION CHECKLIST
Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV

LABORATORY:		
ADDRESS:		
TELEPHONE:	FAX:	EMAIL:
DATE OF EVALUATION:	DATE OF REPORT:	LAST EVALUATION:

LABORATORY REPRESENTED BY:	TITLE:
LABORATORY EVALUATION OFFICER:	SHELLFISH SPECIALIST:
	REGION:
OTHER OFFICIALS PRESENT:	TITLE:

Items which do not conform are noted by:

C – Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "'1"

PART I – QUALITY ASSURANCE			
Code	REF		Item Description
			1.1 Quality Assurance (QA) Plan
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check '1 those that apply) a. Organization of the laboratory. b. Staff training requirements. c. Standard operating procedures. d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established. e. Laboratory safety. f. Internal performance assessment.
C	5		**1.1.2 QA Plan is implemented.**
			1.2 Educational/Experience Requirements
C	State's Human Resources Department		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	USDA Microbiology & EELAP		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	5		**1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.**
			1.3 Work Area
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
			1.4 Laboratory Equipment
K	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit. [Only applicable if using the sample cleanup procedure]
K	5		1.4.2 The pH meter is calibrated daily when in use. Results are recorded and records are maintained. [Only applicable if using the sample cleanup procedure]
K	8		1.4.3 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment. [Only applicable if using the sample cleanup procedure]

K	8		1.4.4 The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e., pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded. [Only applicable if using the sample cleanup procedure]
K	5, 11		1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure. [Only applicable if using the sample cleanup procedure]
K	6, 2		1.4.6 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8		1.4.7 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.8 Refrigerator temperature is maintained between 0 and ~~4~~ 6 ºC~~.~~
K	8		1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	4,15		1.4.10 Freezer temperature is maintained at -10 ºC or below.
K	8		1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	13		**1.4.12 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.13 All glassware is clean.
K	4		1.4.14 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 1.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 20 µL injections, d. temperature controlled column compartment capable of controlling temperature at 40 ºC, e. ultraviolet detector/diode array detector able to achieve the required sensitivity at a wavelength (A) of 242 nm, and f. a data collection system (e.g., computer, integrator).
K	2		1.4.15 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	4		1.4.16 A solid phase extraction (SPE) vacuum manifold capable of holding 3 mL cartridges is used. [Only applicable if using the sample cleanup procedure]
O	4		1.4.17 A centrifuge capable of holding 50 mL polypropylene tubes is used.
			1.5 Reagents and Reference Solution Preparation and Storage
C	4, 15		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	8		1.5.2 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)

K	8		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	4, 15		**1.5.4 The mobile phase system used to analyze domoic acid consists of: 10% aqueous acetonitrile (v/v) and 0.1% trifluoroacetic acid (TFA).**
O	4		1.5.5 Mobile phase is filtered before use if the HPLC does not have a degasser.
C	7		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
K	4, 15		1.5.7 A cartridge wash solution is made up of 1 volume acetonitrile to 9 volumes of water (i.e., 10% aqueous acetonitrile). [Only applicable if using the sample cleanup procedure]
K	4		1.5.8 Citrate buffer (0.5 M, pH 3.2) is made up by dissolving 40.4 g citric acid monohydrate and 14 g triammonium citrate in 400 mL water, then adding 50 mL acetonitrile and diluting the total to 500 mL with water [or equivalent buffer]. [Only applicable if using the sample cleanup procedure]
C	**7**		**1.5.9 NRC CRM Zero-Mus or a negative control is used as a blank to ensure that there is no carry over between samples/standards. Source of the negative control:_____**
C	**7**		**1.5.10 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.11 All standards used are within expiration date.**
C	**2**		**1.5.12 All standards are prepared either gravimetrically or using positive displacement pipettes.**
C	**4, 15**		**1.5.13 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 50% aqueous methanol). Dilution with toxin-free, cartridge wash solution (aqueous acetonitrile) is allowed if using the diluted crude sample or the sample cleanup procedure.**
C	**7**		**1.5.14 Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.15 Quality Control shellfish tissues are stored frozen.**
			1.6 Collection and Transportation of Samples
O	6, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	6, 1		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**

K	14, 2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	2		1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.
PART II – EXAMINATION OF SHELLFISH FOR ASP TOXINS			
			2.1 Preparation of Sample
C	6, 1		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., 3 geoduck gut balls).**
O	6		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	6		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8 Pieces of shell and drainage are discarded.
C	2, 6		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
			2.2 Sample Extraction
K	4,6		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	4		**2.2.2 Four (4) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**4**		**2.2.3 The sample homogenate is extracted with 16 mL extraction solvent (1:1 methanol:water [also referred to as 50% aqueous methanol]).**
K	4, 15		2.2.4 Homogenate/extract mixture is centrifuged and filtered before analysis.
K	4, 15		2.2.5 The filtered extract is injected into the HPLC or loaded into the autosampler immediately.

K	4		2.2.6 When crude samples are diluted~~, dilutions are made by diluting 1 mL of filtered sample supernatant into a 5 mL volumetric flask and diluted with water to 5 mL~~.
K	4, 15		2.2.7 Crude extracts are sealed tightly and stored at -10 ºC or below.
			2.3 Sample Cleanup (Optional)
O	4, 15		2.3.1 Three (3) mL SAX cartridges (500 mg silica derivatized with quaternary ammonium silane) are used for cleanup.
K	4		2.3.2 The SAX cartridge is conditioned with 6 mL methanol, followed by 3 mL water, followed by 3 mL extraction solvent (1:1 methanol:water).
C	**4, 15**		**2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.**
K	4, 15		2.3.4 Five (5) mL of filtered extract is loaded onto the cartridge and flowed slowly (~1 drop/s) until sample meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.5 Five (5) mL of wash solution (1:9 acetonitrile:water) is loaded to the cartridge and washed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4		2.3.6 0.5 mL of citrate buffer (0.5 M, pH 3.2) is loaded to the cartridge and flowed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.7 A 2 mL volumetric tube is placed under the cartridge and any domoic acid is eluted into the tube by loading and flowing as much citrate buffer as needed slowly (~ 1 drop/s) until the 2 mL mark is reached on the tube.
C	**4, 15**		**2.3.8 The solution is thoroughly mixed before withdrawing an aliquot for analysis.**
K	4, 15		2.3.9 The cleaned up extract is injected into the HPLC or loaded into the autosampler immediately.
			2.4 Analysis
C	2		2.4.1 **A standard calibration curve (of at least six concentrations) is performed ~~daily~~. Results are recorded and records are maintained.**
K	4, 15		2.4.2 Twenty (20) µL of extract is injected for analysis.
K	2		2.4.3 Samples are stored in the sample compartment of the autosampler at 4 °C during analysis. Otherwise samples must be analyzed within 9 hours if the autosampler is held at room temperature.
K	4, 15		2.4.4 A column heater is used and the temperature is maintained at 40 °C during the analysis.
C	4		2.4.5 **The appropriate analytical column is used:** **25 cm x 4.6 mm id packed with 5 µm Vydac 201TP octadecylsilica or equivalent.**
K	2		2.4.6 The column is stored following the manufacturer's instructions when not in use.
O	2		2.4.7 If a precolumn in-line filter and/or a compatible guard column (e.g., 201GCC54T) are/is used, rejection criteria are established to determine when to change the filter/guard column.

C	**2**		2.4.8 **Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.**
			2.5 System Suitability
C	**2**		**2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for domoic acid.**
C	3		**2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of L-tryptophan and domoic acid.**
K	2		2.5.3 Peak asymmetry is routinely monitored to evaluate the performance of the column. Results are recorded and records maintained.
C	2		**2.5.4 The column is replaced when a measure of peak asymmetry becomes <~~0.9~~0.5 or >1.3.**
C	2,4		**2.5.5 Daily injection schedules must include the adequate frequency of injection standards and extraction blanks based on an assessment of individual standard toxin variability and lack of carry over.**
C	2		**2.5.6 Repeated injections of calibrated standards/samples agree within ±5% (as determined through the use of the coefficient of variation).**
			2.6 Calculation of Sample Toxicity
C	4, 15		2.6.1 **The toxicity of the individual toxins is calculated as follows:** μg/g domoic acid (DA) = DA injected $\times \frac{V}{W} \times (F)$ **where:** **DA injected = the concentration inμg/ml of the extract injected;** **V = total volume of homogenate and extraction solvent (mL);** **W = weight (g) of tissue homogenate extracted (e.g., ~~4~~ g); and** **F = dilution factor (e.g., if SAX cleanup or crude sample dilution are performed).** **The concentration of DA injected may be determined using the nearest standard or the equation of the day's standard curve.**
C	4, 15		2.6.2 **Calculated domoic acid concentrations include the sum of domoic acid and epimer peaks, when the epimer represents 5% or more of the peak area.**
C	12		2.6.3 **Any value at or above 20 ppm (mg/kg or µg/g) domoic acid is actionable.**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice. 21 CFR 58.
3. AOAC Official Method 991.26 Domoic Acid in Mussels. Liquid Chromatography Method. First Action 1991. Final Action 1999.
4. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1995. J. AOAC Int. 78(2): 543-554.

5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
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12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
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LABORATORY:			DATE OF EVALUATION:
SHELLFISH LABORATORY EVALUATION CHECKLIST SUMMARY OF NONCONFORMITIES			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
AMNESIC SHELLFISH POISON (ASP or domoic acid) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the ASP (domoic acid) Component

1. **Conforms Status: The ASP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The ASP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The ASP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥ 4**.
   - b. or the total # of Key nonconformities is **≥ 6.**
   - c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform – Provisionally Conforms – Conforms

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____ Date:_____

LEO Signature: Date:

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Jackie Knue
Affiliation	State of Alaska Environmental Health Laboratory
Email	Jacqueline.knue@alaska.gov
Proposal Subject	Paralytic Shellfish Poisoning (PSP HPLC-PCOX) HPLC Method Laboratory Evaluation Checklist
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklist
Text of Proposal/ Requested Action	The requested action is to edit the text of the attached checklist for the HPLC method for detecting domoic acid and to append the checklist to the list of NSSP Laboratory Evaluation Checklists at the end of .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Public Health Significance	The attached checklist provides the quality assurance and method requirements that laboratory evaluation officers will use to evaluate laboratories implementing the HPLC method for domoic acid to support the NSSP. The checklist documents the number of critical, key or other nonconformities and how overall laboratory status for the method is determined
Cost Information	None
Action by 2023 Laboratory Committee	Recommended referral of Proposal 23-115 to an appropriate committee as determined by the Conference Chairperson.
Action by 2023 Task Force I	Recommended adoption of the Laboratory Committee recommendation on Proposal 23- 115
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 23-115.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 23-115

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5100 PAINT BRANCH PARKWAY
COLLEGE PARK, MD 20740-3835
TEL. 240-402-2151/2055/4960 FAX 301-436-2601

SHELLFISH LABORATORY EVALUATION CHECKLIST

LABORATORY:		
ADDRESS:		
TELEPHONE:	FAX:	EMAIL:
DATE OF EVALUATION:	DATE OF REPORT:	LAST EVALUATION:

LABORATORY REPRESENTED BY:	TITLE:
LABORATORY EVALUATION OFFICER:	SHELLFISH SPECIALIST:
	REGION:
OTHER OFFICIALS PRESENT:	TITLE:

Items which do not conform are noted by:

C – Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "√"

PART I – QUALITY ASSURANCE			
Code	**REF**		**Item Description**
			1.1 Quality Assurance (QA) Plan
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check √ those that apply) a. Organization of the laboratory. b. Staff training requirements. c. Standard operating procedures. d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established. e. Laboratory safety. f. Internal performance assessment. g. External performance assessment.
C	**5**		**1.1.2 QA Plan is implemented.**
			1.2 Educational/Experience Requirements
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	**State's Human Resources Department**		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	**USDA Microbiology & EELAP**		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	**5**		**1.2.5 LC-Operator must be competent in the operation and maintenance of a basic liquid chromatography system.**
			1.3 Work Area
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
			1.4 Laboratory Equipment.
O	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit.
K	6		1.4.2 pH paper in the appropriate range (i.e. 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	10		1.4.3 pH electrodes consist of pH half-cell and reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).

K	5		1.4.4 pH meter is calibrated daily when in use. Results are recorded and records are maintained.
K	8		1.4.5 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	8		1.4.6 A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.
K	5, 11		1.4.7 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	6		1.4.8 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8, 9		1.4.9 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.11 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	1		1.4.12 Freezer temperature is maintained at -20 ºC or below.
K	8		1.4.13 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**13**		**1.4.14 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.15 All glassware is clean.
K	3		1.4.16 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. binary mobile phase system delivering a pulse-free flow of 0.5-2.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 5-30 µL injections, d. temperature controlled column compartment capable of controlling temperature between 10 – 50 ºC, and e. fluorescence detector able to achieve the required sensitivity at an excitation wavelength (λ) of 330 nm and emission of 390 nm.
K	3, 4		1.4.17 The post-column reaction system used is equipped with the following: a. reactor module capable of maintaining 85 ºC, b. dual reagent pumps capable of delivering accurate flows of 0.4 mL/min, and c. if applicable, a reaction coil (knitted or equivalent) having a total volume of 1 mL and a length of 5 m x 0.5 mm.
K	6		1.4.18 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.19 A boiling water bath with sufficient volume to cover the sample/acid mixture is used for extraction.
O	3		1.4.20 Centrifuge capable of holding 50 mL polypropylene tubes.
K	3		1.4.21 Microcentrifuge capable of holding 1.5 mL microcentrifuge tubes and generating a minimum of 16000 g or equivalent is used.
			1.5 Reagents and Reference Solution Preparation and Storage
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
C	**8**		**1.5.2 Water is glass distilled or deionized and exceeds 0.5 megaohm resistance or is less than 2 µSiemens/cm conductivity at 25 ºC to be tested and recorded monthly for resistance or conductivity and the results are recorded.**
K	8		1.5.3 Water is analyzed for residual chlorine monthly and is at a nondetectable level

			(≤0.1 ppm) Results are recorded and records are maintained.
K	8		1.5.4 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained.
K	8		1.5.5 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.6 The binary mobile phase system used to analyze the GTX and STX toxins consists of:** **1.5.6.1 Mobile Phase A, which contains 11 mM heptane sulfonate and 5.5 mM phosphoric acid (H_3PO_4), pH 7.1.** **1.5.6.2 Mobile Phase B, which contains 11 mM heptane sulfonate, 16.5 mM H_3PO_4 and11.5% acetonitrile (MeCN), pH 7.1.**
C	**3**		**1.5.7 The binary mobile phase system used to analyze the C toxins consists of** **1.5.7.1 Mobile Phase A, which contains 2 mM tetrabutyl ammonium phosphate, pH 5.8.** **1.5.7.2 Mobile Phase B, which contains 2 mM tetrabutyl ammonium phosphate in 4% acetonitrile, pH 5.8.**
C	**3**		**1.5.8 The post-column oxidant consists of 100 mM H_3PO_4 and 5 mM periodic acid (H_5IO_6), pH 7.8.**
C	**3**		**1.5.9 The post-column acid used is 0.75 M nitric acid (HNO_3).**
C	**3**		**1.5.10The heptane sulphonate used in mobile phase A and mobile phase B to analyze for GTX and STX toxins is prepared the day of use or refrigerated for up to one week.**
C	**3**		**1.5.11The pH of mobile phases and the post-column oxidant are adjusted as follows:** **a. Mobile phase A and mobile phase B for the GTX and STX toxins are adjusted to 7.1 with ammonium hydroxide (NH_4OH),** **b. Mobile phase A and mobile phase B for the C toxins are adjusted to 5.8 in one direction only with 10% acetic acid (HOAc) if too basic or 1% NH_4OH if too acidic, and** **c. The post-column oxidant is adjusted to 7.8 with 5 M sodium hydroxide (NaOH).**
O	3		d. Mobile phases and post-column reagents are filtered before use if the HPLC does not have a degreaser.
C	**3,7**		**1.5.12Only certified reference materials are used for standard solutions. Source of the reference standard:**
C	**7**		**1.5.13NRC Zero-Mus or a negative control matched matrix is used as a matrix blank as appropriate. Source of the negative matrix:**
C	**7**		**1.5.14All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.15All standards used are within expiration date.**
C	**3**		**1.5.16All standards are prepared gravimetrically.**
K	3		1.5.17Intermediate mixes of primary standards are made up in 0.003 M HCl for the GTX/STX toxins or pH 5 glass distilled/deionized water for the C toxins labeled with the date of preparation and the expiration date and stored appropriately. The pH of the glass distilled/deionized water is adjusted when necessary by the dropwise addition of 10% acetic acid (HOAc).
C	**3**		**1.5.18Working standards are made up from primary standard or intermediate mixes by dilution with toxin-free, deproteinated, matrix matched extracts.**
C	**7**		**1.5.19Zero-Mus is stored according to manufacturer's instructions.**

C	**2**		**1.5.20Quality Control shellfish tissues are stored frozen.**
C	**7**		**1.5.21Working standards are labeled with the date of preparation, stored appropriately and used within 3 months of preparation.**
			1.6 Collection and Transportation of Samples
O	6		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**6**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	14		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**6**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR PSP TOXINS			
			2.1 Preparation of Sample
C	**6**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	6		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**6**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8 Pieces of shell and drainage are discarded.
C	**6**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogentates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
			2.2 Digestion of Sample
K	6		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**3**		**2.2.2 Five (5) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
K	3		2.2.3 The sample homogenate is extracted in a 1:1 w/v ratio with 0.1 M HCl.

K	3		2.2.4 Homogenate/acid mixture is vortexed thoroughly before boiling to completely mix the contents.
C	**3**		**2.2.5 To prevent toxin transformation, the pH of the homogentate/acid mixture before boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH.**
C	**3**		**2.2.6 Samples in capped 50 mL polypropylene centrifuge tubes are extracted in a boiling water bath for 5 minutes.**
K	3		2.2.7 The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of 5 M HCl. Any sample with a pH of less than 2.0 is discarded and extracted again.
K	3		2.2.8 The homogenate/acid mixture is allowed to separate by gravity or by centrifugation.
			2.3 Deproteination
C	**3**		**2.3.1 ~~500~~ 1000 µL of sample extract is deproteinated with ~~25~~ 50 µL of 30% trichloroacetic acid, vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
C	**3**		**2.3.2 The pH of the deproteinated extract is adjusted with 35 µL of ~~1~~2.0 M NaOH vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
K	3		2.3.3 An aliquot of the deproteinated supernatant is filtered through a 0.2 µm filter.
			2.4 Analysis
C	2		2.4.1 **A standard calibration curve (of at least six concentrations) is performed upon initial instrument set up, following any major hardware maintenance activity, or when the continuing calibration verification (CCV) indicates significant drift (> 30% for individual toxin) from the calibration. Results are recorded and records are maintained.**
K	3		2.4.2 10 µL is injected for GTX/STX toxins and 5 µL is injected for C-toxins.
K	3		2.4.3 Samples are stored in the sample compartment of the autosampler at 4 °C during analysis. Otherwise samples must be analyzed within 20 hours if the autosampler is held at room temperature.
K	3		2.4.4 A column heater that is capable of maintaining 30-40 °C for the GTX/STX toxins and 10-20 °C for the C toxins is used in the analysis.
C	**3**		**2.4.5 The appropriate analytical column is used.** **a. GTX/STX Toxins: Agilent Zorbax Bonus-RP column, 4.6 mm x 150 mm, 3.5 µm or equivalent.** **b. C Toxins: Thermo BetaBasic 8, 4.6 mm x 250 mm, 5 µm or equivalent.**
			2.5 System Suitability
K	2		2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for each individual toxin.

C	**3**		**2.5.2 The resolution and retention time criteria that must be met are:** **a. For GTX and STX toxins, the matrix peak must be at least 70% baseline resolved between GTX3 and GTX2.** **b. For GTX and STX toxins, GTX5 must be at least 40% baseline resolved between dcGTX3 and dcGTX2.** **c. For GTX and STX toxins, dcSTX and STX must be at least 70% baseline resolved.** **d. For GTX and STX toxins, the retention time of GTX4 must be between 5 and 7 minutes.** **e. For the C toxins, C2 must be at least 70% baseline resolved between C1 and C2.** **f. For the C toxins, the retention time of C1 must be between 4 and 7 minutes.**
C	**2**		**2.5.3 Daily injection schedules must include the adequate frequency of injection standards based on an assessment of individual standard toxin variability. Variability in peak response must be less than 10% for calculation of toxicity in samples.**
			2.6 Calculation of Toxicity
C	**4**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** $\mu\text{gSTXdiHCleq}/100\text{g} = \mu\text{M} \times \frac{372.2}{1000\text{mL}} \times \frac{\text{Fvol}}{\text{Ext.vol}} \times \left(\frac{\text{Wt} + \text{Vol}}{\text{Wt}}\right) \times \text{ReTx} \times 100$ **14.** **Where:** **µM = Concentration of toxin in the extract, in µM;** **Fvol = Final volume of the deproteinized extract (e.g. 560 µL);** **Ext.vol = Volume of crude extract used (e.g. 500 µL);** **Wt = Weight of sample used;** **Vol = Volume of acid extractant used (e.g. 5 mL); and** **ReTx = Relative toxicity of toxin vs. Saxitoxin.**

Relative Toxicity Values

Toxin	ReTx	Toxin	ReTx
GTX1	0.9940	NEO	0.9243
GTX2	0.3592	STX	1.0000
GTX3	0.6379	dcSTX	0.5131
GTX4	0.7261	C1	0.0060
GTX5	0.0644	C2	0.0963
dcGTX2	0.1538	C3	0.0133
dcGTX3	0.3766	C4	0.0576

15.

C	**3**		**2.6.2 The individual toxicities for each toxin are summed to obtain the overall sample toxicity in µg STX equivalents/100 g (µg/100 g).**
C	**12**		**2.6.3 Any value at or above 80 µg STX equivalents /100 g of meat is actionable.**

REFERENCES:

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
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6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
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13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.

LABORATORY: **DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS

LABORATORY	DATE
LABORATORY REPRESENTATIVE:	

PARALYTIC SHELLFISHPOISON COMPONENT: PARTS I AND II

A. Results

Total # of **Critical (C)** Nonconformities	_____
Total # of Key (K) Nonconformities	_____
Total # of Critical, Key, and Other (O) Nonconfomities	_____

B. Criteria for Determining Laboratory Status of the PSP, PCOX Component

1. **Conforms Status: The PSP, PCOX component of this Laboratory is in conformity with** NSSP requirements if a ll of the following apply.
   a. No Critical nonconformities.
   b. and <6 Key nonconformities.
   c. and <12 Total nonconformities.

2. **Provisionally Conforms Status:** The PSP, PCOX component of this laboratory is determined to be provisionally conforming to NSSP requirements if a ll of the following apply.
   a. the number of critical nonconformities is ≥ 1 but **< 4**.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

3. **Does Not Conform Status** : The PSP, PCOX component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is **≥ 4**.
   b. or the total# of Key nonconformities is **≥ 6.**
   c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date: _____

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	George Trevelyan
Affiliation	Grassy Bar Oyster Company, Inc.
Email	gboysterco@gmail.com
Proposal Subject	**Guidance for calculating the 90th percentile for end-product depurated shellfish**
Specific NSSP Guide Reference	**Section IV Guidance Documents; Chapter II Growing Areas; Section .17 Calculating the 90th percentile for end-product depurated shellfish**
Text of Proposal/ Requested Action	Process verification in depuration is performed continuously to ensure that the microbial contaminant load is being effectively reduced. Two (2) indices of performance, the geometric mean and the ninetieth (90th) percentile have been developed to describe the effectiveness of the depuration process. Critical limits for these parameters have been established empirically by shellfish species. For soft clams (*Mya arenaria*), a geometric mean of fifty (50) and a ninetieth (90th) percentile of 130 have been set. For hard clams, oysters, manila clams and mussels, a geometric mean of twenty (20) and a ninetieth (90th) percentile of seventy (70) have been adopted. Geometric means and ninetieth (90th) percentiles are determined daily or as end- product results become available from the analysis of the most recent ten (10) consecutive harvest lots per species, per restricted harvest area used. If the critical limits for either the geometric mean and/or the ninetieth (90th) percentile are exceeded, the process is considered to be unverified; and, additional sampling requirements must be instituted to ensure effective process control. End-product depurated shellfish samples are analyzed using two (2) different methods of recovery, a pour plate procedure and a single dilution MPN test. Calculation of the ninetieth (90th) percentile for these samples is complicated by the fact that fecal coliforms recovered by the MPN and ETCP methods follow different statistical distributions. To accommodate these differences and maintain a high likelihood for detecting an unacceptable amount of process variability without having to change or alter the formula used requires the use of nonparametric or "distribution free statistics." Using "distribution free statistics," the position of the ninetieth (90th) percentile for end-product depurated shellfish samples is calculated by arraying the fecal coliform count data in ascending order and applying the formula (n + 1)P/100.

	As an example of the use of this formula, the Model Ordinance requires that the ninetieth (90th) percentile of the fecal coliform analytical data be calculated from the most recent ten (10) consecutive harvest lots for each shellfish species depurated from each restricted harvest area. Fecal coliform count data, whether from the ETCP or MPN procedure for these ten (10) lots must be arrayed from the smallest to the largest value using the arithmetic (not logarithmically transformed) count data. Applying the formula, n would be greater than or equal to ten (10) for the ten (10) most recent consecutive harvest lots required by the Model Ordinance. P, the percentile of interest would be ninety (90). Using the minimum sample set of n=10, ~~Multiplying~~ multiplying the formula out gives the position of the ninetieth (90th) percentile in the arrayed data. Performing these calculations, 10 + 1 = 11, 11 x 90 = 990/100 = 9.9. Thus, the ninetieth (90th) percentile for end-product depurated shellfish data when n=10 is the value of the 9.9th sample in the ten (10) sample array. Using ~~the~~ ten (10) samples ~~as required by the Model Ordinance~~, the ninetieth (90th) percentile for end- product depurated shellfish samples would always be the value of the 9.9th sample in the ascending array of the arithmetic count data. To calculate this value from the arrayed data, interpolation between samples nineand ten (10) is necessary. This is best illustrated using several samples. Example 1… Example 2… Example 3… In cases where more than ten samples have been analyzed in the most recent ten consecutive harvest lots for each species depurated or for each harvest area used, the geometric mean and estimated 90th percentiles may be calculated using the methodologies below in examples 4 and 5. Example 4 (attached) Example 5 (attached)
Public Health Significance	Incorrectly calculating the 90th percentile can lead to erroneous decisions that could affect public health. For instance, both the California Dept of Public Health and the FDA mis-calculated the 90th percentile for a data set in which n=36. They insisted, based on the examples given in the NSSP Guide, that the 90th percentile was **always found between the 2 largest numbers** in the data set, even when n is large, which is incorrect.
Cost Information	This clarification to the NSSP Guide, with additional examples, will make it easier to correctly calculate this depuration performance index and should reduce confusion and disagreements, which could save time and money.
Action by 2023 Task Force I	Recommended referral of Proposal 23-123 to an appropriate committee as determined by the Conference Chairperson.

Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 23-123.
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 23-123

Example 4

Oysters from a restricted harvest area produced the following end-product depurated fecal coliform counts which have been arrayed in ascending order for ease of calculation. In this case, there are 30 samples (n=30) because all 10 lots were under conditional protocol and required 3 end product samples each.

Sample #	FC Count (MPN/100 grams)
1	3.9 (<4)
2	3.9 (<4)
3	3.9 (<4)
4	3.9 (<4)
5	3.9 (<4)
6	3.9 (<4)
7	3.9 (<4)
8	3.9 (<4)
9	3.9 (<4)
10	3.9 (<4)
11	3.9 (<4)
12	4
13	4
14	4
15	4
16	7
17	7
18	12
19	12
20	12
21	16
22	16
23	16
24	22
25	28
26	35
27	35
28	56
29	101 (>100)
30	101 (>100)

In this example, n=30. Applying the formula (n+1)P/100 gives the position of the 90th percentile in the arrayed data: 30+1=31, 31*90=2790/100=27.9. Thus the 90th percentile for a thirty (30) sample array is the 27.9th sample in the array. The value for the 27.9th sample in the array is interpolated by subtracting the value of sample #27 which is 35 from the value of sample #28 which is 56 to give 21. 21 is then multiplied by 0.9 to give 18.9 which is added to the value of sample #27 which is 35 to give 53.9. Rounding this value off to 54, the value for the ninetieth (90th) percentile becomes 54.

Example 5

Once again, oysters from a restricted harvest area produced the following end-product depurated fecal coliform counts which have been arrayed in ascending order for ease of calculation. But in this case, there are 24 samples (n=24) because 6 of the lots were under conditional protocol and required 3 end product samples each, and the other 4 lots, under routine verification, required just one end product sample each.

Sample #	FC Count (MPN/100 grams)
1	3.9 (<4)
2	3.9 (<4)
3	3.9 (<4)
4	3.9 (<4)
5	3.9 (<4)
6	3.9 (<4)
7	3.9 (<4)
8	3.9 (<4)
9	4
10	4
11	4
12	7
13	7
14	7
15	7
16	12
17	12
18	12
19	16
20	28
21	28
22	36
23	101 (>100)
24	101 (>100)

In this example, n=24. Applying the formula (n+1)P/100 gives the position of the 90th percentile in the arrayed data: 24+1=25, 25*90=2250/100=22.5. Thus the 90th percentile for a twenty four (24) sample

array is the 22.5th sample in the array. The value for the 22.5th sample in the array is interpolated by subtracting the value of sample #22 which is 36 from the value of sample #23 which is 101 to give 65. 65 is then multiplied by 0.5 to give 32.5 which is added to the value of sample #22 which is 36 to give 68.5. Rounding this value off to 69, the value for the ninetieth (90$^{th)}$ percentile becomes 69.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Updated Marina and Mooring Area Guidanc
Specific NSSP Guide Reference	Section IV. Guidance (Mooring Area)
Text of Proposal/ Requested Action	MARINA and MOORING AREA GUIDANCE - **DRAFT** The following guidance is provided to ensure the uniform application of the National Shellfish Sanitation Program (NSSP) Model Ordinance (MO) criteria, as adopted by the Interstate Shellfish Sanitation Conference (ISSC), for the evaluation and classification of shellfish growing waters in and around docks, marinas, and boat mooring areas. BACKGROUND A marina policy was developed at the ISSC conference held in August of 1986. It was recognized that a marina is a potential pollution source in a shellfish growing area, and that a closure zone is required to prevent the harvest of shellfish for human consumption in and around occupied marinas and mooring areas. The purpose of the policy was to establish a uniform national approach to marina and mooring area closures. At the July 1988 ISSC conference, approval was given to incorporate the marina policy into the definition and growing area classification sections of the NSSP MO. The 1989 "*Evaluation of Marinas by State Shellfish Sanitation Control Officials*", better known as the **1989 Marina Guideline,** was released in order to further clarify the new marina policy adopted into the 1990 NSSP Manual of Operations Part I Sanitation of Shellfish Growing Areas. The 1989 Marina Guideline was originally intended for the U.S. Food and Drug Administration (FDA) and State Shellfish Control Authorities (Authority) to use as guidance when classifying growing areas in and around marina facilities. The 1989 Marina Guideline has been used in all the FDA growing area training courses since inception as a reference on implementation of the NSSP MO marina criteria. As a result of actions taken at the 2019 biennial conference, "marina" and "mooring area" were separated into two (2) definitions (NSSP MO Section I. B.). In addition, the NSSP MO Section II. Chapter IV. @.06 was created to allow for mooring areas to be classified as conditionally approved and conditionally restricted in the open status if a detailed pollution assessment

	is conducted at the frequencies required by the NSSP MO Section II. Chapter IV. @.01 A. (2.), C., and D. indicating a significant reduced risk from pollution sources and if there is a Conditional Area Management Plan (CAMP) in place with sufficient controls to protect human health. The justification for this change suggests that there may be a different level of human health risk associated with how a mooring area, as a pollution source, may be managed compared to a marina. Boats are considered a potential pollution source due to the capability to discharge human sewage into a growing area. As technology has improved and the management of mooring areas have evolved with the implementation of the Federal No Discharge Zone (NDZ) program and availability of boat waste pump out boats and facilities, there is the potential, with enough oversight and management controls in place, to limit the capacity for overnight occupancy and sewage discharge from boats in a mooring area compared to a marina. This updated marina and mooring area guidance document is intended to serve as guidance for the FDA when evaluating state growing area classification programs and as guidance for authorities regarding the classification and management of marinas and mooring areas in accordance with the NSSP MO requirements. GUIDANCE This guidance will provide clarification for the pollution assessment, classification, dilution calculation, and conditional area classification management of marinas and mooring areas, in and adjacent to, shellfish growing areas. Boats congregated into a marina or mooring area are operated and inherently occupied by people at some time and therefore, have the potential to discharge human sewage and graywater into associated shellfish growing areas. As a result, every public or private watercraft, barge, houseboat, or boat, that has the potential to produce an overboard discharge from a marine toilet or discharge graywater, should be considered a potential pollution source in the evaluation of shellfish growing areas. Since marine toilets may provide only limited or no treatment, human sewage discharges from boats may contain bacteria and viruses attributed to human sewage and graywater. For this reason, discharges of graywater and marine toilets represent a greater public health risk than other discharges

	of sanitary waste, and since these discharges can be sporadic, it may represent a greater public health risk than the FC sources typically detected by routine bacteriological monitoring. Since many marina facilities and mooring areas are in or adjacent to shellfish growing areas, and waste discharges are not uniformly distributed in the water column, detection of low levels of coliforms from waste discharges by current pollution monitoring methods may not provide sufficient information to properly classify the waters in or adjacent to a marina or mooring area. Therefore, each marina and mooring area pollution assessment, dilution analysis, classification, and closure zone should be considered on a site-by-site basis, given the potential significant public health risk combined with the unique characteristics of each site. As a result, a classification other than approved or restricted is required for the area within a marina or mooring area. This requirement is based on the public health requisite that waters receiving sporadic waste discharges from marine toilets or discharge of graywater are not suitable for the direct harvest of shellfish destined for human consumption or for relay or depuration. A pollution assessment and dilution determination must be used for classifying and making status determinations for marinas and mooring areas and adjacent shellfish growing areas. MARINAS Per the 2019 Revision of the NSSP MO Section I. B.: **Definition: Marina -** *any water area with a structure (docks, basin, floating docks, etc.) which is used for docking and constructed to provide temporary or permanent docking space for more than ten (10) boats.* MARINA PROPER Per the NSSP MO Section II. Chapter IV. @.05 A, the marina proper shall be classified as: conditionally approved, conditionally restricted, or prohibited. A *pollution assessment* shall also be conducted in order to support the conditionally approved or conditionally restricted classification. The FDA's interpretation is that the marina pollution assessment is not intended to allow direct harvesting in the marina proper while more than 10 boats are present, but to document the seasonality

and the presence of boats for the development of a Conditional Area Management Plan (CAMP) and to assess the marina proper as a pollution source, gather information for the dilution analysis, and provide documentation in the sanitary survey.

If more than 10 boats are not present during certain seasons (as in some geographical areas) the marina proper may be reclassified or changed to the open status if already classified as conditionally approved or conditionally restricted to permit harvest. During such periods the Authority must document that the area meets the specific NSSP MO criteria for the classification allowing harvest in the CAMP.

ADJACENT WATERS

Per the NSSP MO Section II. Chapter IV. @.05 B., waters adjacent to a marina proper may be impacted by pollution associated with the marina. Therefore, when more than 10 boats are present, a dilution analysis shall be used to determine if there is any impact to the adjacent growing area waters. The dilution analysis shall be based on the volume of water in the vicinity of the marina proper.

If the dilution analysis predicts a theoretical fecal coliform (FC) loading greater than (>) 14 FC/100 ml, the waters adjacent to the marina shall be classified as: conditionally approved, restricted, conditionally restricted, or prohibited. If the dilution analysis predicts a theoretical FC loading less than (<) 14 FC/100 ml, the waters adjacent to the marina may be classified as: approved or conditionally approved.

In reference to NSSP MO Section II. Chapter IV. @.05 B. (3), the dilution analysis around a marina proper shall incorporate the following factors. The recommendations provided represent guidance for how the authority may meet the intent of each requirement:

(a) Slip occupancy rate for the marina:

This is the quantity of waste potentially originating in a marina and depends on the number of people who are present in the marina. The fewer boats that are found to be occupied, the smaller the expected impact from the marina proper. The NSSP MO provides for establishing an occupancy rate for each marina. The slip occupancy rate of the marina should be documented by actual observation of

marina operations during the time of highest usage such as weekends or holidays. Document the overall number of boats in a marina proper and the number of boats being occupied as well as the number of people on each boat. Document the number of slips in the marina proper.

(b) **An actual or assumed rate of boats which will discharge untreated waste**:
Document the number of boats with a marine sanitation device (MSD) type used (i.e., MSD Type I, II, or III) in the marina. If the authority uses an assumed rate of discharge, that rate should be supported by data gathered during the pollution assessment of the marina.

(c) An occupancy per boat (number of ersons per boat):
If the authority chooses not to determine a specific occupancy per boat rate by investigation, the authority shall assume a minimum occupancy rate of two (2) persons per boat (NSSP MO Section II. Chapter IV. @.05 B. (6)).

Document the number of boats with liveaboard capability as well as the number of people on liveaboard boats in the marina. This inventory should be taken during the expected high usage times such as weekends and holidays. The inventory should have continuity so that changes in population during high occupancy times will be documented. Regional differences in boat usage, and the percent of high usage, will vary.

(d) A fecal coliform discharge rate of 2 x 10^9 for the theoretical fecal coliform contribution per person per day.

(e) Assume that the wastes are completely mixed in the volume of water in and around the marina.

(f) **Documentation, verification and enforcement of Federal No Discharge Zones and locally well enforced no discharge and occupancy by-laws and regulations**:
Provide documentation of the NDZ: enforcement records, vessel inspection records, marina use agreements, available educational material, and graywater regulations.

Document in the management plan how vessels are inspected to ensure that boats equipped with an MSD that is not properly sealed to prevent discharge of sewage into the water is documented and enforced. Document Memorandums of Understanding or Agreements with local towns, municipalities, and patrol enforcement agencies defining each agency's responsibility in administering and enforcing the NDZ.

(g) Availability and documented use of pump out boats or facilities:

Document the availability and number of pump out facilities and boats available to the marina. Document use and maintenance records, operation procedures, ease of use, hours of operation, pump out log, previous spills, and the individual responsible for pump out operations. The pump out log should include: date, boat name and length, approximate number of gallons pumped, and initials of the operator.

Document enforcement records and boat inspection records. Document the procedures used if there is a waste spill. Document the frequency of when inspections are conducted to ensure pump-out stations are properly maintained and compliant with Clean Vessel Act (CVA) grant requirements. The records of inspections must be maintained and available for review.

MOORING AREAS

Per the 2019 Revision of the NSSP MO Section I. B.:

Definition: Mooring Areas - *any water area that is used to provide temporary or permanent anchorage for more than twenty (20) boats. Mooring areas do not include any structures for docking boats.*

MOORING AREA PROPER

Per the NSSP MO Section II. Chapter IV. @.06, a designated mooring area, where there is anchoring or mooring of boats, which is in or adjacent to a shellstock growing area shall be classified as: conditionally approved, restricted, conditionally restricted, or prohibited.

Prior to the Authority establishing a classification of conditionally

approved, conditionally restricted, or restricted in the mooring area proper, a *pollution assessment* supporting the classification will need to be conducted by the authority. The NSSP MO provides flexibility so that if the *pollution assessment* determines that the mooring area has controls in place and is not considered a pollution source and it is thoroughly documented in the CAMP, the area may be classified as conditionally approved or conditionally restricted and placed in the open status with boats present.

The following factors shall be considered and documented when conducting a *pollution assessment* to determine the classification of the mooring area and adjacent waters in accordance with the NSSP MO requirements.

POLLUTION ASSESSMENT

The NSSP MO Section II. Chapter IV. @.06 A. (1) requires that a *pollution assessment* supporting the classification of mooring areas be conducted by the authority. In accordance with the 1986 ISSC Marina Policy and the 1989 Marina Guidance, the basis for occupancy and discharge rates should reflect worst case conditions and the inventory should be taken during the expected high usage times such as weekends and holidays.

The *pollution assessment* shall include the following factors according to the NSSP MO Section II. Chapter IV. @.06 A. (1). The recommendations provided for each factor represents suggested guidance for how the authority may meet the intent of each required component of the *pollution assessment*:

(a) **Boat Type and Usage**:

- Documentation of the boat type and usage should be considered from a public health perspective and the risk of the potential for overboard discharge from both treated and untreated sewage as well as graywater.
  - Document the type and size of boats in the mooring or anchorage area such as cabin cruiser, houseboat, cuddy cabin, runabout, commercial fishing vessel, skiff, daysailer, etc.
  - Document the number of boats in

each type and size category.

- Document the usage of boats such as overnight, weekend, day use, as well as commercial, or recreational.
- The boat type and usage information may be used in a mooring area management strategy to separate out boats that might pose more of a human health risk into a different conditionally managed area using separate performance standards.

(b) **Density of Boats**:

- Document the geographic location of the mooring area and include a map defining the mooring area boundaries.
- If boats are geographically managed by type and use, document this management strategy using a map that defines the mooring area management areas.
- Document the density of boats as the number of boats per a unit of area (For example: 100 boats per 1 sq. mile).
- Each individual mooring or anchorage area in a growing area should be accounted for and evaluated and where multiple mooring areas are present in a growing area, the authority should evaluate the impact of those individual mooring areas on the growing area from a holistic or cumulative impact. As an example, using best human health protection management practices, it may not be appropriate to separate a single group of multiple mooring boats (greater than 20 boats) into numerous separate mooring areas of 20 or less boats.

(d) **Accessibility to boats which could reduce likelihood of overnight occupancy**:

- In reference to the term "parking lot" mooring area, such as a location where boats are temporarily moored for short periods of

	time, but not occupied overnight, document the factors which could reduce or increase the likelihood of overnight occupancy in the mooring area proper. • Provide a detailed justification explaining how accessibility to boats in the mooring area increases or decreases the likelihood of overnight occupancy. This may include how the access of the boats in the mooring area are managed and how accessible boats are to overnight occupancy. • Document the municipal mooring area regulation(s), town charter(s), municipal regulation(s), and records documenting enforcement of said regulation(s) and charter(s) that limits or mandates no overnight occupancy. • Document how boat owners access their vessels, such as through launch service (hours of operation), personal dinghy, etc. • Provide and maintain records from the municipal or state enforcement agencies when overnight occupancy regulations are enforced or violated. (d) **Occupancy Rates**: • Document the number of mooring balls/buoys and the number of boats allowed on each. • Document the overall number of boats in a mooring area and the number of boats being occupied as well as the number of people on each boat. If the mooring area is considered a "parking lot", such as a location where boats are temporarily moored for short periods of time but not occupied overnight, provide documentation to that effect, including justification for use. • Document any transient mooring areas

	and their boat capacity. (e) **Seasonal Use Pattern**: • Document if there is a seasonal boat use pattern. • Document what the seasonal boat use pattern is including the seasonal dates as to when more than 20 boats are present in the mooring area. (f) **An actual or assumed rate of boats which will discharge untreated waste**: • Conduct and document an onsite assessment of the mooring area and document the type and number of boats that have the potential for discharging treated or untreated sewage including graywater. • Document boats with marine heads and include the number and location of boats with each type of MSD (Type: I, II, or III). (g) **Documentation, verification, and enforcement of Federal No Discharge Zones (NDZ), and locally well enforced no discharge and occupancy regulations or by-laws:** • Provide documentation of the NDZ: enforcement records, boat inspection records, mooring area use agreements, available educational material, graywater discharge regulations, and occupancy records during high-use times. • Document how boats equipped with a MSD, not properly sealed to prevent discharge of sewage into the water, are inspected. • Provide any Memoranda of Understandings or Agreements with local towns, municipalities, and patrol enforcement agencies. Define each agency's responsibility in administering and enforcing the NDZ; including references to the statue, regulation, or charter that confers authority to enforce the NDZ. (e) Document the CAMP communication

requirements (contact tree) in case an emergency closure is warranted.

(h) **Availability and documented use of shore-based pump out facilities and pump out boats**:

- Document the availability and number of pump out facilities and pump out boats available to the boats in the mooring area proper.
- Document pump out practices, pump out procedures, educational information, and employee/operator training.
- Document the use and maintenance records, operation procedures, ease of use, hours of operation, pump out log, previous spills, and who is responsible for the pump out operations. The pump out log should include date, boat name and length, approximate number of gallons pumped, and initials of the operator.
- Document enforcement records and boat inspection records.
- Document the procedures if there is a waste spill.
- Document the frequency as to when inspections are conducted to ensure pump-out stations are properly maintained and compliant with Clean Vessel Act grant requirements; with records of past inspections maintained and available for review.

The NDZ is only one factor to consider when conducting a *pollution assessment* to classify a growing area with a mooring area(s) as conditionally approved or conditionally restricted in the open status with boats present. The FDA does not consider the NDZ designation to be a standalone *pollution assessment*, control mechanism, or justification for classifying a mooring area(s) as conditionally approved or conditionally restricted in the open status. As stated in the NSSP MO language, documentation, verification, and enforcement of the NDZ and locally well enforced no discharge and occupancy regulations or by-laws will be necessary for the *pollution assessment* and for review during FDA growing area program

	evaluations. In addition, Section 312 of the Clean Water Act (CWA) contains the principal framework for domestically regulating sewage discharges from boats and is implemented jointly by the U.S. Environmental Protection Agency (EPA) and the U.S. Coast Guard (USCG). Sewage, treated or untreated, is prohibited in an NDZ. The NSSP utilizes the CWA definition of sewage. **Definition: Sewage - human body wastes and the waste from toilets and other receptacles intended to receive or retain body wastes.** Graywater is not defined as "sewage" and is not prohibited under the NDZ requirements. Graywater may contain high levels of human bacteria and viruses and poses a significant human health risk when present and this should also be considered in the *pollution assessment*. CONDITIONAL AREA MANAGEMENT PLAN (CAMP) FOR THE MOORING AREA PROPER CLASSIFIED AS CONDITIONALLY APPROVED OR CONDITIONALLY RESTRICTED IN THE OPEN STATUS Per the NSSP MO Section II. Chapter IV. @.06 A. (1), a *pollution assessment* of the mooring area proper is required to determine if the mooring area can be classified as conditionally approved or conditionally restricted. Per the NSSP MO Section II. Chapter IV. @.06 A. (2), after the mooring area proper pollution assessment determines that the mooring area proper is not a pollution source and it is documented in the CAMP, the growing area may be placed in the open status. The CAMP for each mooring area placed in a conditional classification is based on the information gathered during the *pollution assessment*. The CAMP will establish a strict set of criteria or performance standards, which must be met for the growing area to remain in the open status. Failure to meet the criteria or performance standards automatically places the growing area in the closed status, with immediate notice to the CAMP participants, affected industry, and the public. Performance Standards for a Mooring Area CAMP should include:

	• Establishment of a Memorandum of Understanding and/or an agreement to the conditions of the CAMP by the one (1) or more authorities involved including: mooring area management organizations, local municipalities, other local, State and Federal agencies, enforcement, harbor master, or other organizations which may be involved in the management and enforcement of the mooring area proper, pump out operations, and NDZ management and enforcement. • A written CAMP for the mooring area(s) and associated growing area being placed in the conditional classification, which includes a description of the mooring area(s) with a map showing the mooring area(s) boundaries. • A sanitary survey that shows the growing area will be in the open status of its conditional classification and provide a description of the factors determining the growing area's suitability for being classified conditionally approved or conditionally restricted with supporting information and data. • A description of the *pollution assessment* for the mooring area documenting how the reduction of an illicit human sewage (treated or untreated) and graywater discharge will be prevented and what management strategies are in place including, documenting boat types and uses, inspection of boat MSDs, documentation of pump out boats and facilities, NDZ regulations, education, management, and enforcement. • A description of the plan for monitoring water quality including what will be sampled and the location of sample stations on a map, numbers of sample stations, and frequency monitored. • A description of how the closed status for the conditional classification will be implemented which must include:

	○ A clear statement indicating when the performance standards are not met, the growing area will immediately be placed in the closed status; ○ A requirement to notify the authority or authorities that management plan performance standards have not been met, including: ▪ The name of the agency or other party responsible for notifying the authority; ▪ The anticipated response time between the performance standards not being met and notification of the authority; and ▪ The procedures for prompt notification including contingencies such as night, weekend, and absences of key personnel; • A description of implementation and enforcement, including: ▪ The response time between the notification to the authority of the failure to meet performance standards and activation of the legal closure of the growing area by the authority; ▪ The procedures and methods to be used to notify the shellfish industry; and ▪ The procedures and methods to be used to notify the patrol agency (enforcement agency) including: ▪ The name of the responsible patrol agency; ▪ The anticipated response time between the Aathority's legal closure of the growing area and notification of closure to the patrol agency; and ▪ A description of the patrol agencies anticipated activities to enforce the closed status of an area.

- A description of the criteria that must be met prior to reopening a mooring area or growing area in the closed status, including the need to determine that:
    - The performance standards established in the management plan are again compliant;
    - The flushing time for pollution dissipation is adequate;
    - A time interval has elapsed which is sufficient to permit reduction of human pathogens as measured by the coliform indicator group in the shellstock;
    - Where necessary, the bacteriological quality of the water must be verified; and
    - Shellstock feeding activity is sufficient to achieve reduction of pathogens to levels present prior to the pollution event.

- A commitment to a reevaluation of the management plan, at least annually, using the reevaluation requirements in the NSSP MO, or other regulations/rules required as necessary.
- A designation in the CAMP whether the shellstock may be harvested for relaying or depuration in a conditionally approved (closed status) or whether the harvested shellstock are to be relayed or depurated in a conditionally restricted area (open status).

ADJACENT WATERS
Per the NSSP MO Section II. Chapter IV. @.06 B., waters adjacent to a mooring area proper may be impacted by pollution associated as a result. Based on the pollution assessment conducted in NSSP MO Section II. Chapter IV. @.06 A., if the authority determines that the mooring area proper is a pollution source, a dilution analysis shall be used to determine if there is any impact to the adjacent waters. The dilution analysis shall be based on the volume of water in the vicinity of the mooring area proper.

If the dilution analysis predicts a theoretical FC loading greater than (>) 14 FC/100 ml, the waters adjacent to the mooring area shall be classified as: conditionally approved, restricted, conditionally restricted, or prohibited. It the dilution analysis predicts a theoretical FC loading less than (<) 14 FC/100 ml, the waters adjacent to the marina may be classified as: approved or conditionally approved.

	The dilution analysis shall include the following factors according to the NSSP MO Section II. Chapter IV. @.06 B. The recommendations provided, represents guidance for how the authority may meet the intent of each requirement: (a) **An occupancy rate for the mooring area**: Consider that the quantity of waste potentially originating in a mooring area depends on the number of people who are present in the mooring area. The fewer boats that are found to be occupied, the smaller the expected impact from the mooring area. The occupancy rate of the mooring area should be documented by actual observation of mooring area operations during the time of highest usage such as weekends or holidays. Document the overall number of boats in a mooring area and the number of boats being occupied as well as the number of people on each boat. Document the number of mooring balls and buoys in the mooring area. (b) **An actual of assumed rate of boats which will discharge untreated waste**: Document the number of boats with installed toilets and document the MSD type used (MSD Type I, II, or III) in the mooring area having the capability to discharge to the environment. If the authority uses an assumed rate of discharge, that rate should be supported by data gathered during the pollution assessment of the mooring area. **(c) An occupancy per boat (i.e., number of persons per boat):** If the authority chooses not to determine a specific occupancy per boat rate by investigation in specific areas or sites, the authority shall assume a minimum occupancy rate of two (2) persons per boat (NSSP MO Section II. Chapter IV. @.06 B. (6)). Document the number of people on liveaboard boats in the mooring area. This inventory should be taken during the expected high usage times such as weekends and holidays. The inventory should have continuity so that changes in population during high occupancy times can be documented. Regional differences exist regarding boat usage; therefore, the percent of high usage will vary. **(d) A fecal coliform discharge rate of 2 x 10^9 for the**

theoretical fecal coliform contribution per person per day.

(e) Assume that the wastes are completely mixed in the volume of water in and around the marina. Document the average depth of the area based on bathymetry charts and the volume of dilution water needed if complete mixing is assumed.

DILUTION ANALYSIS

The NSSP MO Section II. Chapter IV. @.05 and @.06 states that a dilution analysis will be used for making classification and closure determinations for waters adjacent to each marina proper and mooring area proper (if a pollution assessment determines the mooring area may be a pollution source). The information collected from a pollution assessment will help in determining the potential pollution impact and classification and size of the classification area or closure zone.

This dilution analysis requirement is based on the public health requisite that waters receiving waste discharges from marine toilets from marinas and mooring areas are not suitable for the direct harvest of shellfish destined for human consumption.

The intentional or unintentional direct discharge of treated or untreated human sewage and graywater discharge from a boat into a marina or mooring area is considered a point source and a high human health risk and therefore, pursuant to the NSSP MO Section II. Chapter IV. @.03 E. (5) (a), "An area classified as prohibited shall be established adjacent to each sewage treatment plant outfall or any other point source outfall of public health significance."

The estimated per capita discharge of fecal coliforms, coupled with the estimated population in the marina or mooring area, can be used to determine the classification and estimate a closure zone. Closures for existing or proposed marinas and mooring areas should be developed assuming two (2) persons per boat, and a 2×10^9 fecal coliform (FC) contribution per person per day, unless actual persons per boat or occupancy and discharge rates are documented by surveys conducted for individual marinas or mooring areas on a case-by-case basis. The authority should assume 100% boat slip and mooring ball occupancy unless the actual occupancy rate is documented through observation or credibly estimated. This documentation shall be maintained as specified by the NSSP MO,

Chapter I, for reevaluation of sanitary survey information.

Similarly, any expansion, modification, or change to the operation of a marina or mooring area will necessitate the reevaluation of the marina or mooring area occupancy rate.

In determining the above loading rates, a minimum factor should be considered to provide protection against intentional or unintentional waste discharges from boats in the marina or mooring area.

The theoretical waste discharge based on the occupancy and discharge rate, will be completely mixed in and around the marina or mooring area. The marina or mooring area closure zone shall be calculated to reduce the assumed bacterial load to 14 FC/100 ml, in the volume of water in the vicinity of the marina or mooring area. If the results of hydrographic studies are used, the estimated fecal coliform contribution can be distributed throughout the volume of water calculated to flow by the site in 24 hours.

Dilution hydrographic studies may be used to determine the water volume available for dilution and limits of travel of discharges from a marina. The area to be closed shall provide sufficient water volume for calculations to show that theoretical discharges from the marina or mooring area are diluted to 14 FC/100 ml of water. In situations where there are no hydrographic studies, the closed or prohibited area is to be established on a volumetric basis as though the wastes are completely mixed and uniformly distributed in and around the marina or mooring area. The closed area volume is typically based on average water depth and shall be sufficient to dilute the assumed waste load to a value of 14 FC/100 ml.

EXAMPLE CALCULATIONS

The following examples show how various factors are to be considered in closure area determinations around marinas or mooring areas:

CASE 1: No Documentation of Occupancy or Discharge Rates

Number of Boat Slips **50**

Number of People **2 x 50 =100**

Number of Fecal Coliforms (FC) **100 x 2 x 10^9 = 200 x 10^9 Dilution**

Volume Required **200 x 10^9 FC**

(14 FC/100 mL) x (1000 mL/liter)

Volume = 1.4 x 10^9 liters (5.0 x 10^7 cu ft)

Average Depth in Vicinity of Marina **3 meters (10ft)**

Closed Area Required **1.4 x 10^9 liters**

(3 meters) x (1000 liters/cubic meter)

A = 4.7 x 10^5 square meters (5.0 x 10^6 sq ft)

Radius of Half Circle Prohibited/Closed Area

2/ (4.7 10^5)

R = 550 meters (1800 ft)

CASE 2: Boat Slip Occupancy, Population, Holding Tanks and Pumpout Facilities Documented	
Number of Boat Slips	50
Slip Occupancy- Holiday Weekends	40 (80%)
Boats with No Holding Tanks*	16 (16/40 = 40%)
Average People per Boat	1.5
Number of People	1.5 x .40 x .80 x 50 =24
Number of Fecal Coliform (FC)	24 x 2 x 10^9 = 48 x 10^9
Dilution Volume Required	48 x 10^9 FC (14 FC/100 mL) x (1000 mL/liter) V = 3.4 x 10^8 liters (1.2 x 10^7 cu ft)
Average Depth in Vicinity of Marina	3 meters (10ft)
Closed Area Required	3.4 x 10^8 liters (3 meters) x (1000 liters/cubic meter) A = 1.1 x 10^5 square meters (1.2 x 10^6 sq ft)
Radius of Half Circle Closed Area	2/ππππ (1.1 xxxx 105) R = 265 meters (870ft)

	• Assumes pumpout facilities are consistently used, increase percentage if otherwise **REFERENCES** 1. Interstate Shellfish Sanitation Conference Marina Policy. August 1986. 2. Evaluation of Marinas by State Shellfish Sanitation Control Officials. Guideline 1.0. June 1989. 3. National Shellfish Sanitation Program Manual of Operations, Part I. 1988 revision. 4. Department of Health and Human Services NE Technical Unit. 1986. Hydrographic Studies of the Great Salt Pond, Block Island, Rhode Island. 5. Geldreich, Edwin, et al. Bacteria in the Feces of 295 (March). 1962. The distribution of Coliform Bacteria in the Feces of Warm-Blooded Animals. JWPCF 34(3), 6. U.S. Environmental Protection Agency (EPA), Region IV. 1985. Coastal Marina Assessment Handbook. 7. U.S. Department of Health and Human Services, Northeast Technical Services Unit. 1983. Hydrographic Studies of the Kiawah River, South Carolina. 8. Title 33 Code of Federal Regulations, Section 159.7 https://www.govregs.com/regulations/expand/title33_chapterI_part159_subpartA_section159.7#title33_chapterI_part159_subpartA_sectio n159.1 9. National Shellfish Sanitation Program (NSSP), Model Ordinance (MO). 2019 Revision 10. U.S. Environmental Protection Agency (EPA), Office of Waste Management. 2011. Graywater Discharges from Vessels. U.S. Environmental Protection Agency (EPA), Federal No Discharge Zone (NDZ) Link: https://www.epa.gov/vessels-marinas-and- ports/vessel-sewage-no-discharge-zones
Public Health Significance	The 2019 NSSP MO included new language separating out marinas and mooring areas. The adopted language does not have descriptive details as to how the new mooring area language will be evaluated by the FDA. Given that marinas and mooring areas may be considered a potential pollution source and high risk if mooring areas are not assessed correctly, the proposed updated marina and mooring area guidance is presented to help provide the guidance on how to meet those new requirements

Cost Information	N/A
Action by 2023 Task Force I	Recommended referral of Proposal 23-124 to an appropriate committee as determined by the Conference Chairperson.
Action by 2023 General Assembly	Adopted the recommendation of Task Force I on Proposal 23-124,
Action by FDA July 7, 2023	Concurred with Conference action on Proposal 23-124

Proposal No. 27-100
Date Received:

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Laboratory Committee
3. Affiliation	
4. Address Line 1	4801 Hermitage Rd. Ste 102
5. Address Line 2	
6. City, State, Zip	Richmond, VA 23227
7. Phone	804-330-6380
8. Fax	
9. Email	issc@issc.org
10. Proposal Subject	Modify Approved Laboratory Methods Table In Guidance Documents
11. Specific NSSP Guide Reference	Section IV – Guidance Documents, Ch. III – Laboratory, @.02 Methods, .01 Approved NSSP Laboratory Tests
12. Text of Proposal/ Requested Action	See Attached
13. Public Health Significance	The ISSC Laboratory Committee (LC) circulated a questionnaire to ISSC Membership in 2022 requesting feedback on the existing Approved Laboratory Methods Table included in the Guidance Documents section of the NSSP Guide. Based on the responses to that questionnaire, the LC has been working since that time to update and improve the Lab Methods Table to make it more accurate and user friendly. The updated Methods Table has been reorganized by method application, as most questionnaire responses indicated that determining the application/approved uses of laboratory methods was the primary reason why this table was utilized. Additionally, the following changes were made to the table in response to feedback received: 1) Method names were updated for consistency purposes and to match the terminology used in the Laboratory Evaluation Checklists. 2) The associated indicators and approved matrix categories were updated and corrected to match original approvals. 3) Limitations and conditions of use for each method have been more clearly spelled out in the updated table. 4) Method references have been corrected and refined, and additional details have been added to make them easier to access. 5) An introduction was added providing information on how to use the table, where to access pertinent program documentation, and how to access original copies of the method reference documents via the ISSC website. 6) Method applications for biotoxin methods were updated to match the Biotoxin Management Strategies described in the Model Ordinance text. 7) The market shellfish application was removed, as there are no longer any market shellfish standards included in the Model Ordinance. 8) The *Vibrio cholera* method (qPCR for CTX Gene) was removed from the table, as it was never officially adopted by the ISSC.
14. Cost Information	N/A

Action by 2025 Executive Board	Adopted the recommendation of 2025 Laboratory. Interim Approval on October 15, 2025

NSSP Laboratory Methods Table

All laboratory methods to be used within the NSSP must be validated, reviewed, and found to be fit for purpose following the procedures described in the ISSC Constitution, Bylaws, and Procedures – Procedure XV. Once they have been reviewed and adopted by the ISSC, these laboratory methods are included in the table below, and may be used for the analysis of shellfish, shellfish growing waters, shellfish harvest waters, or wastewater as described in Model Ordinance Ch. III, @.02. Prior to newly adopting any of these methods within a laboratory, that laboratory is required to conduct a method verification, as described in Section IV – Guidance Documents, Ch. III, @.02 - Methods, .02.

The table below describes which laboratory methods can be used for each different program application, as well as what matrix and other use limitations might be in place for each particular method. Shellfish matrix categories, as described in the "Approved Matrix Categories" column, can be found in Section IV – Guidance Documents, Ch. III, @.02 - Methods, .03. The references included in the "Source Method Reference" column can be accessed on the ISSC website at https://www.issc.org/laboratory-method-references.

Microbiological Methods

Application: Growing Area Classification and Management				
Method Name	**Indicator**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Multiple Tube Fermentation Technique for Seawater (APHA Decimal Dilution MPN)	Total Coliforms	Seawater	None	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; Media: pp. 7-15, Method: pp. 28-32
Multiple Tube Fermentation Technique for Seawater (12-tube single dilution MPN)	Total Coliforms	Seawater	Per NSSP Interpretation 23-III-@.02-100, Cannot Be Used With Systematic Random Sampling Strategy	A Simpler Multiple Fermentation Tube Test for Monitoring the Bacteriological Quality of Shellfish Harvest Waters, Proceedings of the 8th National Shellfish Sanitation Workshop (1974), pp. 123-126
Multiple Tube Fermentation Technique for Seawater using MA-1 (Decimal Dilution MPN)	Fecal Coliforms	Seawater	None	AOAC Official Methods of Analysis, 15th Ed., Association of Official Analytical Chemists (1990), pp. 436 - 437
Multiple Tube Fermentation Technique for Seawater using MA-1 without salicin (Decimal Dilution MPN)	Fecal Coliforms	Seawater	Any lab adopting this method must complete a study demonstrating equivalency to original A-1 method using a minimum of 120 samples, collected across seasons (i.e. 4 sets of 30 samples: winter, spring, summer, fall). Results must not be significantly different as shown by a T-Test, and data must be submitted to FDA for concurrence before adopting.	Karolus J, Cumbo M, Boehler S, Savina L. (2003). Modification of an approved medium for fecal coliform detection in seawater: A-1 medium minus salicin. *J Food Prot.* 66(1):120-1.
Multiple Tube Fermentation Technique for Seawater using MA-1 (12-tube single dilution MPN)	Fecal Coliforms	Seawater	Per NSSP Interpretation 23-III-@.02-100, Cannot Be Used With Systematic Random Sampling Strategy	National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish Section VIII - FDA Manual of Interpretations, Interpretation # 23-III-@.02-100
Multiple Tube Fermentation Technique for Seawater or Shellfish (APHA Decimal Dilution MPN)	Fecal Coliforms	Seawater, All Shellfish	None	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; For Seawater - Media: pp. 8-15, Method: pp. 35-36 For Shellfish - Media: pp. 8-15, Method: pp. 40-46
Multiple Tube Fermentation Technique for Seawater (12-tube single dilution MPN)	Fecal Coliforms	Seawater	Per NSSP Interpretation 23-III-@.02-100, Cannot Be Used With Systematic Random Sampling Strategy	National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish Section VIII - FDA Manual of Interpretations, Interpretation # 23-III-@.02-100
Membrane Filtration Technique for Seawater using mTEC	Fecal Coliforms	Seawater	None	Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. (1987). Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, *Journal WPCF,* 59(8).
Standard Plate Count for Shellfish Meats	All Bacteria	All Shellfish	None	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; Media: pp. 7-15, Method: pp. 40-44, 46

Application: Growing Area Classification and Management (Continued)				
Method Name	**Indicator**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Male Specific Coliphage for Wastewater	MSC	Wastewater	Can only be used to test wastewater, including influent, pre-treatment effluent, and final effluent	SLV - Proposal 15-114, as approved at the 2023 ISSC Biennial Meeting
Male Specific Coliphage for Shellfish Meats	MSC	Oysters, Hard Clams, Soft Clams	Can only be used to test shellfish from areas impacted by wastewater treatment plant outfalls or sewage spills	SLV Oysters/Soft Clams - Proposal 05-114, as approved at the 2009 ISSC Biennial Meeting SLV Hard Clams - Proposal 13-120, as approved at the 2013 ISSC Biennial Meeting
MPN Real-time PCR method for pathogenic *Vibrio parahaemolyticus* detection	*Vibrio parahaemolyticus* (tdh & trh)	Oysters	For reopening of growing areas closed as a result of V.p. illnesses	Kinsey TP, Lydon KA, Bowers JC, Jones JL. (2015). Effects of Dry Storage and Resubmersion of Oysters on Total *Vibrio vulnificus* and Total and Pathogenic (tdh+/trh+) *Vibrio parahaemolyticus* Levels. *J Food Prot.* 78(8):1574-80. SLV - Proposal 15-111, approved at the 2015 ISSC Biennial Meeting
MPN Real-time PCR method for total *Vibrio parahaemolyticus* detection	*Vibrio parahaemolyticus* (tlh)	Oysters	For reopening of growing areas closed as a result of V.p. illnesses	Kinsey TP, Lydon KA, Bowers JC, Jones JL. (2015). Effects of Dry Storage and Resubmersion of Oysters on Total *Vibrio vulnificus* and Total and Pathogenic (tdh+/trh+) *Vibrio parahaemolyticus* Levels. *J Food Prot.* 78(8):1574-80. SLV - Proposal 15-113, approved at the 2015 ISSC Biennial Meeting
Alkaline Phosphatase Probe method for *Vibrio parahaemolyticus* detection	*Vibrio parahaemolyticus* (tlh, tdh)	All Shellfish	For reopening of growing areas closed as a result of V.p. illnesses. This method only tests for tlh and tdh, it does not test for trh; NSSP Model Ordinance requires both tdh and trh results for reopening.	Direct Plating Procedure for the Enumeration of Total and Pathogenic *Vibrio parahaemolyticus* in Oyster Meats. David W. Cook, Angelo DePaola, and Susan A. McCarthy, Published by the ISSC in 1999 Bacteriological Analytical Manual United States Food and Drug Administration Chapter 9, Procedures, Other *Vibrios - V. parahaemolyticus* - A(1-2) & B(4)

Application: Controlled Relaying				
Method Name	**Indicator**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Multiple Tube Fermentation Technique for Seawater or Shellfish (APHA Decimal Dilution MPN)	Fecal Coliforms	All Shellfish	None	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; Media: pp. 8-15, Method: pp. 40-46
Male Specific Coliphage for Shellfish Meats	MSC	Oysters, Hard Clams, Soft Clams	Can only be used to test shellfish from areas impacted by wastewater treatment plant outfalls or sewage spills	SLV Oysters/Soft Clams - Proposal 05-114, as approved at the 2009 ISSC Biennial Meeting SLV Hard Clams - Proposal 13-120, as approved at the 2013 ISSC Biennial Meeting

Application: Wet Storage				
Method Name	**Indicator**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Multiple Tube Fermentation Technique for UV treated process water (10-tube, 10 mL portions, single dilution MPN)	Total Coliforms	Seawater	UV Treated Seawater Only; Single Dilution, Ten Tubes, 10-mL of sample in each tube	American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater,* 18th Edition. APHA/AWWA/WEF; 9221 - Multiple Tube Fermentation Technique for Members of the Coliform Group; pp. 9-45 - 9-51
Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES	Total Coliforms	Seawater	UV Treated Seawater Only	American Public Health Association (APHA). 1998. *Standard Methods for the Examination of Water and Wastewater,* 20th Edition. APHA/AWWA/WEF; 9222B - Standard Total Coliform Membrane Filter Procedure SLV - Proposal 11-111, as approved at the 2011 ISSC Biennial Meeting
Nephelometer Turbidity	Turbidity	Seawater	UV Treated Seawater Only; Method is not evaluated	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA.

Application: Depuration				
Method Name	**Indicator**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Multiple Tube Fermentation Technique for UV treated process water (10-tube, 10 mL portions, single dilution MPN)	Total Coliforms	Seawater	UV Treated Seawater Only	American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater,* 18th Edition. APHA/AWWA/WEF; 9221 - Multiple Tube Fermentation Technique for Members of the Coliform Group; pp. 9-45 - 9-51
Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES	Total Coliforms	Seawater	UV Treated Seawater Only	American Public Health Association (APHA). 1998. *Standard Methods for the Examination of Water and Wastewater,* 20th Edition. APHA/AWWA/WEF; 9222B - Standard Total Coliform Membrane Filter Procedure SLV - Proposal 11-111, as approved at the 2011 ISSC Biennial Meeting
Multiple Tube Fermentation Technique for Shellfish Meats (APHA Decimal Dilution MPN)	Fecal Coliforms	All Shellfish	Zero Hour Shellfish Only	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; Media: pp. 8-15, Method: pp. 40-46
Multiple Tube Fermentation Technique for Shellfish Meats (12-tube single dilution MPN)	Fecal Coliforms	All Shellfish	End Product Shellfish Only	National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish Section VIII - FDA Manual of Interpretations, Interpretation # 23-III-@.02-100
Elevated Temperature Coliform Plate Method for Clams	Fecal Coliforms	Hard Clams, Soft Clams	None	Cabelli VJ, Heffernan WP. (1970). Accumulation of Escherichia coli by the Northern Quahaug. *Appled Microbiology.* 19(2).
Male Specific Coliphage for Shellfish Meats	MSC	Oysters, Hard Clams, Soft Clams	Can only be used to test shellfish from areas impacted by wastewater treatment plant outfalls or sewage spills	SLV Oysters/Soft Clams - Proposal 05-114, as approved at the 2009 ISSC Biennial Meeting SLV Hard Clams - Proposal 13-120, as approved at the 2013 ISSC Biennial Meeting

Application: Post Harvest Processing Samples				
Method Name	**Indicator**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Vibrio MPN (with biochemical or Probe Confirmation)	*Vibrio vulnificus*	All Shellfish	For confirmation, may use method described in BAM, method described in Wright et al, or additional method that Laboratory can demonstrate is equivalent.	Bacteriological Analytical Manual, United States Food and Drug Administration, Chapter 9, Procedures, Other *Vibrios* - *V. vulnificus* - A(1-4) & B(1-3) Wright et al., (1993). Rapid identification of *Vibrio vulnificus* on nonselective media with an alkaline phosphatase-labeled oligonucleotide probe. *Appl Environ Microbiol* 59: 541–546.
Vibrio MPN (with biochemical or Probe Confirmation)	*Vibrio parahaemolyticus*	All Shellfish	For confirmation, may use method described in BAM, method described in McCarthy et al, or additional method that Laboratory can demonstrate is equivalent.	Bacteriological Analytical Manual, United States Food and Drug Administration, Chapter 9, Procedures, Other *Vibrios* - *V. parahaemolyticus* - A(1-5) & B(1) McCarthy SA, DePaola A, Cook DW, Kaysner CA, Hill WE. (1999). Evaluation of alkaline phosphatase- and digoxigenin-labelled probes for detection of the thermolabile hemolysin (tlh) gene of *Vibrio parahaemolyticus. Lett Appl Microbiol.* Jan;28(1):66-70.
Vibrio MPN (with PCR Confirmation)	*Vibrio parahaemolyticus*	All Shellfish	Following method described in BAM and in Bej et al, or additional method that Laboratory can demonstrate is equivalent.	Bej AK, Patterson DP, Brasher CW, Vickery MC, Jones DD, Kaysner CA. (1999). Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of tl, tdh and trh. *J Microbiol Methods.* Jun;36(3):215-25. Bacteriological Analytical Manual United States Food and Drug Administration Chapter 9, Procedures, Other *Vibrios* - *V. parahaemolyticus* - A(1-5) & B(4a11)
MPN Real-time PCR method for pathogenic *Vibrio parahaemolyticus* detection	*Vibrio parahaemolyticus* (tdh & trh)	Oysters	None	Kinsey, Thomas P., Lydon, Keri A., Bowers, John C., Jones, Jessica L. (2015). Effects of Dry Storage and Resubmersion of Oysters on Total *Vibrio vulnificus* and Total and Pathogenic (tdh+/trh+) *Vibrio parahaemolyticus* Levels, *Journal of Food Protection,* 78(8). Pages 1574-1580. SLV - Proposal 15-111, as approved at the 2015 ISSC Biennial Meeting
MPN Real-time PCR method for total *Vibrio parahaemolyticus* detection	*Vibrio parahaemolyticus* (tlh)	Oysters	None	Kinsey, Thomas P., Lydon, Keri A., Bowers, John C., Jones, Jessica L. (2015). Effects of Dry Storage and Resubmersion of Oysters on Total *Vibrio vulnificus* and Total and Pathogenic (tdh+/trh+) *Vibrio parahaemolyticus* Levels, *Journal of Food Protection,* 78(8). Pages 1574-1580. SLV - Proposal 15-113, as approved at the 2015 ISSC Biennial Meeting
MPN Real-time PCR method for *Vibrio vulnificus* detection	*Vibrio vulnificus*	Oysters	None	Kinsey, Thomas P., Lydon, Keri A., Bowers, John C., Jones, Jessica L. (2015). Effects of Dry Storage and Resubmersion of Oysters on Total *Vibrio vulnificus* and Total and Pathogenic (tdh+/trh+) Vibrio parahaemolyticus Levels, *Journal of Food Protection,* 78(8), Pages 1574-1580. SLV - Proposal 19-126, as approved at the 2019 ISSC Biennial Meeting

Biotoxin Methods

Application: Growing Area Management (Strategy B: Routine Shellfish Toxicity Monitoring)				
Method Name	**Biotoxin Type**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Paralytic Shellfish Toxins (PSP) by Mouse Bioassay Neurotoxic Shellfish Toxins (NSP) by Mouse Bioassay	PSP, NSP	All Shellfish	None	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; For PSP - pp. 57-61 For NSP - pp. 61-65
Paralytic Shellfish Toxins (PSP) by Receptor Binding Assay	PSP	Mussels	None	SLV - Proposal 13-114, as approved at the 2013 ISSC Biennial Meeting
Paralytic Shellfish Toxins (PSP) by Receptor Binding Assay	PSP	Hard Clams, Scallops	Results can only be used for screening and precautionary closures	SLV - Proposal 13-114, as approved at the 2013 ISSC Biennial Meeting
Paralytic Shellfish Toxins (PSP) by HPLC-PCOX	PSP	Mussels, Soft-shell Clams, Oysters, Scallops	None	SLV - Proposal 09-104, as approved at the 2009 ISSC Biennial Meeting
Diarrhetic Shellfish Toxins (DSP) by LC MS/MS	DSP	Hard Clams	None	SLV - Proposal 17-103, as approved at the 2017 ISSC Biennial Meeting
Amnesic Shellfish Toxins (domoic acid, ASP) by HPLC-UV	ASP	All Shellfish	None	Quilliam, Michael A., Xie, Mie, Hardstaff, William R. (1995). Rapid Extraction and Cleanup for Liquid Chromatographic Determination of Domoic Acid in Unsalted Seafood, Journal of AOAC Iinternational, 78(2). Pages 543–554.
~~Paralytic Shellfish Toxins (PSP) by Scotia Rapid Test~~ [1]	~~PSP~~	~~All Shellfish~~	~~a) Negative result can be used to maintain an area in the open status~~ ~~b) In an area that is in a closed status, method can be used as a screen to determine when to perform a Mouse Bioassay (i.e., reduce MBA use for samples that test positive by SRT)~~ ~~c) In an area in the open status, positive result shall be used for a precautionary closure~~	~~SLV - Proposal 03-116, as approved at the 2003 ISSC Biennial Meetings~~ ~~SLV - Proposal 05-110, as approved at the 2005 ISSC Biennial Meetings~~
~~Amnesic Shellfish Toxins (domoic acid, ASP) by Reveal 2.0~~ [2]	~~ASP~~	~~Oysters, Hard Clams, Mussels~~	~~Results can only be used for screening and precautionary closures~~	~~SLV - Proposal 13-112, as approved at the 2013 ISSC Biennial Meeting~~
Neurotoxic Shellfish Toxins (Brevetoxins, NSP) by MARBIONC ELISA	NSP	Oysters, Hard Clams	A negative result (≤1.6 ppm for hard clams, ≤1.8 ppm for oysters) can be used to support reopening of a growing area that was placed in the closed status. A positive result (>1.6 ppm for hard clams, >1.8 ppm for oysters) requires additional testing by Mouse Bioassay, or can be used to support the same management decision as a failed Mouse Bioassay result (i.e., the area remains closed to harvest).	SLV - Proposal 17-107, as approved at the 2017 ISSC Biennial Meeting

[1] Proposal 27-102: Request to rescind Scotia Rapid Test received Interim Approval on October 15, 2025

[2] Proposal 27-101: Request to rescind Reveal 2.0 received Interim Approval on October 15, 2025

Application: Pre-Harvest Shellfish Toxicity Testing (Strategy C)				
Method Name	**Biotoxin Type**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Paralytic Shellfish Toxins (PSP) by Mouse Bioassay Neurotoxic Shellfish Toxins (NSP) by Mouse Bioassay	PSP, NSP	All Shellfish	None	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; For PSP - pp. 57-61 For NSP - pp. 61-65
Paralytic Shellfish Toxins (PSP) by Receptor Binding Assay	PSP	Mussels	None	SLV - Proposal 13-114, as approved at the 2013 ISSC Biennial Meeting
Paralytic Shellfish Toxins (PSP) by HPLC-PCOX	PSP	Mussels, Soft-shell Clams, Oysters, Scallops	None	SLV - Proposal 09-104, as approved at the 2009 ISSC Biennial Meeting
Diarrhetic Shellfish Toxins (DSP) by LC MS/MS	DSP	Hard Clams	None	SLV - Proposal 17-103, as approved at the 2017 ISSC Biennial Meeting
Amnesic Shellfish Toxins (domoic acid, ASP) by HPLC-UV	ASP	All Shellfish	None	Quilliam, Michael A., Xie, Mie, Hardstaff, William R. (1995). Rapid Extraction and Cleanup for Liquid Chromatographic Determination of Domoic Acid in Unsalted Seafood, Journal of AOAC Iinternational, 78(2). Pages 543–554.
Neurotoxic Shellfish Toxins (Brevetoxins, NSP) by MARBIONC ELISA	NSP	Oysters, Hard Clams	A negative result (≤1.6 ppm for hard clams, ≤1.8 ppm for oysters) can be used for management decisions for shellfish toxicity testing. A positive result (>1.6 ppm for hard clams, >1.8 ppm for oysters) requires additional testing by Mouse Bioassay, or can be used to support the same management decision as a failed Mouse Bioassay result (i.e., the area remains closed to harvest).	SLV - Proposal 17-107, as approved at the 2017 ISSC Biennial Meeting

Application: Shellfish Lot Testing (Strategy D) This strategy can be used when growing areas are in controlled access status.				
Method Name	**Biotoxin Type**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Paralytic Shellfish Toxins (PSP) by Mouse Bioassay Neurotoxic Shellfish Toxins (NSP) Mouse Bioassay	PSP, NSP	All Shellfish	None	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; For PSP - pp. 57-61 For NSP - pp. 61-65
Paralytic Shellfish Toxins (PSP) by Receptor Binding Assay	PSP	Mussels	None	SLV - Proposal 13-114, as approved at the 2013 ISSC Biennial Meeting
Paralytic Shellfish Toxins (PSP) by HPLC-PCOX	PSP	Mussels, Soft-shell Clams, Oysters, Scallops	None	SLV - Proposal 09-104, as approved at the 2009 ISSC Biennial Meeting
Diarrhetic Shellfish Toxins (DSP) by LC MS/MS	DSP	Hard Clams	None	SLV - Proposal 17-103, as approved at the 2017 ISSC Biennial Meeting
Amnesic Shellfish Toxins (domoic acid, ASP) by HPLC-UV	ASP	All Shellfish	None	Quilliam, Michael A., Xie, Mie, Hardstaff, William R. (1995). Rapid Extraction and Cleanup for Liquid Chromatographic Determination of Domoic Acid in Unsalted Seafood, Journal of AOAC Iinternational, 78(2). Pages 543–554.
Neurotoxic Shellfish Toxins (Brevetoxins, NSP) by MARBIONC ELISA	NSP	Oysters, Hard Clams	A negative result (≤1.6 ppm for hard clams, ≤1.8 ppm for oysters) can be used for management decisions for lot testing. A positive result (>1.6 ppm for hard clams, >1.8 ppm for oysters) requires additional testing by Mouse Bioassay, or can be used to support the same management decision as a failed Mouse Bioassay result (i.e., the product is not released).	SLV - Proposal 17-107, as approved at the 2017 ISSC Biennial Meeting

Application: Pre-Harvest Shellfish Toxicity Screening and Lot Testing (Strategy E: formerly Onboard Screening/Dockside Testing Program) These strategies can be used when growing areas are in controlled access status.				
Method Name	**Biotoxin Type**	**Approved Matrix Categories**	**Limitations and Conditions of Use**	**Source Method Reference**
Paralytic Shellfish Toxins (PSP) by Abraxis Shipboard ELISA	PSP	Hard Clams	Pre-harvest screening only; Method can only be used in conjunction with the extraction method described in Proposal 05-111, and only as part of the onboard screening/dockside testing program in the Northeast Region.	Extraction SLV - Proposal 05-111, as approved at the 2011 ISSC Biennial Meeting Method SLV - Proposal 09-107, as approved at the 2011 ISSC Biennial Meeting
~~Paralytic Shellfish Toxins (PSP) by Scotia Rapid Test~~[1]	~~PSP~~	~~All Shellfish~~	~~Pre-harvest screening only~~	~~SLV - Proposal 03-116, as approved at the 2003 ISSC Biennial Meetings~~ ~~SLV - Proposal 05-110, as approved at the 2005 ISSC Biennial Meetings~~
~~Amnesic Shellfish Toxins (domoic acid, ASP) by Reveal 2.0~~[2]	~~ASP~~	~~Oysters, Hard Clams, Mussels~~	~~Pre-harvest screening only~~	~~SLV - Proposal 13-112, as approved at the 2013 ISSC Biennial Meeting~~
Paralytic Shellfish Toxins (PSP) by Receptor Binding Assay	PSP	Hard Clams, Scallops	Pre-harvest screening only	SLV - Proposal 13-114, as approved at the 2013 ISSC Biennial Meeting
Paralytic Shellfish Toxins (PSP) by Receptor Binding Assay	PSP	Mussels	Pre-harvest screening and lot testing	SLV - Proposal 13-114, as approved at the 2013 ISSC Biennial Meeting
Neurotoxic Shellfish Toxins (Brevetoxins, NSP) by MARBIONC ELISA	NSP	Oysters, Hard Clams	Pre-harvest screening and lot testing	SLV - Proposal 17-107, as approved at the 2017 ISSC Biennial Meeting
Paralytic Shellfish Toxins (PSP) by Mouse Bioassay Neurotoxic Shellfish Toxins (NSP) Mouse Bioassay	PSP, NSP	All Shellfish	Pre-harvest screening and lot testing	American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition. APHA; For PSP - pp. 57-61 For NSP - pp. 61-65
Paralytic Shellfish Toxins (PSP) by HPLC-PCOX	PSP	Mussels, Soft-shell Clams, Oysters, Scallops	Pre-harvest screening and lot testing	SLV - Proposal 09-104, as approved at the 2009 ISSC Biennial Meeting
Diarrhetic Shellfish Toxins (DSP) by LC MS/MS	DSP	Hard Clams	Pre-harvest screening and lot testing	SLV - Proposal 17-103, as approved at the 2017 ISSC Biennial Meeting
Amnesic Shellfish Toxins (domoic acid, ASP) by HPLC-UV	ASP	All Shellfish	Pre-harvest screening and lot testing	Quilliam, Michael A., Xie, Mie, Hardstaff, William R. (1995). Rapid Extraction and Cleanup for Liquid Chromatographic Determination of Domoic Acid in Unsalted Seafood, Journal of AOAC Iinternational, 78(2). Pages 543–554.

[1] Proposal 27-102: Request to rescind Scotia Rapid Test received Interim Approval on October 15, 2025

[2] Proposal 27-101: Request to rescind Reveal 2.0 received Interim Approval on October 15, 2025

Proposal No. 27-101
Date Received:

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting
(Tab to go to next field)

1. a. ☒ Growing Area
   b. ☐ Harvesting/Handling/Distribution
   c. ☐ Administrative

2. Submitter	ISSC Laboratory Committee
3. Affiliation	
4. Address Line 1	4801 Hermitage Rd. Ste 102
5. Address Line 2	
6. City, State, Zip	Richmond, VA 23227
7. Phone	804-330-6380
8. Fax	
9. Email	issc@issc.org
10. Proposal Subject	Request to rescind the Reveal 2.0 for ASP Method
11. Specific NSSP Guide Reference	Section IV, Chapter III, @.02 Methods, .01 Approved NSSP Laboratory Tests

12. Text of Proposal/ Requested Action

4. Approved Limited Use Methods for Marine Biotoxin Testing

	Biotoxin Type: Amnesic Shellfish Poisoning (ASP)	Biotoxin Type: Paralytic Shellfish Poisoning (PSP)	Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP)	Application: Growing Area Survey & Classification Sample Type: Shellfish	Application: Onboard Testing Program Sample Type: Shellfish	Application: Controlled Relaying Sample Type: Shellfish	Application: Controlled Harvest End Product Testing
Abraxis Shipboard ELISA[2]		X			X		
SRT[1]		X		X	X	X	
~~Reveal 2.0 ASP[3]~~	~~X~~			~~X~~	~~X~~	~~X~~	
~~RBA[4]~~RBA[3]		X		X	X	X	
MARBIONC Brevetoxin ~~ELISA[5]~~ELISA[4]			X	X	X	X	X

Footnotes:

1. Scotia Rapid Test for PSP, formerly Jellett Rapid Testing Ltd.
   a. Method can be used to determine when to perform a mouse bioassay in a previously closed area.
   b. A negative result can be substituted for a mouse bioassay to maintain an area in the open status.
   c. A positive result shall be used for a precautionary closure.
2. Saxitoxin (PSP) ELISA Kit. Method can be used in conjunction with rapid extraction method using 70% isopropanol (rubbing alcohol): 5% acetic acid (white vinegar) 2.5:1. ISSC Summary of actions, Proposal 05.111 (page 15) and 09-107 (page 140).

~~3. Reveal 2.0 ASP. Neogen Corporation. Screening Method for Qualitative Determination of Domoic Acid Shellfish. ISSC 201 Summary of Actions Proposal 13-112.~~

~~4.~~3. Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination. Dr. Fran Van Dolah. Method for Clams and Scallops for the Purpose of Screening and Precautionary Closure for PSP. ISSC 2013 Summary of Actions Proposal 13-114.

~~5.~~4. MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within this parameters:
   a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.
   b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.8 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method. ISSC 2017 Proposals for Consideration, Proposal 17-107 (page 466).

13. Public Health Significance

The Neogen Corporation has discontinued production on all biotoxin test kits, and the last of the Reveal 2.0 for ASP kits that were produced prior to that discontinuation have all passed their expiration date. There are no plans for

	Neogen or any other corporation to resume production of these kits. With this discontinuation, the only remaining laboratory method available within the NSSP for testing for ASP is the HPLC method cited in this same method table in Section 2. Approved Methods for Marine Biotoxin Testing.
14. Cost Information	N/A
Action by 2024 Executive Board	Adopted the recommendation of 2024 Laboratory Committee. Interim Approval on April 16, 2025

Proposal No. 27-102
Date Received:

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting
(Tab to go to next field)

1. a. ☒ Growing Area
   b. ☐ Harvesting/Handling/Distribution
   c. ☐ Administrative

2. Submitter	ISSC Laboratory Committee
3. Affiliation	
4. Address Line 1	4801 Hermitage Rd. Ste 102
5. Address Line 2	
6. City, State, Zip	Richmond, VA 23227
7. Phone	804-330-6380
8. Fax	
9. Email	issc@issc.org
10. Proposal Subject	Request to rescind the Scotia Rapid Test for PSP Method
11. Specific NSSP Guide Reference	Section IV, Chapter III, @.02 Methods, .01 Approved NSSP Laboratory Tests

12. Text of Proposal/ Requested Action

4. Approved Limited Use Methods for Marine Biotoxin Testing

	Biotoxin Type: Amnesic Shellfish Poisoning (ASP)	Biotoxin Type: Paralytic Shellfish Poisoning (PSP)	Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP)	Application: Growing Area Survey & Classification Sample Type: Shellfish	Application: Onboard Testing Program Sample Type: Shellfish	Application: Controlled Relaying Sample Type: Shellfish	Application: Controlled Harvest End Product Testing
Abraxis Shipboard ~~ELISA[2]~~ ELISA[1]		X			X		
~~SRT[1]~~		~~X~~		~~X~~	~~X~~	~~X~~	
Reveal 2.0 ~~ASP[3]~~ ASP[2]	X			X	X	X	
~~RBA[4]~~RBA[3]		X		X	X	X	
MARBIONC Brevetoxin ~~ELISA[5]~~ELISA[4]			X	X	X	X	X

Footnotes:

~~1. Scotia Rapid Test for PSP, formerly Jellett Rapid Testing Ltd.~~
~~a. Method can be used to determine when to perform a mouse bioassay in a previously closed area. A negative result can be substituted for a mouse bioassay to maintain an area in the open status.~~
~~b. A positive result shall be used for a precautionary closure.~~

~~2.~~1. Saxitoxin (PSP) ELISA Kit. Method can be used in conjunction with rapid extraction method using 70% isopropanol (rubbing alcohol): 5% acetic acid (white vinegar) 2.5:1. ISSC Summary of actions, Proposal 05.111 (page 15) and 09-107 (page 140).

~~3.~~2. Reveal 2.0 ASP. Neogen Corporation. Screening Method for Qualitative Determination of Domoic Acid Shellfish. ISSC 201 Summary of Actions Proposal 13-112.

~~4.~~3. Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination. Dr. Fran Van Dolah. Method for Clams and Scallops for the Purpose of Screening and Precautionary Closure for PSP. ISSC 2013 Summary of Actions Proposal 13-114.

~~5.~~4. MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters:

a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.

b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.8 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method. ISSC 2017 Proposals for Consideration, Proposal 17-107 (page 466).

13. Public Health Significance

The Scotia Rapid Testing company has gone out of business and is no longer producing these test kits. There are still several options available for PSP testing

	within the NSSP, but the discontinuation of this particular test kit eliminates one rapid, easy-to-use option from the list of approved limited use methods.
14. Cost Information	N/A
Action by 2025 Executive Board	Adopted the recommendation of 2024 laboratory Committee. Interim Approval on April 16, 2025

Proposal No. 2 7 - 1 0 3

Add Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	ISSC Laboratory Committee
3. Affiliation	ISSC
4. Address Line 1	4801 Hermitage Rd
5. Address Line 2	
6. City, State, Zip	Richmond,VA 23227
7. Phone	(804)330-6380
8. Email	issc@issc.org
9. Proposal Subject	Checklist – RBA PSP
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	Corrections to Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP) Checklist
12. Public Health Significance	N/A
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A
Action by 2024 Executive Board	Adopted the recommendation of 2024 Laboratory Committee. Interim Approval on October 30, 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3		1.1.1 Written Plan (Check √ those items which apply).
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			f. Internal performance assessment.
			g. External performance assessment.
C	**2**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
C	**6**		**1.2.5 Training regarding radiation laboratory safety, handling and disposal practices is documented and records are maintained.**
C	**15**		**1.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitoxin in this assay. Alternatively, the laboratory uses less than 50 µCi per year and adheres to the American Radiolabeled Chemical (ARC) exemption status.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**

		1.4 Laboratory Equipment	
C	**4**	**1.4.1**	**Any lab equipment that may come into contact with [^{3}H]-STX at any point in the preparation or assay procedures must be specially labelled and must remain in the work area designated for low-level radiation work.**
O	5	1.4.2	The pH meter has a standard accuracy of 0.1 pH units.
K	7	1.4.3	The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8	1.4.4	The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1	1.4.5	The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.
K	1	1.4.6	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	9	1.4.7	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.
K	6	1.4.8	pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6	1.4.9	The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3	1.4.10	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
K	2	1.4.11	Refrigerator temperatures are maintained between 0 and 4 °C. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.
K	1	1.4.12	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**4, 6, 10**	**1.4.13**	**Freezer temperature used to store [^{3}H] STX standard, rat brain membrane tissue preparation, interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -80 °C or below. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.**

K	6, 10		1.4.14 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.15 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
O	8		1.4.16 All glassware is clean.
C	**3**		**1.4.17 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.18 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
C	**6**		**1.4.19 Micropipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.**
C	**11**		**1.4.20 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.21 Minimum radiation safety equipment and protocols include the following: A wipe-test is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
		1.5 Reference Solution Reagent Storage, Preparation and Security	
C	**12**		**1.5.1 [^{3}H] STX standard is stored in a freezer at -80 °C or below.**
C	**10**		**1.5.2 Concentration of [^{3}H] STX standard is calculated from the lot information provided by the supplier with each batch.**
K	6		1.5.3 Unopened diHCl STX standard may be stored at room temperature or refrigerated.
C	**10**		**1.5.4 Preparation of MOPS assay buffer includes the following:** **a. 100 mM MOPS/L.** **b. 100 mM choline chloride/L.** **c. pH adjustment to 7.4 with NaOH.** **e. refrigerated storage at 4 °C.** **d. Maintained ice cold while in use.**
C	**10**		**1.5.6 Bulk standard curve dilutions are stored at 4 °C for up to one (1) month.**
K	1		1.5.7 Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice)*. b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____ c. Water contains <100 CFU/mL using the heterotrophic plate count method.

		1.6 Rat Brain Membrane Tissue Preparation and Storage		
C	**10**		**1.6.1**	**MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to MOPS/choline chloride fresh on the day of use.**
C	**10**		**1.6.2**	**The cerebral cortex of 6-week old Sprague-Dawley rats is used in membrane tissue preparations, placed in iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL) and homogenized with no visible chunks remaining in the homogenate. This procedure is repeated until twenty (20) rat brains have been processed.**
C	**10**		**1.6.3**	**The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**
K	10		1.6.4	The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).
K	10		1.6.5	The resuspended rat brain tissue preparations are pooled and the centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue**.**
K	10		1.6.6	The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.
K	10		1.6.7	The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed for 20 seconds to obtain a homogeneous membrane tissue preparation.
C	**10**		**1.6.8**	**Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months.**
		1.7 Rat Brain Membrane Tissue Protein Receptor Determination		
C	**10**		**1.7.1**	**The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube method) or equivalent.**
C	**10**		**1.7.2**	**The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined.**
K	10		1.7.3	Dilutions of the protein/receptor concentration of the rat brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous.
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS - RBA				
		2.1 Collection and Transportation of Samples		
C	**5**		**2.1.1**	**A representative sample of shellfish is collected.**
K	5		2.1.2	Shellfish samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	5		2.1.3	Shellfish samples are labeled with the collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**5**		**2.1.4**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**

K	6, 13		2.1.5	Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
		2.2 Preparation of Samples for Analysis – Homogenization		
C	**5, 6**		**2.2.1**	**At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2	The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3	Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4	The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8	Pieces of shell and drainage are discarded.
C	**5, 6**		**2.2.4**	**Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender <u>with all freeze-thaw liquid</u> for homogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender <u>with all freeze-thaw liquid</u> for homogenization.**
K	6, 13		2.2.5	Homogenates should be extracted immediately. If homogenates must be stored, they should be frozen.
		2.3 Preparation of Samples for Analysis – Extraction		
K	5, 10		2.3.1	0.1 M HCl is used for extractions.
K	5, 10		2.3.2	At least five (5) grams of tissue +/- 0.1g is extracted using a 1:1 mass to volume ratio of 0.1 M HCl.
C	**10**		**2.3.3**	**The pH of the sample is checked and adjusted as necessary to between 3.0– 4.0.**

C	**10**		**2.3.4**	**Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**
C	**6**		**2.3.5**	**The sample is promptly brought to a boil at 99.0 +/- 1.0 °C and gently boiled for 5 minutes.**
O	6		2.3.6	The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.7	The sample is allowed to cool to room temperature.
C	**10**		**2.3.8**	**The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	5, 10		2.3.9	The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water).
K	10		2.3.10	The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; then b. an aliquot of the sample is centrifuged at 3000 x g for 10 minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.11	The sample extract is analyzed immediately, refrigerated at 4 °C in a sealed container for up to 24 hours, or frozen at -20 °C.
		2.4 Sample Assay		
K	6		2.4.1	One analyst performs the entire plate set-up for the assay.
K	6		2.4.2	Microtubes containing dilutions and samples are vortexed immediately before dispensing.
K	10		2.4.3	The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).
C	**10**		**2.4.4**	**The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension.**
K	10		2.4.5	Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.
C	**10**		**2.4.6**	**The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.7	Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane.
K	10		2.4.8	All wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, in order to ensure even pressure and filtration across the plate.
C	**10**		**2.4.9**	**Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate).**
K	10		2.4.10	[^{3}H] STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.
C	10		**2.4.11**	**An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).**
K	10		2.4.12	Standard curve fitting is calculated using appropriate software program.

C	**10**		**2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.15 The IC_{50} is in acceptable range (2.0 nM +/- 30%). If the IC_{50} is outside this range, the assay results are rejected and the assay must be repeated**
C	**10**		**2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.**
K	4		2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.
		2.5 Calculation of Sample Toxicity	
C	**10**		**2.5.1 When more than one dilution falls within B/B_0 of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**
C	**10**		**2.5.2 Sample toxicity is calculated as follows:** **(nM STX equiv.) x (sample dilution) x (210 µL total volume/35 µL sample = mM STX equivalent in extract** **(nM STX diHCl equiv. in extract) x 1L/1000 mL x 372 ng/nmol x1 µg/1000 ng =µg STX diHCl equiv./mL** **µg STX diHCl equiv./mL x mL extract/g shellfish x 1000 g/kg =µg STX diHCl equiv./kg**
C	**14**		**2.5.3 Any value equal to or greater than 80 µg STX diHCl equiv./100 g) of sample is actionable.**
C	**14**		**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:**

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LABORATORY:

DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
RECEPTOR BINDING ASSAY FOR PSP COMPONENT: (Part I-II)	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical (C), Key (K), and Other (O) Nonconformities	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the RBA for PSP:

1. **Conforms Status:** The RBA for PSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The RBA for PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. The number of Critical nonconformities is ≥ 1 but < 4;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status**: The RBA for PSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is ≥4;
   b. or total # of Key nonconformities is ≥ 6;
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☒ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	New Federal Waters Definitions
10. Specific NSSP Guide Reference	**Section I. Purposes & Definitions** Definitions
11. Text of Proposal/ Requested Action	**Definitions** A. General. The definitions provided below are consistent in intent with the NSSP. B. Definition of Terms. **(1) Adequately Iced …** **…** **(23) Container …** **(24) Contract Harvester List** refers to a list of harvesters holding an approved NOAA SIP contract for harvesting shellstock from federal waters. (~~24~~25) **Corrosion Resistant Materials …** **…** (~~81~~82) **National Shellfish Sanitation Program (NSSP) …** **(83) NOAA** means the National Oceanic and Atmospheric Administration. **…** (~~116~~118) **Shellfish Gardening …** **(119) Seafood Inspection Program (SIP)** is a NOAA program established to ensure the safety and quality of seafood by providing inspection services to the seafood industry for fish, shellfish, and fishery products. **…** (~~117~~120) **Shellfish Standardization Inspector …** **…** (~~124~~127) **Standardization …** **(128) State of Landing Authority** means the NSSP member state where product harvested in federal waters lands and is received by the primary processor. **…** (~~125~~129) **Swing Deficiency …** **…** (~~131~~135) **Wet Storage …**

12. Public Health Significance	The proposed update to develop a federal waters chapter introduces new terms to the Model Ordinance that need defined for clarification.
13. Cost Information	No cost.
14. Changes Needed to Guidance (if proposed change is adopted)	TBD

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Definition of mooring areas
10. Specific NSSP Guide Reference	Section I Purposes & Definitions
11. Text of Proposal/ Requested Action	**(74) Marina** means any water area with a structure (docks, basin, floating docks, etc.) which is used for docking and constructed to provide temporary or permanent docking space for more than ten (10) boats. **(79) Mooring Area** means any water area that is used to provide temporary or permanent anchorage for more than ten ~~twenty~~ (2~~1~~0) boats ~~with marine sanitation devices~~. Mooring areas do not include any structures for docking boats.
12. Public Health Significance	**Background** The current NSSP Model Ordinance (2023) defines a **mooring area** as: *"any water area that is used to provide temporary or permanent anchorage for **more than twenty (20) boats with marine sanitation devices**. Mooring areas do not include any structures for docking boats."* By contrast, a **marina** is defined as: *"any water area with a structure (docks, basin, floating docks, etc.) which is used for docking and constructed to provide temporary or permanent docking space for **more than ten (10) boats**."* FDA proposes reverting the mooring area threshold to **>10 boats** and **eliminating the MSD qualifier**. The following provides the science-based public health rationale. The 10-boat threshold proposed for mooring areas is grounded in established Federal science. EPA's *Guidance Specifying Management Measures for Sources of Nonpoint Pollution in Coastal Waters* (EPA-840-B-92-002, January 1993), issued under Coastal Zone Act Reauthorization Amendments (CZARA), explicitly applies management measures to "any mooring field where 10 or more boats are moored"

	— the same scientific baseline from which the NSSP's marina definition derives. The 2023 NSSP amendment, which doubled the threshold to >20 boats and added an MSD qualifier, departed from this Federal standard without a supporting public health rationale. Restoring the >10-boat threshold eliminates an irrational public health regulatory disparity: marinas and mooring areas concentrate equivalent numbers of boats in fixed water areas and generate equivalent per-boat fecal coliform loading. The only difference between them is the presence of docking structures — a factor with no established relationship to pathogen risk. The public health consequences of the current definition are direct and documentable. The NSSP's own dilution analysis methodology calculates fecal coliform loading based on boat count and occupancy — not MSD presence — at a rate of 2×10^9 fecal coliforms per person per day. A cluster of 11–20 boats at an unqualified mooring area generates loading sufficient to shift adjacent shellfish growing area classifications, yet the current definition excludes this range from required sanitary survey consideration entirely. EPA CZARA guidance identified boating activities as a contributing source in the closure of millions of acres of East Coast shellfish-growing waters. Shellfish bioaccumulate fecal pathogens — including norovirus and hepatitis A virus — at concentrations far exceeding surrounding waters, creating direct, foreseeable risk of foodborne illness when growing area classifications do not account for all proximate pollution sources. This proposal closes that gap.
13. Cost Information	No cost.
14. Changes Needed to Guidance (if proposed change is adopted)	TBD

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Model Ordinance Effectiveness Review Committee
3. Affiliation	ISSC
4. Address Line 1	4801 Hermitage Rd
5. Address Line 2	
6. City, State, Zip	Richmond,VA
7. Phone	804-330-6380
8. Email	issc@issc.org
9. Proposal Subject	Model Ordinance Effectiveness
10. Specific NSSP Guide Reference	Chapter I. Shellfish Sanitation Program Requirements for the Authority
11. Text of Proposal/ Requested Action	**@.01 Administration** A. **Scope.** (1) **The Authority shall establish a statewide shellfish safety and sanitation program to regulate:** (a) **The classification of shellfish growing areas;** (b) **The harvesting of shellfish;** (c) **Shellfish processing procedures and facilities;** (d) **Product labeling;** (e) **Storage, handling and packing;** (f) **Shellfish shipment in interstate commerce;** (g) **Shellfish dealers; and** (h) **Bivalve aquaculture.** (2) **All foreign countries shipping shellfish into the United States will have a memorandum of understanding or an equivalency agreement with the United States.** (3) **The regulatory responsibility for growing area and harvest control in federal waters will be the responsibility of the FDA and NOAA.** B. **State Laws and Regulations. The Authority shall have laws and regulations which provide an adequate legal basis for the safety and sanitary control of all program elements including but not limited to the elements outlined in @.01 A. Federal Agencies shall have laws and regulations which provide an adequate legal basis for the safety and sanitary control of growing areas and harvest control.** C. **Records. The Authority shall maintain records to demonstrate the effective administration of a statewide shellfish safety and sanitation program. These records shall be made available~~maintained in a central file and made available physically and/or electronically~~ to any interested person upon request, consistent with appropriate State and Federal law.** D. **Shared Responsibilities. If more than one agency is involved in the administration of the statewide shellfish safety and sanitation**

program, memoranda of agreement shall be developed between the agencies to define each agency's responsibilities. In the case of Federal Waters, if more than one agency is involved in the administration of the shellfish safety and sanitation program, memoranda of agreement shall be developed between the agencies to define each agency's responsibilities.

E. Administrative Procedures.

(1) The Authority shall have administrative procedures sufficient to:

(a) Regulate shellfish harvesting, sale, processing and shipment;

(b) Ensure that all shellfish shipped in interstate commerce originate from a dealer located within the State from which the shellstock are harvested or landed, unless the Authority has a memorandum of understanding with the Authority in another State to allow dealers from its State to purchase the shellstock;

(c) Detain, condemn, seize, and embargo shellfish; and

(d) Assure compliance with Shellfish Plant Inspection Standardization.

(2) In the case of Federal Waters, the FDA and NOAA shall have administrative procedures sufficient to regulate growing areas and harvest control.

F. Epidemiologically Implicated Outbreaks of Shellfish-Related Illness.

The Authority shall:

(1) Have a written protocol with the appropriate State agencies responsible for collecting epidemiological information related to reported foodborne illnesses. The protocol shall outline the procedure to ensure that all shellfish related illnesses are reported to the shellfish Authority(s); and

(2) Have written procedures for investigating incidents of ~~shellfish~~ foodborne illness~~disease~~.

G. Commingling.

(1) Except for any shellfish included in the Authority's commingling plan, the Authority shall not permit the commingling of shellfish.

(2) If the Authority permits shellfish product commingling, the Authority shall develop a commingling management plan. The plan shall:

(a) Minimize the commingling dates of harvest and growing areas;

(b) Define a primary dealer;

(c) Limit the practice of commingling to primary dealers;

(d) Limit commingling to shellfish harvested from specific growing areas within the State as identified by the Authority and purchased directly from harvesters; and

(e) Define how the commingled shellfish will be identified.

H. **Personnel training requirements for implementing the NSSP**

(1) **Shellfish Dealer Inspections**

(a) **Shellfish Standardization Officer (SSO) shall successfully complete:**

(i) the FDA standardization training course;

(ii) seafood HACCP; and

(iii) the field evaluation by a FDA standardization officer.

(b) **Shellfish Standardization Inspector (SSI) shall successfully complete:**

(i) the FDA standardization training course;

(ii) seafood HACCP; and

(iii) the field evaluation by a FDA standardization officer or the SSO.

(2) **Growing Area Classification**

(a) **Sanitary Survey Officer shall successfully complete:**

(i) the FDA growing area course; and

(ii) have a minimum of one (1) year of on the job experience in a NSSP growing area classification program within the shellfish sanitation program.

(3) **Patrol Enforcement**

(a) **Officers responsible for the patrol of shellfish growing areas shall obtain the following training:**

(i) basic law enforcement before assuming patrol duties;

(ii) shellfish control regulations before assuming independent patrol duties; and

(iii) updated shellfish control regulations at an interval deemed appropriate by the Authority.

(4) **Laboratory**

(a) **Laboratory Evaluation Officer (LEO) shall successfully complete:**

(i) the FDA Laboratory Evaluation Officer training course;

(ii) field standardization by a FDA LEO; and

(iii) have a minimum of two (2) years of shellfish laboratory experience or a laboratory background with a minimum of three (3) years of bench level experience with the method types that will be evaluated.

I. **Request for Emergency Consideration. In the event of a declared public health emergency or natural or man-made disaster, including activation of the State Emergency Response Plan, if the Authority is not in a position to operate the program in full compliance with NSSP program requirements, the Authority shall immediately notify the ISSC and the FDA. The FDA shall immediately conduct discussions with the Authority to reach a mutually acceptable resolution.**

Additional Guidance: Section IV Guidance Documents Chapter III. Section.02

@.02 Dealer Certification

A. **General**

(1) **A person requesting certification shall be subject to a comprehensive, onsite inspection and meet the criteria in Section B. or Section C., as appropriate. The plant inspection shall be conducted by the SSO or SSI, using the appropriate inspection form, within the 120-day period immediately prior to the issuance or renewal of the certification.**

(2) **Certification shall be given only to persons who meet the established requirements established for certification.**

(3) **All certifications shall expire annually. The month selected for certification expiration shall be at the discretion of the Authority.**

(4) **The Authority shall issue only a unique~~one~~ certification number to a dealer for a location. A person or dealer may obtain more than one certification if each business is maintained as a separate entity.~~:~~**

~~(a) Maintained as a separate entity; and~~

~~(5) Is not found at the same location.~~

~~(6)~~(5) **The Authority may permit separate certified dealers to share a facility.**

~~(7) The certification number issued to each dealer by the Authority shall be unique.~~

~~(8)~~(6) **Adequate records documenting each dealer's compliance with certification requirements shall be maintained for at least three (3) years. These records shall include:**

(a) **Inspection reports of dealers;**

(b) **Notification letters and enforcement actions;**

(c) **Shellfish sample results and follow-up actions taken;**

(d) **Records of complaints or inquiries and follow-up actions taken; and**

(e) **Administrative hearing transcripts and records.**

B. **Initial Certification.**

(1) **Initial certification shall be given only to persons who meet the following requirements:**

(a) **HACCP requirements:**

(i) **A HACCP plan accepted by the Authority;**

(ii) **No critical deficiencies;**

(iii) **Not more than two (2) key deficiencies; and**

(iv) **Not more than two (2) other deficiencies.**

(b) **Sanitation and additional Model Ordinance Requirements**

(i) **No critical deficiencies;**

(ii) **Not more than two (2) key deficiencies; and**

(iii) **Not more than three (3) other deficiencies.**

(2) **The initial certification shall include a compliance schedule to correct any deficiencies not corrected by the**

dealer during the inspection.

~~(2)~~**(3) Provide proof of training for employees in accordance with Chapter X .04A 2.(c).**

C. **Renewal of Certification.**

(1) **A dealer shall make application for certification renewal annually at the time specified by the Authority. The Authority shall not renew the certification for any dealer until the dealer:**

(a) **Meets the requirements of Section B. 1. (a) and Section B. 1. (b). The number of deficiencies allowed under Section B. 1. (a) and Section B. 1. (b) shall include carry over deficiencies from an existing compliance schedule approved by the Authority and new deficiencies identified during the certification renewal inspection; and**

(b) Agrees to a compliance schedule to address any new deficiencies not corrected by the dealer during the inspection.

~~(b)~~**(c) Provides proof of training for employees in accordance with Chapter X .04A 2.(c).**

D. **Revocation or Suspension of Certification.**

(1) **The Authority shall not allow any dealer whose certification has been suspended or revoked under Section H. to deal in shellfish.**

(2) **The Authority shall not issue certification to a dealer whose certification has been suspended or revoked to deal in shellfish until the dealer meets the requirements for initial certification.**

E. **Interstate Certified Shellfish Shippers List (ICSSL).**

(1) **When the Authority certifies a person to become a dealer, the Authority shall notify the FDA for the purpose of having the dealer listed in the ICSSL. The Authority shall include the certification type and any permit designation to be included in the ICSSL. The notice shall be in the format of FDA Form 3038.**

Designations:

Certification	Permit
SP – Shucker Packer	PHP – Post-Harvest Proce
RP – Repacker	AQ – Aquaculture
SS – Shellstock Shipper	WS – Wet Storage
RS – Reshipper	
DP – Depuration	

(2) **The Authority shall notify the FDA by updating Form 3038 for the purpose of having the dealer removed from the ICSSL whenever a dealer's certificate or permit is suspended, revoked or cancelled.~~:~~**

~~**(a) Suspended; or**~~

~~**F. Revoked.**~~

~~**G.**~~**F. Inspections.**

	(1) **After any person is certified, the Authority ~~shall~~should make unannounced inspections of the dealer's facilities:** (a) **During periods of activity; and** (b) **At the following minimum frequencies during the period of certification:** (i) **Within thirty (30) days of beginning activities if the dealer was certified on the basis of a pre-operational inspection;** (ii) **At least monthly for dealer facilities certified as depuration processors;** (iii) **At least triannually for dealer's activities certified as shucker-packer or repacker; and** (iv) **At least semiannually for other dealer activities or annually for seasonal other dealer activities that are certified for six (6) months or less.** (2) **The Authority shall provide a copy of the completed inspection form to the person in-charge at the dealer's operation within a reasonable time of completing the inspection. The inspection form shall contain a listing of deficiencies by area in the operation and inspection item with corresponding citations to this Model Ordinance.** ~~H.~~G. **Performance Based Inspection Program (PIP).** (1) **A PIP may be instituted by the Authority for any dealer who meets the requirements of this section.** (2) **The minimum frequency of inspection under a PIP shall be no less than one (1) inspection per certification period. The recertification inspection may qualify as the required minimum inspection frequency.** (3) **To be eligible for a PIP, the dealer shall have demonstrated a history of satisfactory compliance for the previous three (3) year period. The three (3) year demonstration shall include:** (a) **Full compliance with the minimum inspection frequency shown under Section F.;** (b) **Recertification of the dealer by the Authority;** (c) **Verification that no critical deficiencies, no more than one (1) key deficiency and no more than two (2) other deficiencies have occurred in any one (1) inspection;** (d) **Correction of all identified deficiencies in accordance with the compliance schedule approved by the Authority; and** (e) **No repetition of the identified deficiencies.** ~~I.~~H. **Enforcement.** (1) **General.** (a) **The Authority shall use any combination of administrative hearings, fines, certification cancellations, temporary suspension of operating licenses, embargoes, product condemnations or product**

seizures to accomplish the implementation of this Ordinance.

(b) When a dealer has failed to meet the compliance schedule, the Authority shall:

(i) Consider whether it is appropriate to revise the compliance schedule, suspend or revoke the dealer's certification, or seek other administrative remedies; and

(ii) Document why an option was selected.

(2) Actions Triggered by Inspections.

(a) When any inspection detects a critical deficiency:

(i) The deficiency shall be corrected during that inspection; or

(ii) The dealer must cease production affected by the deficiency.

(b) When the dealer fails to comply with (a) above, the Authority shall immediately begin actions to suspend or revoke the dealer's certification.

(c) Product affected by a critical deficiency shall be controlled to prevent contaminated or adulterated product from reaching consumers. When necessary the Authority shall:

(i) Detain or seize any undistributed lots of shellfish that may have been adulterated;

(ii) Initiate a recall of any distributed shellfish; and

(iii) Immediately notify the enforcement officials for FDA and any other Authorities where the product was distributed.

(d) When any inspection detects any key or other deficiencies not already covered in a compliance schedule, the Authority, working with the dealer, shall develop a compliance schedule to correct the new key or other deficiencies.

(e) When any inspection detects four (4) or more new key deficiencies, the Authority shall consider at least one of the following options and document the reasons for the selection of a particular option:

(i) Revise the existing compliance schedule;

(ii) Suspend or revoke the dealer's certification; or

(iii) Seek other administrative remedies.

@.03 Evaluation of Shellfish Sanitation Program Elements

A. The goal of shellfish program evaluation shall be to monitor program implementation and work with States to determine where problems may exist and how to address them.

1. Shellfish program evaluation methodologies shall:

a. Monitor State program implementation;

b. Assess State program effectiveness; and

c. Evaluate the effectiveness~~validity~~ of the elements of

the NSSP Guide for the Control of Molluscan Shellfish.

2. **The minimum components of shellfish program evaluation shall include:**
   a. **A description of the program activity;**
   b. **A comparison of FDA observations with State observations; and**
   c. **A measurement of conformity of shellfish program activities with elements of the NSSP Guide for the Control of Molluscan Shellfish.**
3. **The focus of data collection shall be on measuring conformity of shellfish program activities with elements of the NSSP Guide for the Control of Molluscan Shellfish.**
4. **The types of data collected shall include the following:**
   a. **Program records;**
   b. **Direct observation made by the evaluator; and**
   c. **Data and information from the Authority or other pertinent sources.**

B. Criteria for evaluation of shellfish sanitation program elements shall be as follows:

1. **Laboratory**
   a. **Requirements for evaluation of shellfish laboratories shall include at a minimum:**
      i. **Records audit of laboratory operations both Quality Systems and Technical methods;**
      ii. **Direct observation of current laboratory operating conditions; and**
      iii. **~~Information collection from the Authority and other pertinent sources concerning laboratory operations.~~Other relevant information collected from the Authority.**
   b. **Laboratory status is determined by the number and types of nonconformities found in the evaluation using NSSP standardized criteria contained in the FDA Shellfish Laboratory Evaluation Checklists found in Section IV Guidance Documents Chapter II. Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.**
      i. **Quality System Evaluation.**

      This checklist includes a conforming and nonconforming status only. All nonconformities must be reconciled prior to scheduling an onsite evaluation of technical methods in NSSP laboratories. As this part of the evaluation specifically refers to the Quality manual and SOPs and other documentation considered the basis for data defensibility, this documentation must be in order prior to further Laboratory Evaluation Officer (LEO) scheduling. The

	Quality Systems evaluation is performed as a desk audit and is in accordance with the checklist found in Section IV Chapter II. ii. **Technical Evaluation: Shellfish Laboratory will be technically evaluated and will be assigned the designation of conforms, provisionally conforms or non-conformance. The criteria used in determining the evaluation designations are included in the NSSP Shellfish Laboratory Evaluation Checklist designated for the specific type of laboratory evaluation being performed. (For more information see Section IV. Guidance Documents Chapter II. Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists.)** c. **Corrective Actions for Conforming Status. A laboratory found to be in conforming status for technical checklists, other than the Quality Systems checklist, has up to ninety (90) days to successfully correct all nonconformities noted in each component evaluated or has an approved action plan in place to deal with the nonconformities noted. After this period, the laboratory's status will be downgraded to nonconforming if any key nonconformities remain to be successfully corrected. As a result, data being generated by the laboratory will no longer be acceptable for use in support of the NSSP for the laboratory component in question.** d. **Corrective Actions for Provisionally Conforming Status. A laboratory found to be in provisionally conforming status for technical methods checklists has up to sixty (60) days to successfully correct all nonconformities found in each provisionally conforming component evaluated or has an approved action plan in place to deal with the nonconformities noted. After this period, the laboratory will be assigned the following status for the laboratory component(s) in question:** i. **Conforms if all the critical and key nonconformities have been successfully corrected in each provisionally conforming component evaluated; or** ii. **Nonconforming if any critical or key nonconformities remain to be successfully corrected in each provisionally conforming component evaluated or if the lab is not able to be evaluated because of a nonconforming Quality System. As a result, data being generated by the laboratory will no longer be acceptable for use in support of the NSSP for the laboratory component**

in question.

e. **Nonconformance.**

i. **Upon a determination of nonconforming status in any of the technical method components, the laboratory has up to thirty (30) days to demonstrate successful correction of all nonconformities found. After this period, if all critical and key nonconformities have been successfully corrected, the status of the laboratory will be upgraded to conforming for the laboratory component(s) in question. However, if any critical or key nonconformities remain to be successfully corrected, the status of the laboratory for the laboratory component(s) in question will continue to be nonconforming; and as a result, data being generated by the laboratory for this/these laboratory components will continue to be unacceptable for use in support of the NSSP.**

ii. **Upon a determination of nonconformance for the Quality Systems component, the laboratory will have to successfully implement a quality system prior to the onsite technical evaluation. Once all nonconformities are reconciled successfully, a technical evaluation for NSSP methods using the appropriate method specific NSSP Shellfish Laboratory Evaluation Checklist will be scheduled with the laboratory.**

iii. **When a laboratory is found to be nonconforming in either the technical or quality component or in both components for failure to successfully implement the required corrective action, or for having repeated critical or key nonconformities in consecutive evaluations, the Authority will ensure that an action plan is developed to correct the situation in an acceptable and expeditious manner or discontinue use of the laboratory to support the NSSP.**

iv. **For each laboratory component evaluated, the laboratory will be reevaluated either on-site or through a thorough desk audit as determined by the FDA Shellfish LEO and the FDA certified State Shellfish LEO if one is utilized by the State. Only a finding of fully conforming in laboratories whose data has ceased to be acceptable to the NSSP will restore its acceptability for use in the NSSP for the laboratory components in question.**

2. **Growing Areas**

Requirements for evaluation of the shellfish growing area program element shall include at a minimum:

a. **Records audit of sanitary survey;**

b. **Bacteriological standards;**
c. **Growing area classification;**
d. **Marine biotoxin control; and**
e. **Marinas.**

3. **Patrol**

a. **Requirements for evaluation of shellfish growing area patrol elements shall include at a minimum:**
   i. **Records audit of past patrol activities;**
   ii. **Direct observation of current patrol activities; and**
   iii. **Information collection from the Authority and other pertinent sources.**

b. **Legal Penalties – Chapter VIII. @.01 A. (2) (c) Are there penalties in place to address illegal harvest?**
Compliance Criteria: The patrol element will be deemed in compliance if laws and regulations exist that provide penalties for controlling harvest from harvest restricted areas. [Critical]

c. **Notification of Harvest Restricted Areas – Chapter VIII. @.01 A. (2) (d)**
Is the industry notified of the boundaries of Harvest Restricted Areas? – Chapter VIII. @.01 A. (3) (b)
Compliance Criteria: The patrol element will be deemed in compliance with this requirement when the appropriate Authority demonstrates that the industry has been notified of the boundaries. [Critical]

d. **Comprehensive Listing of Harvest Restricted Areas – Chapter VIII. @.01 A. (3) (b)**
Does the Patrol Agency have a comprehensive listing of Harvest Restricted areas?
Compliance Criteria: The patrol element will be deemed in compliance with this requirement when it is determined that the Authority has a comprehensive listing of all Harvest Restricted areas. [Critical]

e. **Patrol Policy Document – Chapter VIII. @.01 B. (6).**
   i. **Does the Patrol Agency have a patrol policy document?**
   Compliance Criteria: The patrol element will be deemed in compliance with this requirement when the Authority provides a patrol policy document. [Key]
   ii. **Is the patrol policy document complete?**
   Compliance Criteria: The patrol element will be deemed in compliance with this requirement when it is determined that the patrol policy document includes all items in Chapter VIII. @.01 B. (7) listed below. [Key]
      (a) **Citation of the law providing the legal basis for enforcement authority**
      (b) **Citation of the laws and regulations, including**

	penalties, which are directly related to effective control of illegal harvest activities; (c) **The organizational structure of the unit responsible for patrol activities, including:** (1) **Patrol unit(s) name, address, and phone number;** (2) **The roster and chain of command;** (3) **Area assignments that support the frequencies of patrol delineated in B. (2); and** (4) **A listing of specific vessels, vehicles, and equipment that support the frequencies of patrol delineated in @.01 B. (2);** (d) **Summaries of training in shellfish patrol techniques;** (e) **The methods used to inform officers of growing area classifications and status, and of any special activities licensed in the area;** (f) **A listing of growing areas where patrol is required;** (g) **An identification of any patrol problems;** (h) **The type and frequency of reporting by patrol personnel;** (i) **Copy of agreements with other agencies responsible for shellfish control activities; and** (j) **Citations/summons for the past year. If available, this information may include:** (1) **The number of convictions or dismissals;** (2) **Fines in dollar amount;** (3) **Equipment or property confiscations and forfeitures;** (4) **License suspensions or revocations; and** (5) **Jail sentences; and** (6) **Written warnings.** iii. **Is the patrol policy document updated annually?** **Compliance Criteria: The patrol element will be deemed in compliance with this requirement when the Authority can demonstrate that the patrol policy document is updated every calendar year. [Key]** f. **Officer Training – Chapter I. @.01 H. (3) (a) (i-iii)** **Has the Patrol Agency met the NSSP patrol training requirements?** **Compliance Criteria: The patrol element will be deemed in compliance with this requirement when the Patrol Agency can demonstrate that all officers have met or are scheduled for the training requirements of Chapter VIII. @.01 B. (6) before assuming their patrol duties [Key].** g. **Patrol Frequency – Chapter VIII. @.01 B. (2).** i. **Has the agency determined risk categories for all**

harvest restricted areas? – Chapter VIII. @.01 B. (4)?
Compliance Criteria: The patrol element will be deemed in compliance with this requirement when the Authority assigns risk categories for each harvest restricted area and provides a listing of those categories. [Critical]

ii. **Does a risk management plan exist if required? – Chapter VIII. @.01. B. (3) (c) and (d)**
Compliance Criteria: The patrol element will be deemed in compliance with this requirement when the Patrol Authority has conducted a Risk Management Plan for all areas that are not patrolled at the frequency required in Chapter VIII.
@.01 B. (2). [Critical]

iii. **Has the patrol frequency requirement been met in all areas? – Chapter VIII.**
@.01 B. (3) (b), (c), and (d)
Compliance Criteria: The patrol element will be deemed in compliance as follows:

(a) **When the Authority achieved 95-100% of required patrols in all harvest restricted areas the program is considered to be in conformance with NSSP patrol frequency requirements.**

(b) **When the Authority achieved 80 – 94% of required patrols in all harvest restricted areas the program is considered to be in nonconformance with NSSP patrol frequency requirements. [Key]**

(c) **When the Authority achieved <80% of required patrols in all harvest restricted areas the program is considered to be in major non-conformance with NSSP patrol frequency requirements. [Critical]**

h. **Memorandum of Understanding/Agreements - Chapter VIII. @.01 B. (5). If enforcement of shellfish regulations is shared with another agency(s), is there a formalized MOU/MOA with the other agency(s)?**
Compliance Criteria: The patrol element will be deemed in compliance when the authority has developed a Memorandum of Understanding/Agreement with all Authorities which have delegated patrol responsibilities. [Key]

i. **The following procedures will be implemented when an FDA evaluation identifies deficiencies with the above patrol evaluation criteria.**

i. **The overall Patrol Program element will be assigned one (1) of the following designations:**

(a) **Conformance: The program is in compliance with all of the criteria listed above.**
(b) **Conformance with Deficiencies: The program only has minor deficiencies associated with a key compliance item.**
(c) **Nonconformance: The program has:**
i. **at least one (1) critical deficiency;**
ii. **two (2) or more key deficiencies; or**
iii. **a repeat [Key] deficiency from the previous evaluation.**
(d) **Major Nonconformance: The program has multiple deficiencies, key or critical, that suggests the program has become ineffective to control harvest in harvest restricted waters.**

ii. **During the closeout meeting for patrol evaluation, the Shellfish Specialists shall identify any patrol deficiency to the State patrol agency;**
iii. **Within thirty (30) days of the closeout meeting, the Shellfish Specialist shall provide a written Program Element Evaluation Report (PEER), including supporting documentation, to the State patrol agency;**
iv. **Within thirty (30) days of receiving the PEER, the State patrol agency shall provide a written response that indicates:**
(a) **The item(s) was corrected;**
(b) **A correction plan has been developed with a completion date; or,**
(c) **The reasons why the State disagrees with FDA's finding(s).**
v. **Within fifteen (15) days of receipt FDA shall review the State response, and respond to the State;**
vi. **Any CRITICAL item deficiency shall be corrected within thirty (30) days of acceptance by FDA of the correction plan;**
vii. **Any KEY item deficiency shall be corrected within one (1) year of acceptance by FDA of the correction plan.**
viii. **FDA shellfish specialists shall be responsible for monitoring the progress of State action plans.**
ix. **Patrol Program recommendations addressing improvements not associated with the criteria included in Section I or recommendations addressing improvements beyond the requirements of the Model Ordinance should be submitted to the Authority in correspondence.**

4. Plants

Requirements for evaluation of the shellfish plant inspection program elements shall include at a minimum:

a. **Records audit of past shellfish processing facility**

inspections;

b. **Direct observation of current shellfish processing facility conditions;**

c. **Information collection from the Authority and other pertinent sources concerning shellfish processing facility inspection program.**

d. **Shellfish sanitation program element criteria shall be used to evaluate consecutive full evaluations (not including follow up). If a violation of the same criteria is repeated, the program element is considered out of compliance. This program element compliance will be based on the following criteria:**

i. **All dealers are required to be certified in accordance with the Guide for the Control of Molluscan Shellfish.**

ii. **95% of the certified dealers evaluated must have been inspected by the State at the frequency required by the current Guide for the Control of Molluscan Shellfish.**

iii. **Where compliance schedules are required no more than 10% of the certified dealers evaluated will be without such schedules.**

iv. **States must demonstrate that they have performed proper follow up for compliance schedules for 90% of dealers evaluated, and if the compliance schedules were not met, that proper administrative action was taken by the State.**

v. **All critical deficiencies have been addressed by the State inspector in accordance with the Guide for the Control of Molluscan Shellfish.**

e. **Plant Evaluation Criteria**

i. **Legal Authority – Chapter I @ .01 B.**
The plant sanitation element will be deemed in compliance if administrative laws and regulations exist that provide the administrative authority to implement the Dealer Certification requirements listed in Chapter I @ .01 and @ 02. [Critical]

ii. **Initial Certification – Chapter I @ .02 B and C.**
The Plant Sanitation Element will be deemed in compliance with this requirement when all plants are certified in accordance with criteria listed below:

(a) **HACCP requirements:**

(i) **A HACCP plan accepted by the Authority**

(ii) **No critical deficiencies;**

(iii) **Not more than two (2) key deficiencies;**

(iv) **Not more than two (2) other deficiencies.**

(b) **Sanitation and additional Model Ordinance**

Requirements:
(i) **No critical deficiencies;**
(ii) **Not more than two (2) key deficiencies;**
(iii) **Not more than three (3) other deficiencies.**

iii. **Inspection frequency – Chapter I @ .02 F. and G.**
The Plant Sanitation Element will be deemed in compliance with this requirement when one (1) or less plants inspected doesn't meet the required inspection frequency.

iv. **Compliance schedules.**
The Plant Sanitation Element will be deemed in compliance with this requirement when no more than 10% of the inspection reports~~the certified dealers~~ evaluated are found to be without schedules.

v. **Follow-Up.**
The Plant Sanitation Element will be deemed in compliance with this requirement when the State demonstrates that they have performed proper follow-up for compliance schedules for 90% of inspection reports~~dealers evaluated~~ and if the compliance schedules were not met that administrative action was taken.

vi. **Deficiency Follow-up.**
The Plant Sanitation Element will be deemed in compliance with this requirement when the State demonstrates that all critical deficiencies have been addressed.

vii. **In-Field Plant Criteria.**
Certified plants will be evaluated to determine compliance with the criteria listed below:
(a) **Shucker/packers and repackers HACCP requirements:**
(i) **A HACCP plan accepted by the Authority;**
(ii) **No critical deficiencies; and**
(iii) **Not more than four (4) key deficiencies.**
(b) **Shucker/packers and repackers sanitation and additional Model Ordinance requirements:**
(i) **No critical deficiencies; and**
(ii) **Not more than four (4) key deficiencies.**
(c) **Shellstock shippers and reshippers HACCP requirements:**
(i) **A HACCP plan accepted by the authority;**
(ii) **No critical deficiencies; and**
(iii) **Not more than three (3) key deficiencies.**
(d) **Shellstock shippers and reshippers**

sanitation and additional Model Ordinance requirements

(i) **No critical deficiencies; and**

(ii) **Not more than three (3) key deficiencies.**

viii.

f. **Conformance Designations**

i. **The overall Plant Sanitation Program element will be assigned one (1) of the following conformance designations based on compliance with the criteria listed in Chapter I. @.03 B. 4.:**

(a) **Conformance: The program is in compliance with all of the criteria listed in Chapter I. @.03 B. 4. e. i.-vi. and has 25% or fewer of plants with deficiencies as outlined in Chapter I. @.03 B. 4. e. vii.**

(b) **Provisional Conformance: The program is in compliance with Chapter I. @.03 B. 4. e. i - vi. and has 26% to 42% of plants with deficiencies as outlined in Chapter I. @ .03 B. 4. e. vii. For plant sanitation programs that have 26-42% deficiencies, the Authority can achieve a designation of conformance by successful completion of the actions listed in Chapter I. @.03 B. 4. f. ii. (b).**

(c) **Nonconformance: The program is in compliance with Chapter I. @.03 B. 4. e. i., but does not meet the criteria in Chapter I. @.03 B. 4. e. ii. or iii. or iv. or v. or vi. or has greater than 42% of plants with deficiencies as outlined in Chapter I. @.03 B. 4. e. vii.. Two consecutive FDA audits of Provisional Conformance will result in a conformance designation of Non-Conformance. This conformance designation requires an action plan as outlined in Chapter I. @.03 B, 4. f. ii. (c). the program has been deemed in Provisional Conformance on two consecutive FDA audits.**

(d) **Major Nonconformance: The program has multiple deficiencies. It is non-compliant with Chapter I. @.03 B. 4. e. i., or two (2) or more of Chapter I. @.03 B. 4. e. ii., or iii., or iv., or v., or vi., The failure of a state to develop and implement an acceptable and effective action plan.**

ii. **Each conformance designation will require the actions listed below:**

(a) **Conformance: The Authority will work cooperatively with the individual firms to correct deficiencies or develop deficiency-**

specific compliance schedules in plants audited by FDA.

(b) **Provisional Conformance: For plant sanitation programs that have 26-42% deficiencies, the Authority can achieve a designation of Conformance by successful completion of the actions listed below:**

(i) **Correct deficiencies or develop deficiency-specific compliance schedules in plants audited by FDA within 30 days of the in-field closeout meeting. If there are any disagreements between the Authority and FDA an additional 15 days will be allowed to resolve differences.**

(ii) **The State must take one of the following actions.**

- **Within 30 days, the SSO will conduct an audit of the same number of plants as the original FDA evaluation to determine compliance with Chapter I. @.03 B. 4. e. vii., (The Authority will work with FDA to select the plants.); or**
- **Conduct inspections of all certified dealers within 120 days to identify and correct deficiencies. Within 30 days of completion of the inspections, the SSO will conduct an audit of the same number of plants to determine compliance with Chapter I. @.03 B. 4. e. vii. (The Authority will work with FDA to select the plants.)**

(iii) **Conduct a file review for the purpose of comparing FDA and SSO findings to previous inspections**

(iv) **Determine if inspector re-standardization or additional training is needed.**

(v) **Re-standardize and provide additional training for inspectors as needed.**

Should the SSO audit outlined in Chapter I. @.03 B. 4. f. ii. (b) (ii) above determine that compliance with Chapter I.@.03 B. 4. f. i. (a) the program will be reassigned a conformance designation of Conformance. This reassignment will be

acknowledged in FDA correspondence to the Authority.

Should the SSO audit outlined in Chapter I. @.03 B. 4. f. ii. (b) (ii) determine that the program is not in compliance with Chapter I. @.03 B. 4. f. i. (a), the program will be reassigned a designation of Nonconformance. This reassignment will be acknowledged in FDA correspondence to the Authority.

(c) **Nonconformance: The Authority must develop and complete an action plan that includes a plan to specifically address any deficiencies associated with Chapter I. @.03 B. 4. e. ii-vi. Should the designation of Nonconformance be the result of deficiencies associated with Chapter I. @.03 B. 4. e. vii the action plan shall include the following:**

- **(i) Correct deficiencies or develop deficiency-specific compliance schedules in plants audited by FDA within 30 days of the in-field closeout meeting. Should the state disagree with FDA regarding an identified deficiency(s), an additional 15 days will be allowed for resolution and/or correction of those specific deficiencies.**
- **(ii) Within 10 days of correcting the deficiencies identified in the FDA audit, the Authority shall request re-standardization of state SSO(s) by FDA.**
- **(iii) Within 60 days of SSO re-standardization by FDA, the SSO will conduct an abbreviated re-standardization of all inspectors using a minimum of 3 plants for the purpose of evaluating staff competency.**
- **(iv) Provide additional inspector training as determined by the Authority.**
- **(v) Following re-standardization, the state will conduct a state-wide compliance inspection of all plants (excluding plants audited by FDA). This activity must be completed within 120 days or another timeframe mutually agreed upon by the Authority and FDA.**
- **(vi) Within 30 days of completion of the state-wide compliance effort, the SSO will conduct an audit of the same number of plants to determine**

	compliance with Chapter I. @.03 B. 4. e. (The Authority will work with FDA to select the plants) **(vii) The state SSO will conduct a file review for the purpose of comparing FDA and SSO findings to previous inspections** **Failure to complete an effective action plan will result in a Conformance designation of major Non-Conformance.** **If Non-Conformance is the result of Provisional Conformance failure, an action plan would be required consistent with a conformance designation of Non-Conformance.** (d) **Major Non-Conformance: All determinations of Major Non-Conformance and the identification of deficiencies that pose imminent health concerns will be immediately reported to the ISSC Executive Board for consideration for appropriate action.** g. **FDA will follow the current compliance program for communication with the State agencies.** h. **All deficiencies observed by FDA while conducting the in-plant inspection portion of the evaluation will be documented and included in the compliance determination outlined in Chapter I. @.03 B. 4. e.ii.** a.
12. Public Health Significance	N/A
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	New Federal Waters Chapter
10. Specific NSSP Guide Reference	**Table of Contents** **Section II. Model Ordinance**: Chapter I. @.01; Chapter IV. @.01; Chapter IV. @.03; Chapter VI. @.03; Chapter VI. .07; Chapter VIII. .03; Chapter X. .09; **Section IV. Chapter XVII. .01 Federal Waters Guidance**
11. Text of Proposal/ Requested Action	**Table of Contents** **Section II. Model Ordinance…** Chapter VI. Shellfish Aquaculture Requirements for the Authority …~~@.03 Aquaculture in Federal Waters……64~~ Requirements for the Harvester/Dealer …~~.07 Requirements for the Harvester in Aquaculture in Federal Waters……67~~ Chapter VIII. Control of Shellfish Harvesting Requirements for Harvesters …~~.03 Shellstock Harvesting in Federal Waters……84~~ Chapter X. General Requirements for Dealers …~~.09 Shellfish Harvested from Federal Waters……98~~ Chapter XVIII. Federal Waters Federal Agencies .01 Requirements for the Harvester in Federal Waters .02 Requirements for Shellfish Aquaculture in Federal Waters

.03 Requirements for the Dealer Receiving Shellstock Harvested from Federal Waters

Section II. Model Ordinance

Chapter I. @.01 Administration

A. Scope…

(3) The regulatory responsibility for growing area and harvest control in federal waters will be the responsibility of the FDA and NOAA, in accordance with Section II, Chapter XVIII.

B. State Laws and Regulations. The Authority shall have laws and regulations which provide an adequate legal basis for the safety and sanitary control of all program elements including but not limited to the elements outlined in @.01 A. ~~Federal Agencies shall have laws and regulations which provide an adequate legal basis for the safety and sanitary control of growing areas and harvest control.~~

…

D. Shared Responsibilities. If more than one agency is involved in the administration of the statewide shellfish safety and sanitation program, memoranda of agreement shall be developed between the agencies to define each agency's responsibilities. In the case of federal waters where the state shellfish control Authority, in consultation with FDA and NOAA, chooses to be responsible for all or a portion of the marine biotoxin control of shellfish harvested from federal waters and landed at the associated state of landing certified shellfish dealer, a written agreement shall be developed between FDA, NOAA, and the state of landing Authority defining each agency's roles and responsibilities. ~~if more than one agency is involved in the administration of the shellfish safety and sanitation program, memoranda of agreement shall be developed between the agencies to define each agency's responsibilities.~~

E. Administrative Procedures.

…

~~(2) In the case of Federal Waters, the FDA and NOAA shall have administrative procedures sufficient to regulate growing areas and harvest control.~~

Chapter IV. Shellstock Growing Areas

@.01 Sanitary Survey

…

~~E. Sanitary surveys for Federal waters will be the responsibility of FDA. Sanitary surveys will be conducted in accordance with Chapter IV @.01, as applicable.~~

@.03 Growing Area Classification

…

~~F. FDA is responsible for the classification of growing areas in Federal waters. Federal waters are classified as Approved for shellfish harvesting unless such areas are known to be polluted (i.e., microbiological, chemical, or marine biotoxin hazards) and involve commercial shellfish resources. Should FDA allow harvesting in Federal waters with known marine biotoxin hazards, the FDA will classify the~~

~~harvest area in a manner equivalent to the requirements of Model Ordinance Chapter IV.~~

Chapter VI. Shellfish Aquaculture

Requirements for the Authority…

~~.@.03 Aquaculture in Federal Waters~~
~~A. Federal Agency Responsibilities. Once the appropriate permits for the construction of the aquaculture facility have been obtained,~~
~~(1) NOAA is responsible for establishing a contract, in consultation with FDA, with the aquaculture facility describing requirements of the NSSP including:~~
~~(a) the frequency with which NOAA will audit the aquaculture facility and vessels;~~
~~(b) testing requirements of the aquaculture facility; and~~
~~(c) the generation of product identification for traceability (i.e., tag numbers); and~~
~~(2) FDA is responsible for reviewing the aquaculture facility operational plan prior to the start of operations, as well as the annual inspection of records, to ensure adherence to NSSP requirements. FDA is also responsible for the classification of the growing area(s) associated with the aquaculture facility.~~

Requirements for the Harvester/Dealer
~~….07 Requirements for the Harvester in Aquaculture in Federal Waters~~
~~A. Prior to beginning any aquaculture activities, the person who performs aquaculture or operates an aquaculture facility to raise shellfish in Federal waters for human consumption shall obtain the appropriate permission(s) from Federal agencies as described in @.03.~~
~~B. Operational Plan. Each aquaculture facility shall have a written operational plan as described for Land Based Aquaculture in Section II Chapter VI .05(A). The operational plan shall also include:~~
~~(1)Description of harvest, tagging, handling, storage, transportation, and landing procedures;~~
~~(2)Description of a marine biotoxin management and contingency plan (Section II Chapter IV @.04) to include marine biotoxin sampling consistent with Section II Chapter IV @.04(B)(6) and ensure product segregation and control until biotoxin results confirm the shellfish do not contain biotoxins equal to or exceeding criteria established in Section IV Chapter II .08.;~~
~~(3)Description of a contingency in the event of an emergency situation or condition (e.g., sewage or oil spills); and~~
~~(4)Procedures for implementing product recalls.~~
~~C. Each aquaculture facility shall obtain a review from the FDA to ensure adherence to NSSP requirements prior to its implementation. If the aquaculture facility makes changes to the operational plan, they shall obtain a new review from the FDA to ensure adherence to the NSSP requirements.~~

Chapter VIII. Control of Shellfish Harvesting

…
Requirements for Harvesters

~~.03 Shellstock Harvesting in Federal Waters~~
~~A. The harvester shall obtain a NOAA contract to land commercial shellfish harvested from Federal waters at a state certified dealer. In addition, if applicable, obtain the required NOAA NMFS managed fisheries harvester license(s) and/or permit(s)~~
~~B. Prior to harvesting shellfish in Federal waters from an area in the controlled access status, the harvester shall:~~
~~(1) Enter into agreements or memoranda of understanding between the landing state~~
~~Authority, NOAA and the shellfish dealers receiving the shellfish as necessary to comply with the requirements outlined in NSSP MO, Chapter IV. @.04 B. and in accordance with Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans.~~

Chapter X. General Requirements for Dealers
…
~~.09 Shellfish Harvested from Federal Waters~~
~~A. The dealer shall:~~
~~(1) If receiving shellstock harvested from Federal waters in the controlled access status, be party to agreement or memoranda of understanding between the Authority, NOAA and the individual harvesters as necessary to comply with the biotoxin controls outlined in the NSSP Chapter IV. @.04 B. and in accordance with Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans.~~

Chapter XVIII. Federal Waters

Federal Agencies

Landing shellfish harvested from federal waters (those waters that fall outside of state and local jurisdiction but within U.S. sovereignty and are typically 3 to 200 nautical miles offshore) at a state certified shellfish dealer is considered “interstate commerce” under the Federal Food and Drug Cosmetic Act Sec. 201(b). As such, the regulatory responsibility for shellfish growing area and harvest control in federal waters is the responsibility of the FDA and NOAA.

Per Section II, Chapter I. @.01 D., in consultation with the FDA and NOAA, through a written agreement, a state shellfish control Authority may choose to be responsible for all or a portion of the marine biotoxin control of shellfish harvested from federal waters that is landed at an associated state of landing certified shellfish dealer. In this case, the FDA will evaluate the Authority’s role in federal waters marine biotoxin control during the state program evaluation process.

The NOAA Seafood Inspection Program (SIP) is the primary contact for all commercial shellfish harvesting activities in federal waters. This does not

	supersede the harvester's responsibilities to contact other federal agencies related to federal fisheries permits and aquaculture siting permits. To ensure the safety and quality of seafood, NOAA SIP provides a contract as the mechanism for the control of harvest and traceability for all commercial shellfish grown and harvested from federal waters. It is the responsibility of each shellfish harvester to contact NOAA SIP to apply for and obtain a contract to land shellfish harvested from federal waters at a state certified shellfish dealer. The NOAA SIP contract also provides the unique identifier number that will be used on federal waters shellfish harvester tags. The NOAA SIP will generate and maintain a NOAA SIP Contract Harvester List which can be accessed through the Interstate Shellfish Sanitation Conference (ISSC) website. The FDA is responsible for the classification and status of growing areas in federal waters. The FDA will coordinate with the NOAA SIP to notify harvesters of any microbiological water or shellfish tissue sampling requirements in federal waters through the NOAA SIP contract application process. The FDA is responsible for overseeing marine biotoxin control in federal waters. The FDA will coordinate with the NOAA SIP to notify harvesters of any marine biotoxin management requirements in federal waters through the NOAA SIP contract application process. The FDA, in coordination with the NOAA SIP, will provide a marine biotoxin contingency plan template for all federal waters growing and harvest areas addressing the management of PSP, ASP, NSP, DSP and AZP in the event of the emergence of a toxin-producing phytoplankton that has not historically occurred, or an illness outbreak caused by marine biotoxins, per Section II, Chapter IV. @.04 A. The FDA, in coordination with the NOAA SIP, will determine and delineate shellfish growing and harvest areas in federal waters that meet the criteria and requirement for a marine biotoxin management plan in Section II, Chapter IV. @.04 B. The FDA, in coordination with the NOAA SIP, will provide a marine biotoxin management plan template to each harvester harvesting in an area in federal waters where biotoxins are required to be managed and a marine biotoxin management plan is required. Within the marine biotoxin management plan template, in consultation with the FDA and the NOAA SIP, the harvester will choose which marine biotoxin management strategy to use and which laboratory to use for biotoxin sample analyses. Per Section II, Chapter IV. @.04 B.(4)(d) & (e) and (5), as part of the marine biotoxin management plan, the use of the marine biotoxin management strategy, lot testing or pre-harvest shellfish toxicity screening coupled with lot testing, requires a written agreement or MOU between the state of landing Authority, NOAA SIP, shellfish dealer(s), and the harvester. The written agreement will be reviewed by

	the FDA and the NOAA SIP before implementation of the marine biotoxin management plan. **.01 Requirements for the Harvester in Federal Waters** A. The harvester shall: (1) Obtain a NOAA contract to land commercial shellfish harvested from federal waters at a state certified dealer. In addition, if applicable, obtain the required NOAA NMFS managed fisheries harvester license(s) and/or permit(s). (2) Prior to harvesting shellfish in federal waters from an area in the controlled access status, the harvester shall enter into agreements or memoranda of understanding between the landing state Authority, NOAA, and the shellfish dealers receiving the shellfish as necessary to comply with the requirements outlined in Section II, Chapter IV. @.04 B. (3) Shellstock harvested from growing areas in the controlled access status shall be tagged with restricted shellstock tags (Section II, Chapter IV. @.04 C. (7)). (4) Meet the requirements for Section II, Chapter VIII .02 Shellstock Harvesting and Handling (5) In the event that federal waters shellfish harvesters are required to collect microbiological or marine biotoxin samples, to meet the laboratory requirements for the analysis of regulatory samples from federal waters, the harvester will be responsible for identifying and using a laboratory with an operational status of conforming or provisionally conforming to the requirements set forth by the NSSP and implement NSSP approved and/or approved limited use method for fecal coliform and marine biotoxin analysis (Section II, Chapter I @.03 B. 1.). **.02 Requirements for Shellfish Aquaculture in Federal Waters** A. The aquaculturist shall contact NOAA SIP to apply for and obtain a NOAA SIP contract to land commercial shellfish grown and harvested from federal waters at a state certified shellfish dealer. B. Each shellfish aquaculture facility shall have and implement a written site-specific operational plan ~~as described for Land Based Aquaculture in Section II, Chapter VI .05(A). The operational plan shall also~~ that includes: (1) A description of the design and activities of the culture facility; (2) The specific site and boundaries in which shellfish culture activities will be conducted; (3) The types and locations of any structures, including rafts, pens, cages, nets, or floats which will be placed in the waters; (4) The species of shellfish to be cultured and harvested; (5) The source of seed. All sources of seed produced or collected in prohibited waters shall be sanctioned by the FDA and NOAA SIP. (6) Procedures to assure that no poisonous or deleterious substances are introduced into the activities; (7) A program of sanitation, maintenance, and supervision to prevent contamination of the shellfish products;

(8) Description of harvest, tagging, handling, storage, transportation, and landing procedures;
(9) Description of a marine biotoxin contingency plan and, if required, a marine biotoxin management plan (Section II, Chapter IV. @.04) to include marine biotoxin sampling consistent with Section II, Chapter IV. @.04(B);
(10) Description of a contingency plan in the event of an emergency situation or condition (such as sewage or oil spills);
(11) If it is determined that the aquaculture site attracts sufficient birds and/or marine mammals that their waste presents a human health risk, include a description of the mitigation or deterrent measures to minimize the potential pollution impact of birds and/or mammals; and
(12) Procedures for implementing product recalls.
(13) Maintenance of the required records.

C. Each aquaculture facility shall obtain a review of the operational plan from the FDA and NOAA SIP prior to its implementation. If the aquaculture facility makes changes to the operational plan, they shall obtain a new review from the FDA and NOAA SIP.

.03 Requirements for the Dealer Receiving Shellstock Harvested from Federal Waters

A. The dealer shall:
(1) Only receive shellstock from harvesters in federal waters that have a NOAA contract and are on the Harvester Contract List.
(2) If receiving shellstock harvested from federal waters in the controlled access status with restricted tags, be party to agreement or memoranda of understanding between the Authority, NOAA, and the individual harvesters as necessary to comply with the biotoxin controls outlined in the Section II, Chapter IV. @.04 B.

GUIDANCE

Chapter XVII. Federal Waters

.01 Federal Waters Guidance

A. Introduction

~~Requirements for Federal waters shellfish harvesters, dealers, the State of Landing Authority and FDA and NOAA are listed in multiple sections throughout the NSSP Model Ordinance.~~ The following guidance provides additional information to assist in meeting the~~se~~ requirements for federal waters shellfish harvesters, dealers, the state of landing Authority, FDA, and NOAA.

B. Harvester Requirements

(1) HARVESTER LICENSING AND TRACEABILITY

	The ~~Food and Drug Administration (~~FDA~~)~~ and ~~the National Oceanographic Atmospheric Administration (~~NOAA~~)~~ are the federal agencies responsible for shellfish growing areas and harvest control in federal waters. The state of landing Authority, through written agreements and in coordination with the FDA and NOAA, may also ~~take the lead and/or take~~ choose ~~agree~~ to be ~~have~~ responsible~~ilities~~ for ~~the management, control of harvest, and/or~~ marine biotoxin control associated with commercial shellfish harvested from federal waters and landed in their state. The NOAA Seafood Inspection Program (SIP) is the primary contact for all commercial shellfish harvesting activities in federal waters. This does not supersede the harvester's responsibilities to contact other federal agencies related to federal fisheries permits and aquaculture siting permits. ~~To meet the requirement in the NSSP MO, Chapter VIII .03A. for Federal waters,~~ The NOAA SIP utilizes the NOAA SIP contract that serves as the mechanism for the control of harvest and traceability for all commercial shellfish grown and harvested from federal waters. It is the responsibility of shellfish harvesters to contact the NOAA SIP to obtain a NOAA SIP contract, which is the identified mechanism for authorizing harvesters to land shellfish harvested from federal waters at a state certified dealer. The NOAA SIP contract also provides the unique identifier number that will be used on federal waters shellfish harvester tags. The NOAA SIP contract application process requires that the harvester provide their contact information as well as the intended federal waters harvest and/or aquaculture site location information to the NOAA SIP. Harvester contact information will be used to contact each harvester in the event of an emergency closure (e.g., oil spill, hurricane, severe storm, chemical spill, WWTP spill, or ship discharge) and reopening, status change, classification change, and/or product recall. The NOAA SIP will generate and maintain a NOAA SIP Contract Harvester List which can be accessed through the Interstate Shellfish Sanitation Conference (ISSC) website for reference. The NOAA SIP will coordinate with the FDA regarding meeting the requirements related to the growing area classification, control of harvest, and marine biotoxin control of the intended area of harvest as well as shellfish aquaculture operation and initial siting evaluation. (2) Federal Waters Shellfish Classification

The FDA is responsible for the classification of federal waters shellfish growing areas. ~~(NSSP MO, Section II, Chapter IV @.03 F.). Federal waters are considered generally free from bacterial and chemical pollution and are therefore classified as approved for shellfish harvesting unless such areas are known to be polluted and involve commercial shellfish resources (Verber, 1977).~~ Areas known to be polluted or are considered potential sources of pollution in federal waters may include but are not limited to ocean dump sites designated for the disposal of contaminated wastes, areas where major estuarine complexes discharge large quantities of sewage effluents or other contaminants, wastewater treatment plant effluent pipes, commercial shipping channels and anchorages, and oil platforms.

When applying for the NOAA SIP contract, the harvester will provide the intended harvest location(s) to the NOAA SIP using either the 10-minute latitude and longitude grid number(s), the NOAA National Marine Fisheries Statistical grid, or the latitude(s) and longitude(s). The NOAA SIP will coordinate and provide the FDA with the intended harvest site location(s).

For shellfish harvest areas of concern, the FDA will conduct a site-specific sanitary survey in accordance with NSSP MO, Chapter IV. @.01, as applicable. Once the sanitary survey is completed, the FDA will coordinate with the NOAA SIP to notify the harvester of the sanitary survey findings, any growing area classification and/or status change, and if warranted, any microbiological and/or biotoxin monitoring requirements.

(3) Marine Biotoxins

To meet the NSSP MO, Chapter IV. @.04 requirements, once the harvester notifies the NOAA SIP of the intended harvest location(s) in federal waters, through coordination with the NOAA SIP, the FDA will review available data and determine if marine biotoxins are of concern and which marine biotoxin requirements apply to the harvester for the intended harvest and/or aquaculture site locations. The harvester will then be notified by the NOAA SIP of any marine biotoxin requirements.

If the harvester is harvesting from a location in federal waters where the associated ~~S~~state of ~~L~~landing Authority has agreed to be responsible for marine biotoxin control, the harvester must abide by the ~~S~~state of ~~L~~landing Authority marine biotoxin contingency plan and if applicable, marine biotoxin management plan.

i. MARINE BIOTOXIN CONTINGENCY PLAN

To meet the NSSP MO, Chapter IV. @.04 A. requirements, as a default, each harvester will abide by the FDA/NOAA SIP Marine Biotoxin Contingency Plan that addresses the management of paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP) and azaspiracid shellfish poisoning (AZP) in the event of the emergence of a toxin-producing phytoplankton that has not historically occurred, or an illness outbreak caused by marine biotoxins.

If applicable, in the case where the ~~S~~state of ~~L~~landing Authority chooses to be responsible for the control of marine biotoxins in federal waters, the harvester will follow the ~~S~~state of ~~L~~landing marine biotoxin contingency plan. The FDA will review the federal waters component in the ~~S~~state of ~~L~~landing Authority's marine biotoxin contingency plan during the state program growing area evaluation process.

ii. MARINE BIOTOXIN MANAGEMENT PLAN

To meet the NSSP MO, Chapter IV. @.04 B. requirements ~~(and in accordance with Section IV. Guidance Documents- Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans)~~, the FDA and NOAA SIP will work with other federal and associated state agencies as well as the shellfish industry to collect and review all available data to assist in identifying and delineating shellfish growing areas in federal waters that meet(s) the criteria and requirement for a marine biotoxin management plan. If harvesting in these designated areas, each harvester must utilize the FDA/NOAA SIP Marine Biotoxin Management Plan template and specify and abide by the marine biotoxin management strategy(ies) of choice, intended state of landing, and the laboratory to be used for marine biotoxin sample analysis.

In the case where the ~~S~~state of ~~L~~landing Authority has agreed to be responsible for the management of biotoxins and/or has an established a biotoxin management strategy(ies) for shellfish landed in their state from federal waters, each harvester must coordinate with the ~~S~~state of ~~L~~landing Authority to meet the marine biotoxin management plan requirements.

	In coordination with the NOAA SIP, the FDA will review all harvester marine biotoxin management plans for compliance with NSSP MO, Chapter IV. @.04 B. For marine biotoxin management plans associated with federal waters managed by the ~~S~~state of ~~L~~landing Authority, the FDA will evaluate these management plans during the state of landing growing area program evaluation. In addition, to meet the requirements for marine biotoxin management strategies that include shellfish lot testing or pre-harvest shellfish toxicity screening coupled with lot testing [NSSP MO, Chapter IV. @.04 B.(4)(d) & (e) and (5)] and allow the landing of shellfish harvested in a growing area that is placed in the controlled access status, the harvester will be required to enter into ~~an~~ a written agreement or memoranda of understanding (MOU) between the ~~S~~state of ~~L~~landing Authority, individual growers, individual shellfish dealers, and NOAA SIP. At a minimum, the agreement or MOU- should reference the marine biotoxin management plan and include language indicating that all parties~~signatories~~ agree with and will abide by the marine biotoxin management plan. The FDA and NOAA SIP will review the agreement or MOU for NSSP compliance. To meet the restricted tag requirement of the NSSP MO, Chapter IV. @.04 C. (7), all shellstock harvested from growing areas in the controlled access status shall be tagged with restricted shellstock tags. Information included on the restricted shellstock tag should include specific details defining the restriction. iii. LABORATORY REQUIREMENTS FOR SAMPLE ANALYSES To meet the laboratory requirements for the analysis of regulatory samples from federal waters, the harvester will be responsible for identifying and using a laboratory with an operational status of conforming or provisionally conforming to the requirements set forth by the NSSP and implement NSSP approved and/or approved limited use method for fecal coliform and marine biotoxin analysis. For guidance on available laboratories, the harvester may refer to the Interstate Shellfish Sanitation Conference (ISSC) website for the Domestic NSSP Laboratory List. (4) Vibrio Risk Assessment & Time/Temperature Control The harvester is responsible for meeting the requirements in the NSSP MO, Chapter VIII. @.02 & Chapter II. @.06 &

@.07. To meet this requirement, the harvester must meet the time to temperature matrix found in the NSSP MO, Chapter VIII. @.02 A. (3) or if the risk of *Vibrio parahaemolyticus* or *Vibrio vulnificus* illness has been determined to be reasonably likely to occur, then they must meet the defined Vibrio Control Plan for the area.

(5) Harvester Training

To meet the NSSP MO, Chapter VIII. .01 B. harvester training requirement, each harvester will ~~be provided an electronic harvester training document~~ be provided with training during the application process for the NOAA SIP contract.

(6) Shellfish Aquaculture Operational Plan

~~Per the NSSP MO, Chapter VI .07 B., e~~Each federal waters shellfish aquaculture harvester ~~site~~ is required to develop and maintain a farm site-specific Operational Plan. During the NOAA SIP contract application process, each Operational Plan will be provided to the NOAA SIP by the harvester for review by the FDA and NOAA SIP to ensure that it meets the NSSP requirements. The Operational Plan must at a minimum, include all items from the NSSP MO, ~~Chapter VI. .05 A. and~~ Chapter XVIII .02~~VI. .07 B~~.

(7) Finalize NOAA SIP Contract

Once all the harvester requirements have been reviewed and found to conform with the NSSP MO by the FDA and NOAA SIP, the NOAA SIP contract may be finalized with signatures, an effective date, and the contract number assigned by NOAA SIP to be used as the shellfish harvester's tag number. The finalized NOAA SIP contract will be added to the NOAA SIP Contract Harvester List located on the ISSC website.

C. Dealer Requirements

To meet the requirement for state shellfish dealers listed on the Interstate Certified Shellfish Shippers List (ICSSL) to only accept shellfish harvested from federal waters from a harvester with a NOAA SIP contract, the dealer may go to the ISSC website and review the NOAA SIP Contract Harvester List to verify that a federal waters harvester has a valid NOAA SIP contract.

When receiving shellstock harvested from federal waters in the controlled access status, the dealer must agree to be a party to a written ~~to be a signatory to an~~ agreement or MOU ~~to~~ and abide by the marine biotoxin management plan. In addition, the marine biotoxin management plan will include specific language detailing the use of the

	restricted shellstock tag(s) as well as restrictions that require further processing and testing prior to the distribution of the shellstock into commerce. References ~~1. Verber, 1977, Classification of Offshore Waters, James L. Verber~~ ~~2~~1. NOAA SIP Contract a. NOAA SIP Contract information (click here) b. Harvester Contract List (click here) ~~3~~2. ~~State of landing shellfish contacts (click here)~~ ~~4~~3. ~~FDA/NOAA SIP Marine Biotoxin Contingency and Management Plan (click here)~~ ~~5~~2. NSSP Conforming Laboratories (click here)
12. Public Health Significance	The Model Ordinance was organized by state shellfish control Authority (Authority), harvester, and dealer requirements. The Authority requirements are identified by using the "@" symbol. Federal waters shellfish control requirements are the responsibility of federal agencies (FDA and NOAA) and have over time been inserted throughout the Model Ordinance and under various sections designated as requirements for the Authority. The FDA and NOAA do not fall under that category so, this proposal consolidates the federal waters requirements into one chapter in the Model Ordinance.
13. Cost Information	No cost.
14. Changes Needed to Guidance (if proposed change is adopted)	Chapter XVII. Federal Waters .01 Federal Waters Guidance

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	David Borkman[1], Bryant Lewis[2], Matt Richards[3], Christian M. Petitpas[4]
3. Affiliation	Rhode Island Department of Environmental Management[1]; Maine Department of Marine Resources[2] ; New York Department of Environmental Conservation[3]; Massachusetts Division of Marine Fisheries[4]
4. Address Line 1	235 Promenade Street1; 194 McKnown Point Road[2]; 123 Kings Park Blvd[3]; 706 South Rodney French Boulevard[4]
5. Address Line 2	
6. City, State, Zip	Providence, RI 02908[1]; West Boothbay Harbor, ME 04575[2]; Kings Park, NY 11754[3]; New Bedford, MA 02744[4]
7. Phone	401-537-4203[1] 207-633-4700[2]; 631-444-0491[3]; (508) 742-9766[4]
8. Email	david.borkman@dem.ri.gov [1]; Bryant.j.lewis@maine.gov [2]; matt.richards@dec.ny.gov[3]; christian.petitpas@mass.gov[4]
9. Proposal Subject	Clarification of illness investigation timing and precautionary growing area closure during illness investigation
10. Specific NSSP Guide Reference	Section II, Chapter II, @.01 B (2) and @.01 E (1-3)
11. Text of Proposal/ Requested Action	Section II, Chapter II, @.01 B (2) B. When the state or local epidemiologist in the state in which the outbreak occurs has determined an epidemiological association between an illness outbreak meeting the definition of the NSSP and shellfish consumption, the appropriate Authorities shall: (1) Notify the FDA Shellfish Specialist that a shellfish related outbreak has occurred. (2) Within twenty-four (24) hours initiate the investigation to determine whether the illness is growing area related or is the result of post-harvest contamination, mishandling or illegal harvesting from a closed area. The determination of post-harvest contamination may involve multiple authorities in multiple states. The determination of the illness being growing area related will be conducted by the source state. (3) When the Authority can not complete the determination outlined in Chapter II. @.01 B. within 24 hours, the Authority in the source state shall: (a) Immediately place the implicated portion(s) of the harvest area(s) in a precautionary closed status. Should the Authorities later determine that the illnesses are related to post-harvest contamination, or mishandling, or harvested from a closed area, the suspected growing area can be reopened. (b) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, when the authority deems appropriate. (c) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, when the authority can document a rationale that a recall would be effective. AND Section II, Chapter II, @.01 E (1-3) ~~(3) When the Authority can not complete the determination outlined in Chapter II. @.01 B. within 24 hours, the Authority in the source state shall: (1) Immediately place the implicated portion(s) of the harvest area(s) in a precautionary closed status. Should the Authorities later determine that the illnesses are related to post harvest contamination, or mishandling, or harvested from a closed area, the suspected growing area can be reopened.~~ ~~(2) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, when the authority deems appropriate.~~ ~~(3) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, when the authority can document a rationale that a recall would be effective.~~

12. Public Health Significance	The proposed change clarifies the distinction between precautionary growing area closure and growing area closures during the initial phase of an illness investigation and does not reduce public health protection. Existing language in the 2023 MO requires determination of the cause(s) of shellfish-related illness within 24-hours ("…Authorities shall: Within twenty-four (24) hours determine whether the illness is growing area related or is the result of post-harvest contamination, mishandling or illegal harvesting…" (Section II, Chapter II, @.01 B (2)); and then in Section II, Chapter II, @.01 C requires that, lacking initial evidence of post-harvest contamination, etc., implicated growing areas be immediately placed in the closed status, with a series of actions required to reopen the closed area(s) outlined in Section II, Chapter II, @.01 F. However, in Section II, Chapter II, @.01 E current MO language allows implicated growing area(s) to immediately placed in the precautionary closed status *if the Authority can not make an illness determination within 24 hours* (Section II, Chapter II, @.01 E(1)). The proposed language would require that the Authorities initiate illness investigation within 24 hours and place implicated growing area(s) into the precautionary closed status during that investigation. If subsequent investigation identifies non-growing area causes of contamination (post-harvest handling, illegal harvest, etc.) then precautionarily closed areas can be reopened immediately, the same as stipulated in existing 2023 MO language in Section II, Chapter II, @.01 E (1). If the investigation identifies the growing area as the cause of illness, then the implicated growing area(s) will be changed from the precautionary closed to the closed status immediately upon that determination, with the reopening criteria in Section II, Chapter II, @.01 F applied prior to reopening the implicated closed area(s). The proposal also seeks to move unmodified language of Section II, Chapter II, @.01 E forward so that it becomes Section II, Chapter II, @.01 B (3) (a-c) to clarify the timing of precautionary closure of implicated growing area(s) during the investigatory period. No language changes are proposed for Section II, Chapter II, @.01 E of the current MO; only the above-described relocation of that text for clarity is proposed.
13. Cost Information	No additional costs are associated with the proposed changes.
14. Changes Needed to Guidance (if proposed change is adopted)	Guidance in Section IV. Guidance Documents – Chapter II Risk Assessment and Risk may need minor revision to reflect the distinction between a precautionary closure during the period of investigation versus a growing area closure if the investigation identifies the growing area is responsible for the illness.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Model Ordinance Effectiveness Review Committee
3. Affiliation	ISSC
4. Address Line 1	4801 Hermitage Rd
5. Address Line 2	
6. City, State, Zip	Richmond,VA
7. Phone	804-330-6380
8. Email	issc@issc.org
9. Proposal Subject	Model Ordinance Effectiveness
10. Specific NSSP Guide Reference	Chapter II. Risk Assessment and Risk Management
11. Text of Proposal/ Requested Action	**@.01 Outbreaks of Shellfish-Related Illness** A. **When shellfish are implicated in an illness outbreak involving two (2) or more persons not from the same household (or one (1) or more persons in the case of shellfish toxicity poisoning associated with marine biotoxins), the determination of whether an epidemiological association exists between the illness and the shellfish consumption will be made by the state or local epidemiologist in the state in which the outbreak occurs. The determination will be made by reviewing:** (1) **Food history;** (2) **Whether the disease has the potential or is known to be transmitted by shellfish; and** (3) **Whether the symptoms and incubation period of the illnesses are consistent with the suspected etiologic agent.** **NOTE: For additional guidance refer to the International Association for Food Protection *Procedures to Investigate Food Borne Illness*.** B. **When the state or local epidemiologist in the state in which the outbreak occurs has determined an epidemiological association between an illness outbreak meeting the definition of the NSSP and shellfish consumption, the appropriate Authorities shall:** (1) **Notify the FDA Shellfish Specialist that a shellfish related outbreak has occurred.** (2) **Within twenty-four (24) hours determine whether the illness is growing area related or is the result of post-harvest contamination, mishandling or illegal harvesting from a closed area. The determination of post-harvest contamination may involve multiple authorities in multiple states. The determination of the illness being growing area related will be conducted by the source**

state.

C. **When the Authorities determine that the outbreak is not the result of a post-harvest contamination problem or illegal harvesting from a closed area, the Authority shall determine if the outbreak is from a single source or multiple sources.**

(1) **When the Authority determines that a single source outbreak has occurred, the Authority shall:**

(a) **Immediately place the implicated portion(s) of the harvest area(s) in the closed status;**

(b) **Notify the ISSC and the FDA Shellfish Specialist that a potential health risk is associated with shellfish harvested from the implicated growing area;**

(c) **Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, when a recall is deemed appropriate by the Authority. The recall shall include all implicated products.**

(d) **Transmit to the ISSC and FDA information identifying the dealers shipping the implicated shellfish.**

(e) **The ISSC will notify States and FDA Shellfish Specialists of growing area closures and recalls. In the case of recalls, ISSC will notify States with information identifying dealers shipping the implicated shellfish. Closure and recall notices (not to include dealers) will be posted on the ISSC website. ISSC will maintain an inventory of closure and recall information.**

(2) **When the Authority determines that a multiple source outbreak has occurred, the Authority shall immediately place the implicated portion(s) of the harvest area(s) in a precautionary closure. A specific growing area placed in a precautionary closed status under this section can be immediately re- opened when one (1) or more of the following conditions are met:**

(a) When the investigation, conducted in consultation with epidemiologist(s) in the state(s) in which the outbreak occurs, determines that the shellfish which caused the outbreak did not come from one (1) or more of the implicated growing areas in question based on consumption data provided by victims or other relevant data provided by state investigators. This would include an additional illness(es) that matches one (1) or more of the implicated areas and allows for a more precise identification of the growing area(s) which caused the outbreak.

(b) When an investigation, in accordance with Chapter II @ .01 H, of an implicated growing area identifies an actual or potential pollution source(s) in a specific growing area and no source(s) are identified in other implicated

growing areas, the precautionary closures in other implicated growing areas can be reopened. The reopening can only occur in a growing area after the investigation referenced above does not indicate an actual or potential pollution source that could be the cause of the outbreak.

(c) When the investigation, conducted in consultation with the epidemiologists in the state(s) in which the illnesses occur and the Authorities in the state from which the shellfish were harvested, provides information that may include but shall not be limited to:

(i) Volume or distribution information which would implicate a specific growing area;

(ii) Illness reporting from immediately adjacent growing areas;

(iii) Pollution source investigation in conjunction with growing area evaluation does not identify a pollution source.

(iv) Epidemiological tools that would link cases based on genetic similarity.

(3) When precautionary closures are established to address an illness outbreak involving multiple sources, Authorities will not be required to initiate voluntary recalls until the investigations indicate a single source.

D. **When the appropriate Authorities determine that the illnesses are related to post- harvesting contamination or mishandling, growing area closure is not required. However, the Authority in the state where the post-harvest contamination, mishandling or illegal harvesting from a closed area occurred shall:**

(1) **Notify the ISSC and the FDA Shellfish Specialist of the problem; and**

(2) **Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7 when a recall is deemed appropriate by the Authority . The recall shall include all implicated products.**

(3) **Transmit to the ISSC and FDA information identifying the dealers shipping the implicated shellfish.**

(4) **The ISSC will notify States and FDA Shellfish Specialists of ~~growing area closures and~~ recalls. In the case of recalls, ISSC will notify States with information identifying dealers shipping the implicated shellfish. ~~Closure and r~~Recall notices (not to include dealers) will be posted on the ISSC website. ISSC will maintain an inventory of ~~closure and~~ recall information.**

E. **When the Authority can not complete the determination outlined in Chapter II. @.01 B. within 24 hours, the Authority in the source state shall:**

(1) **Immediately place the implicated portion(s) of the**

harvest area(s) in a precautionary closed status. Should the Authorities later determine that the illnesses are related to post-harvest contamination, or mishandling, or harvested from a closed area, the suspected growing area can be reopened.

(2) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, when the authority deems appropriate.

(3) Promptly initiate recall procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, when the authority can document a rationale that a recall would be effective.

F. **Upon closing an implicated portion(s) of the harvest area(s) for naturally occurring pathogens and/or biotoxins, the Authority:**

(1) Shall follow an existing marine biotoxin contingency/management plan, if appropriate.

(2) Shall collect and analyze samples relevant to the investigation, if appropriate.

(3) Shall keep the area closed until it has been determined that levels of naturally occurring pathogens and/or biotoxins are not a public health concern.

(4) Shall follow the procedure outlined in Chapter II @ .02 A. (10)(a) or (b) for closures resulting from *V.p.* illnesses.

(5) May limit the closure to specific shellfish species when FDA concurs that the threat of illness is species specific.

G. **When the growing area is determined the problem, the Authority shall:**

(1) Place the growing area in the closed status until:

(a) The Authority verifies that the area is properly classified by conducting a review of the growing area to include:

i. current data, in compliance with the NSSP Model Ordinance;

ii. A field review of existing pollution sources;

iii. A review of actual and potential intermittent pollution sources, such as vessel waste discharge and wastewater discharge from treatment plant collection systems. If a previously unknown pollution source can be corrected, the closure period shall be extended to allow for natural depuration following correction of the pollution source; and

iv. Examination of water quality subsequent to the illness outbreak.

(b) It has been determined that the event which caused the contamination no longer exists and sufficient time has elapsed for natural depuration;

(2) Keep the area closed until at least 21 days have passed from the last date of harvest of the implicated shellstock if the illness is consistent with viral etiology; and

(3) Develop a written report summarizing the findings of the

investigation and actions taken.

H. **Whenever an Authority or dealer initiates a recall of shellfish products because of public health concerns, the Authority will monitor the progress and success of the recall. The Authority will immediately notify the FDA, ISSC and the Authorities in other States involved in the recall. The Authority shall submit periodic recall status reports to the FDA Shellfish Specialist consistent with the Recall Enforcement Policy Title 21 CFR Part 7, Subpart C, Section 7.53 (b) (1- 6) until such time that the Authority deems the recall to be completed. Each Authority involved in a recall will implement actions to ensure removal of recalled product from the market, issue public warnings if necessary to protect public health and provide periodic reports to the Authority in the State of product origin regarding recall efforts within their State until such time that the Authority in the State of product origin deems the recall to be completed. FDA will decide whether to audit or issue public warnings after consultation with the Authority/Authorities and after taking into account the scope of the product distribution and other related factors. If the FDA determines that the Authority in any State involved in the recall fails to implement effective actions to protect public health, the FDA may classify, publish and audit the recall, including issuance of public warnings when appropriate.**

I. **Molluscan shellfish product that is recalled as a result of an illness outbreak associated with *V.v., V.p.* or Norovirus may be reconditioned.**

(1) **Validated reconditioning processes for *V.v.* and *V.p.* include subjecting product to validated post-harvest processing (PHP) or placing product into approved, conditionally approved, conditionally restricted, or restricted growing areas for an appropriate period of time, not less than fourteen (14) days, with appropriate controls and documentation to be determined by the Authority.**

(2) **Product associated with a Norovirus outbreak may be reconditioned by returning the product, within ten (10) days of harvest, to the area from which it was harvested for an appropriate period of time. Environmental conditions in the harvest area must be conducive for pumping and feeding. The period of time shall not be less than sixty (60) days. The Authority shall ensure appropriate controls and provide documentation of the activity**

@.02 Shellfish Related Illnesses Associated with *Vibrio parahaemolyticus (V.p.)*

A. **When the investigation outlined in Section @.01 A. indicates the illness(es) are associated with the naturally occurring pathogen *Vibrio parahaemolyticus (V.p.)*, the Authority shall determine the number of laboratory confirmed cases epidemiologically**

associated with the implicated area. States will not be expected to close growing areas based on *V.p.* cases that are reported more than thirty (30) days after harvest or when environmental parameters have changed or monitoring indicates the *V.p.* risk is reduced. Actions taken by the Authority will be based on the number of cases and the span of time as follows.

(1) **When sporadic cases do not exceed a risk of one (1) illness per 100,000 servings or involves at least two (2) but not more than four (4) cases occurring within a thirty (30) day period from an implicated area in which no two (2) cases occurred from a single harvest day, the Authority shall determine the extent of the implicated area. The Authority will make reasonable attempts to ensure compliance with the existing Vibrio Management Plan.**

(2) **When the risk exceeds one (1) illness per 100,000 servings within a thirty (30) day period or when cases exceed four (4) but not more than ten (10) over a thirty (30) day period from the implicated area or two (2) or more cases but less than four (4) cases occur from a single harvest day from the implicated area, the Authority shall:**

- (a) **Determine the extent of the implicated area; and**
- (b) **Immediately place the implicated portion(s) of the harvest area(s) in the closed status; and**
- (c) **As soon as determined by the Authority, transmit to the FDA and receiving States information identifying the dealers shipping the implicated shellfish.**

(3) **When the number of cases exceeds ten (10) illnesses within a thirty (30) day period from the implicated area or four (4) or more cases occurred from a single harvest date from the implicated area, The Authority shall:**

- (a) **Determine the extent of the implicated area; and**
- (b) **Immediately place the implicated portion(s) of the harvest area(s) in the closed status; and**
- (c) **Promptly initiate a voluntary industry recall consistent with the Recall Enforcement Policy, Title 21 CFR Part 7 unless the Authority determines that a recall is not required where the implicated product is no longer available on the market or when the Authority determines that a recall would not be effective in preventing additional illnesses. The recall shall include all implicated products.**
- (d) **Issue a consumer advisory for all shellfish (or species implicated in the illness).**

(4) **When the number of cases and the span of time reach the thresholds outlined above, prior to implementing the controls above, the Authority shall conduct an investigation of the illnesses within seventy-two (72) hours of reaching any one (1) of the thresholds of Chapter II @.02 A. (1), (2) or (3) to determine whether**

	the illness is growing area related or is the result of post-harvest abuse or mishandling such as time temperature abuse. (a) **If the conditions in Chapter II @.02 A. (2) or (3) are met and the investigation cannot be completed within 72 hours, immediately place the implicated portion(s) of the harvest area(s) in a precautionary closed status.** (b) **Should the Authority later determine that the illnesses are related to post harvest abuse or mishandling the implicated harvest area(s) can be immediately reopened.** **(5) When the investigation outlined in Model Ordinance Chapter II. @.02 A. (4) demonstrates that the illnesses are related to post- harvesting contamination or mishandling, growing area closure is not required. However, the Authority shall:** (a) **Notify the ISSC and the FDA Shellfish Specialist of the problem; and** (b) **Determine the appropriateness of initiating a voluntary recall by firms. If a firm or firms is requested by the Authority to recall, the firm will use procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7. The recall shall include all implicated products.** (c) **Transmit to the ISSC and FDA information identifying the dealers shipping the implicated shellfish; Should closures and recalls be necessary the ISSC will notify States and FDA Shellfish Specialists of growing area closures and recalls. In the case of recalls, ISSC will notify States with information identifying dealers shipping the implicated shellfish. Closure and recall notices (not to include dealers) will be posted on the ISSC website. ISSC will maintain an inventory of closure and recall information.** (6) **When the investigation outlined in Model Ordinance Chapter II. @.02 A.(4) does not indicate a post-harvest contamination problem, or illegal harvesting from a closed area, the Authority shall:** (a) **Follow the procedures outlined in Chapter II @.02 A. (1), (2) and (3).** (b) **Notify the ISSC and the FDA Shellfish Specialist that a potential health risk is associated with shellfish harvested from the implicated growing area;** (c) **If a recall is required by Chapter II @.02 A. (3)** i. **Transmit to the ISSC and FDA information identifying the dealers shipping the implicated shellfish.**

ii. **The ISSC will notify States and FDA Shellfish Specialists of growing area closures and recalls. In the case of recalls, ISSC will notify States with information identifying dealers shipping the implicated shellfish. Closure and recall notices (not to include dealers) will be posted on the ISSC website. ISSC will maintain an inventory of closure and recall information.**

(7) **When the State Authority investigating the laboratory confirmed *V.p.* cases does not provide information to identify a single growing area and multiple growing areas are implicated, the State Authorities in the states with implicated growing areas shall evaluate to determine if the illness should be attributed to the implicated area(s). Evaluations may include but are not limited to:**

(a) Vibrio levels in the growing area around the time and date of harvest

(b) Comparison of other single source illnesses attributed to a growing area(s) involved in a multiple source outbreak. The purpose of this comparison would be to determine if a common growing area can be identified.

(c) Environmental conditions which could increase the risk of *V.p.* at the time of harvest. This could include conditions such as water temperature, air temperature and tidal stage.

(d) Genetic typing of clinical isolates implicates a common growing area or rules out implicated growing areas

(8) **If the evaluation in (7) provides sufficient information to implicate a single area, then the Shellfish Authority shall take actions outlined in A. (6), above.**

(9) **When a growing area has been closed as a result of *V.p.* cases, the Authority shall keep the area closed for the following periods of time to determine if additional illnesses have occurred:**

(a) **The area will remain closed for a minimum of fourteen (14) days when the risk exceeds one (1) illness per 100,000 servings within a thirty (30) day period or cases exceed four (4) but not more than ten (10) cases over a thirty (30) day period from the implicated area or two (2) or more cases but less than four (4) cases occur from a single harvest date from the implicated area.**

(b) **The area will remain closed for a minimum of twenty-one (21) days when the number of cases exceeds ten (10) illnesses within thirty (30) days or four (4) cases occur from a single harvest date from the implicated area**

(10) **Prior to reopening an area closed as a result of @.02 A. (9)(a) or (b), the Authority shall:**

(a) **Collect and analyze samples to ensure that tdh does**

not exceed 10/g and trh does not exceed 10/g; or other such values as determined appropriate by the Authority based on studies.

(i) **Samples shall be collected to be representative of the area and shellfish types.; and**

(ii) **Multiple sample collection events shall span the closure time period in @.02 A. (9)(a) or (b) and be collected at intervals necessary to determine trends in the implicated area.**

;or

(b) **Ensure that environmental conditions have returned to levels not associated with *V.p.* cases.**

(11) **Shellfish harvesting may occur in an area closed as a result of *V.p.* illnesses when the Authority implements one (1) or more of the following controls:**

(a) **PHP using a process that has been validated to achieve a two (2) log reduction in the levels of total *V.p.* for Gulf and Atlantic Coast oysters and/or hard clams and a three (3) log reduction for Pacific Coast oysters and/or hard clams;**

(b) **Restricting oyster and/or hard clam harvest to product that is labeled for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing;**

(c) **Other control measures that based on appropriate scientific studies are designed to ensure that the risk of *V.p.* illness is no longer reasonably likely to occur, as approved by the Authority.**

@. 03 Annual Assessment of *Vibrio vulnificus* and *Vibrio parahaemolyticus* Illnesses and Shellfish Production

A. **The Authority shall assess annually *Vibrio vulnificus (V.v.)* and *Vibrio parahaemolyticus (V.p.)* CIDT cases or culture confirmed illnesses associated with the consumption of molluscan shellfish. The assessment will include a record of all *V.v.* and *V.p.* shellfish-associated illnesses reported within the State and from receiving States, the numbers of illnesses per event, and actions taken by the Authority in response to the illnesses.**

B. **The Authority shall collect by month and report annually to the ISSC the volume of shellfish harvested in the State. The report shall include the volume of shellfish harvested for each species. Where available the volume breakdown of the production data will be reported by utilization type (raw, shucked, PHP, etc.).**

@.04 Presence of Human Pathogens in Shellfish Meats

A. **Finding. Upon determination that human pathogens are present in shellfish meats, the Authority shall investigate the harvesting, the distribution, and the processing of the shellfish.**

B. **Growing Area Investigation.**

(1) **The Authority shall review the following factors:**

(a) **The documentation to trace the shellfish to its source;**

(b) **The classification assigned to the growing area and whether the sanitary survey data supporting that classification is current; and**

(c) **The probability of illegal harvesting from areas classified as restricted or prohibited, or in the closed status.**

(2) **The Authority shall not be required to~~shall~~ take ~~no~~ further action when the Authority determines that:**

(a) **The growing area is properly classified;**

(b) **No evidence that illegal harvesting is taking place; and**

(c) **There is an absence of evidence~~no reason to believe~~ that the growing area is the source of the pathogens.**

(3) **When the Authority determines that the growing area is not properly classified, the Authority shall take immediate action to:**

(a) **Change the existing classification to the correct classification; or**

(b) **Close the growing area until the correct classification can be determined; and**

(c) **Determine whether to initiate a voluntary recall by firms. If a firm or firms is requested by the Authority to recall, the firm will use procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7. The recall shall include all implicated products.**

(4) **When the Authority determines that the growing area may be the source of pathogens and the pathogens exceed tolerance levels, the Authority shall request a voluntary recall by firms. The firms will use procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7. The recall shall include all implicated products.**

(5) **When the Authority determines that illegal harvesting is taking place, the Authority shall determine whether to initiate a voluntary recall by firms. The firm will use procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7. The recall shall include all implicated products.**

C. **Distribution and Processing Investigation.**

(1) **The Authority shall evaluate the distribution and processing of the shellfish. This investigation may include collection of additional meat samples.**

(2) **The Authority shall not be required to take ~~no~~ further**

action when the Authority determines that there is no reason to believe a problem exists in the distribution or processing of the shellfish.

(3) **When the Authority determines that a problem exists in the distribution or processing of the shellfish, the Authority shall take immediate steps to correct the problem and determine whether to initiate a voluntary recall by firms. If a firm(s) is requested by the Authority to recall, the firm will use procedures consistent with the Recall Enforcement Policy, Title 21 CFR Part 7. The recall shall include all implicated products.**

D. **Risk Management and Tolerance Levels.**

(1) **Pathogen Present. When a growing area continues to demonstrate the presence of human pathogen isolates in shellfish meats in the absence of illness, the Authority shall perform a risk assessment to determine the correct classification for an area.**

(2) **Established Tolerance Levels.**

(a) **When the established tolerance level for a particular pathogen isolate is not exceeded, the Authority:**

(i) **Shall maintain a written summary of its finding and the data supporting its finding in its central file; and**

(ii) **May leave the growing area in its present classification.**

(b) **When the established tolerance level for a particular pathogen isolate is known and there are no known outbreaks of shellfish associated disease caused by that pathogen in a particular growing area, the Authority shall:**

(i) **Leave the area in the open status of its classification when the tolerance level is not exceeded; and**

(ii) **Place the area in the closed status of its classification when the tolerance level is exceeded.**

(c) **When the tolerance level is exceeded, the Authority may:**

(i) **Maintain the growing area in the closed status of its current classification;**

(ii) **Reclassify the growing area to the restricted or prohibited classification; or**

(iii) **Reclassify the growing area to the conditionally restricted classification and establish a management plan.**

(d) **Any management plan based on shellstock exceeding established tolerance levels shall:**

(i) **Meet all appropriate requirements for a management plan for the conditionally approved or conditionally restricted classification;**

(ii) **Specify the additional criteria associated with the particular pathogen isolate that the growing area must meet to be in the open status of its classification;**

(iii) **Document the scientific basis for the additional criteria;**

(iv) **Provide for periodic retesting of the shellfish meats; and**

(v) **Provide for the growing area to be placed in the closed status if the criteria are exceeded.**

(3) **Established Tolerance Levels Not Known.**

(a) **When an established tolerance level does not exist for the particular pathogen isolated, the Authority shall assess the public health significance of the levels of the pathogen found in the growing area shellfish meats. The Authority may consider FDA recommended action levels or levels of concern in this determination. When the Authority determines that:**

(i) **The levels are acceptable, the growing area ~~shall~~ may remain in the open status of its classification; or**

(ii) **The levels are unacceptable; the growing area shall be placed in the closed status of its classification.**

(b) **If a growing area is placed in the closed status, the Authority may elect to**

(i) **Maintain that status indefinitely;**

(ii) **Reclassify the area to the restricted or prohibited classification; or**

(iii) **Reclassify the area to the conditionally restricted classification and establish a management plan. The management plan shall meet the requirements of Section D. (2) (d).**

@.05 Presence of Toxic Substances in Shellfish Meats

A. **Upon determination that toxic substances, including but not limited to heavy metals, chlorinated hydrocarbons, and natural toxins are present in levels of public health significance in shellfish meats, the Authority shall investigate the harvesting, distribution, and processing of shellfish and take necessary corrective action in accordance with the procedures described in Section @.04.**

B. **When a growing area continues to demonstrate the presence of toxic substances in the absence of illness, the Authority shall perform a risk assessment to determine the correct classification of the area. The risk assessment and subsequent risk management shall follow the procedures outlined in Section @.04 D., Risk Management and Tolerance Levels.**

@.06 *Vibrio vulnificus* Control Plan

A. **Risk Evaluation**

Each shellfish producing State that is not currently implementing

	a *V.v.* Control Plan shall conduct a *V.v.* risk evaluation annually. The evaluation shall consider each of the following factors, including seasonal variations in the factors, in determining the risk of *V.v.* infection from the consumption of shellfish harvested from the State's growing waters. **(1) In conducting the risk evaluation, the Authority will at a minimum consider the following:** **(a) The number of *V.v.* cases etiologically confirmed and epidemiologically linked to the consumption of commercially harvested shellfish from the State; and** **(b) Levels of *V.v.* in the growing waters and in shellfish, to the extent that such data exists; and** **(c) The quantity of harvest from the area and its uses i.e. shucking, half shell, PHP.** **B. States which have previously met the illness threshold requiring a *V.v.* Control Plan will continue to maintain and implement a *V.v.* Control Plan.** **C. All States not currently implementing a *V.v.* Control Plan shall develop and implement a *V.v.* Control Plan if the risk evaluation indicates two (2) or more etiologically confirmed, and epidemiologically linked *V.v.* illnesses from consumption of commercially harvested oysters that originated from the growing waters of that State within the previous ten (10) years** **D. The Authority shall develop a *V.v.* Contingency Plan should the risk evaluation indicate any etiologically confirmed shellfish-borne *V.v.* illness from the growing waters of that State but the number of cases does not reach the threshold established in @.06 C.** **E. Control Plan** **(1) The *V.v.* Control Plan shall include the following:** **(a) Identification of triggers which address factors that affect risks. The triggers will be used to indicate when control measures are needed. One (1) or more of the following triggers will be used:** **(i) The water temperatures in the area;** **(ii) The air temperatures in the area;** **(iii) Salinity in the area;** **(iv) Harvesting techniques in the area; and** **(v) Other factors which affect risk which can be used as a basis for reducing risk.** **(b) Implementation of one (1) or more of the following control measures to reduce the risk of *V.v.* illness:** **(i) Labeling oysters as being for shucking by a certified dealer or for approved post-harvest processing to control the *Vibrio vulnificus* hazard when the Average Monthly Maximum Water Temperature exceeds 70 °F.** **(ii) Subjecting all oysters intended for the raw, half-shell market to Authority approved PHP when the Average Monthly Maximum Water Temperature exceeds 70 °F.**

(iii) **Reducing time of exposure to ambient air temperature prior to delivery to the initial certified dealer based on modeling or sampling, as determined by the Authority in consultation with FDA. For the purpose of time to temperature control, time begins once the first shellstock harvested is no longer submerged. When this control measure is selected, State *V.v.* plans will include controls when water temperature promotes *V.v.* levels and risk of illness increases. The controls will minimize risk to less than three (3) illnesses per 100,000 servings when Average Monthly Maximum Water Temperature exceeds 80 °F. Authority approved Best Management Practices (BMPs) will be applied to minimize *V.v.* growth to the extent possible when Average Monthly Maximum Water temperature exceeds 70 °F but is less than or equal to 80 °F. BMPs will ensure that when the water temperature exceeds 70 °F but is less than or equal to 75 °F risk is minimized to less than 1.75 illnesses per 100,000 servings and when water temperature exceeds 75 °F but is less than or equal 80 °F the risk will not exceed 2.5 illnesses per 100,000 servings. These risks per serving will be determined using the FDA developed *V.v.* calculator. A State is in compliance with the NSSP when it effectively implements the controls established in its plan using the FDA calculator to determine the risk per serving for the established water temperatures.**

(iv) **The State Authority may implement alternative controls that will reduce the risk to a level comparable to the risk per serving identified above in @.06 E. (1) (b) (iii) when water temperatures exceed 70 °F.**

(c) **A state has the option to implement a *Vibrio vulnificus* Control Plan that includes time-temperature harvesting controls when Average Monthly Maximum water temperatures are below 70ºF. If the state implements this option, shellstock intended for raw consumption shall comply with the matrix below:**

Action Level	Water Temperature	Max Expos

Level 1	**<65ºF**	**36 hours**
Level 2	**65ºF - 70ºF (18ºC - 23ºC)**	**14 hours**

(d) All shellstock harvested according to a *Vibrio vulnificus* control plan shall be cooled to an internal temperature of 55ºF (12.7ºC) or less within 10 hours of being placed into temperature control.

(e) When pre-harvest culture practices have the potential to elevate Vibrio levels in market size product intended for immediate harvest, the Authority shall establish Vibrio control measures and include the measures in the State Vibrio Control Plan. Such control measures may be implemented on a State-wide, regional, geographic, or farm or growing area-specific basis. When shellfish are re-immersed as a control measure the Authority should consider inclusion of record keeping requirements such as means of shellfish segregation/identification procedures, date re-immersed in water and date of final harvest. The Authority may require growers to have a control plan approved by the Authority.

(2) Control Plan Evaluation

(a) The State Authority will conduct annual evaluations of the plan. At a minimum the Authority will consider:

(i) The annual number of *Vibrio vulnificus* cases associated with the State's growing waters and the amount of shellstock sold for half shell consumption to determine risk per servings for each temperature period.

(ii) Environmental changes which could affect total *V.v.* in shellfish pre and post-harvest.

(iii) Industry compliance with existing controls.

(iv) The Authorities enforcement of industries' implementation of the controls.

(b) For the purposes of determining Authority compliance the FDA will conduct an annual Vibrio evaluation of the Authority to determine the following:

(i) Authority compliance with *V.v.* Risk Evaluation as required in Chapter II. @.06 A.

(ii) For States requiring the development of *V.v.* Plans, compliance with Control Plan requirements of Chapter II. @.06 E. (1). The evaluation should determine:

(a) Appropriate identification of triggers to determine when control measures are needed.

(b) **Did the Authority implement one (1) or more of the control measures required in Chapter II. @.06 E. (1) (b).**

(c) **For Authorities implementing Chapter II. @.06 E. (1) (b) (i) and (ii), were the controls implemented adequately.**

(d) **For Authorities implementing Chapter II. @.06 E. (1) (b) (iii) (time and temperature control), did the Authority establish controls consistent with water temperature and was the FDA developed *V.v.* calculator used correctly.**

(iii) **For Authorities required to develop *V.v.* Contingency Plans the evaluation should determine:**

a. **Did the risk evaluation indicate the need for a Contingency Plan.**

b. **For States requiring the development of a Contingency Plan, does the plan include the regulatory steps to be implemented should the number of illnesses reach the threshold for a *V.v.* Plan**

(c) **Should the findings of the State evaluation indicate that the Authority was in compliance with the items audited in (2) (b) and the observed risk per servings exceeded established risk per serving for one (1) or more water temperature, the Authority will be deemed in compliance with the NSSP Model Ordinance. The FDA will include this finding in a report to the ISSC.**

(d) **The results of the State and FDA risk per serving evaluations will be shared with the ISSC Vibrio Management Committee for use in conducting trend evaluations as stated in the ISSC Constitution, Bylaws, and Procedures.**

F. **Contingency Plan**

(1) **The Contingency Plan shall include a detailed plan outlining the regulatory steps that will be implemented should the number of illnesses reach the threshold established for development and implementation of a *V.v.* Control Plan.**

(2) **Contingency Plan Evaluation**
In consultation with FDA the Authority will evaluate the adequacy of their Contingency Plan.

@.07 *Vibrio parahaemolyticus* Control Plan

The goal of the Control Plan is to reduce the probability of occurrence of *Vibrio parahaemolyticus* (*V.p.*) illness during periods that have been historically associated with annual illnesses. The Plan is to be

implemented as part of a comprehensive program which includes all the time and temperature requirements contained in the Model Ordinance.

A. **Independent Species Specific Risk Evaluation.**
Every State from which oysters or hard clams (*Mercenaria mercenaria*) are harvested shall conduct a *V.p.* risk evaluation annually. The evaluation shall consider each of the following factors, including seasonal variations in the factors, in determining whether the risk of *V.p.* infection from the consumption of oysters or hard clams harvested from an area (hydrological, geographical, or growing) is reasonably likely to occur: (For the purposes of this section, "reasonably likely to occur" shall mean that the risk constitutes an annual occurrence)
(1) **The number of *V.p.* cases epidemiologically linked to the consumption of oysters or hard clams commercially harvested from the State;**
(2) **Levels of total and tdh+ *V.p.* in the area, to the extent that such data exists;**
(3) **The water temperatures in the area;**
(4) **The air temperatures in the area;**
(5) **Salinity in the area;**
(6) **Harvesting techniques in the area; and**
(7) **The quantity of harvest from the area and its uses i.e. shucking, half-shell, PHP.**

B. **Independent Species Specific Control Plan**
(1) **If a State's *V.p.* risk evaluation determines that the risk of *V.p.* illness from the consumption of oysters or hard clams harvested from a growing area is reasonably likely to occur, the State shall develop and implement a *V.p.* Control Plan.**
(2) **If a State has a shellfish growing area in which harvesting occurs at a time when average monthly daytime water temperatures exceed those listed below, the State shall develop and implement a *V.p.* Control Plan. The average water temperatures representative of harvesting conditions (for a period not to exceed thirty (30) days) that prompt the need for a Control Plan are:**
(a) **Waters bordering the Pacific Ocean: 60 °F.**
(b) **Waters bordering the Gulf of Mexico and Atlantic Ocean (NJ and south): 81 °F.**
(c) **Waters bordering the Atlantic Ocean (NY and north): 60 °F.**
(d) **However, development of a Plan is not necessary if the State conducts a risk evaluation, as described in Section A. that determines that it is not reasonably likely that *V.p.* illness will occur from the consumption of oysters or hard clams harvested from those areas.**
(i) **In conducting the evaluation, the State shall evaluate the factors listed in Section A. for the area during**

	periods when the temperatures exceed those listed in this section; (ii) **In concluding that the risk is not reasonably likely to occur, the State shall consider how the factors listed in Section A. differ in the area being assessed from other areas in the State and adjoining States that have been the source of shellfish that have been epidemiologically linked to cases of *V.p.* illness; or** (3) **If a State has a shellfish growing area that was the source of oysters or hard clams that were epidemiologically linked to an outbreak of *V.p.* within the prior five (5) years, the State shall develop and implement a *V.p.* Control Plan for the area.** (4) **For States required to implement *V.p.* Control Plans, the Plan shall include the administrative procedures and resources necessary to accomplish the following:** (a) **Establish one (1) or more triggers for when control measures are needed. These triggers shall be the temperatures in Section B. (2) where they apply, or other triggers as determined by the risk evaluation.** (b) **Implement one (1) or more control measures to reduce the risk of *V.p.* illness at times when it is reasonably likely to occur. The control measures may include:** (i) **PHP using a process that has been validated to achieve a two (2) log reduction in the levels of total *V.p.* for Gulf and Atlantic Coast oysters and hard clams and a three (3) log reduction for the Pacific Coast oysters;** (ii) **Closing the area to oyster and/or hard clam harvest;** (iii) **Restricting oyster and/or hard clam harvest to product that is labeled for shucking by a certified dealer, or other means to allow the hazard to be addressed by further processing;** (iv) **Limiting time from harvest to refrigeration to no more than five (5) hours, or other times based on modeling or sampling, as determined by the Authority in consultation with FDA;** (v) **Limiting time from harvest to refrigeration such that the levels of total *V.p.* after the completion of initial cooling to 60 °F (internal temperature of the oysters or hard clams) do not exceed the average levels from the harvest water at time of harvest by more than 0.75 logarithms, based on sampling or modeling, as approved by the Authority;** (vi) **Other control measures that based on appropriate scientific studies are designed to ensure that the risk of *V.p.* illness is no longer reasonably likely to occur, as approved by the Authority.** (c) **Require the original dealer to cool oysters and/or hard clams to an internal temperature of 50 °F (10 °C) or below within ten (10) hours or less as determined by the**

	Authority after placement into refrigeration during periods when the risk of *V.p.* illness is reasonably likely to occur. The dealer's HACCP Plan shall include controls necessary to ensure, document and verify that the internal temperature of oysters and/or hard clams has reached 50 °F (10 °C) or below within ten (10) hours or less as determined by the Authority of being placed into refrigeration. When deemed appropriate by the Authority an exception may be permitted for hard clams to allow for tempering. Oysters and/or hard clams without proper HACCP records demonstrating compliance with this cooling requirement shall be diverted to PHP or labeled as being for shucking by a certified dealer or for approved post-harvest processing to control the *Vibrio parahaemolyticus* hazard. **(d) Evaluate the effectiveness of the Plan.** **(e) Modify the Control Plan when the evaluation shows the Plan is ineffective, or when new information is available or new technology makes this prudent as determined by the Authority.** **(f) Optional cost benefit analysis of the *V.p.* Control Plan.** **(5) When pre-harvest culture practices have the potential to elevate *V.p.* levels in market size product intended for immediate harvest, the Authority shall establish *V.p.* control measures and include the measures in the State Vibrio Control Plan. Such control measures may be implemented on a State-wide, regional, geographic, or farm or growing area-specific basis. When shellfish are re-immersed as a control measure the Authority should consider inclusion of record keeping requirements such as means of shellfish segregation/identification procedures, date re-immersed in water and date of final harvest. The Authority may require growers to have a control plan approved by the Authority.** **C. The Time When Harvest Begins** **For the purpose of time to temperature control, time begins once the first shellstock harvested is no longer submerged.**
12. Public Health Significance	N/A
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Chris Schillaci
3. Affiliation	East Coast Shellfish Growers Association (ECSGA)
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6. City, State, Zip	New Bedford, MA 02740
7. Phone	(401) 783-3360
8. Email	chris@ecsga.org
9. Proposal Subject	Consideration of Illnesses Among Non-Consumers of Shellfish in Outbreak Investigations
10. Specific NSSP Guide Reference	NSSP Guide for the Control of Molluscan Shellfish, 2023 Revision. Section II. Model Ordinance, Chapter II. Risk Assessment and Risk Management, @.01 Outbreaks of Shellfish-Related Illness, B. (new (3) and (4)), C. (2) (new (d)), and E. (1).
11. Text of Proposal/ Requested Action	Amend Section II. Model Ordinance, Chapter II. Risk Assessment and Risk Management, @.01 Outbreaks of Shellfish-Related Illness, as follows. New language is shown in red double underline. Deleted language is shown in blue strikethrough. Existing text not shown is unchanged (indicated by ellipses). **1. Add new paragraphs B.(3) and B.(4):** B. When the state or local epidemiologist ... the appropriate Authorities shall: (1) Notify the FDA Shellfish Specialist that a shellfish related outbreak has occurred. (2) Within twenty-four (24) hours determine whether the illness is growing area related or is the result of post-harvest contamination, mishandling or illegal harvesting from a closed area. ... (3) The Authority, in consultation with the epidemiologist(s) in the state(s) in which the outbreak occurs, may determine that the outbreak is not attributable to shellfish when the investigation documents that: (a) Cases within the same illness cluster (the same event, establishment, or common exposure setting and time period) include one (1) or more persons who did not consume shellfish; (b) The illnesses among persons who did not consume shellfish are caused by, or are clinically consistent with, the same etiologic agent as the illnesses among persons who consumed shellfish and, where laboratory data are available, share the same genotype, strain, or sequence type; (c) The onset dates of the illnesses among persons who did not consume shellfish are consistent with exposure to a common source during the same exposure period and are not consistent with secondary (person-to-person) transmission from persons who consumed shellfish; and (d) The epidemiological evidence, including food histories and, where

	available, food-specific attack rates, identifies a source other than shellfish (such as an ill food worker, another food item, or environmental contamination) or does not support an association between illness and shellfish consumption. (4) When the Authority makes the determination described in B.(3): (a) Growing area closure and recall of shellfish are not required; (b) Any precautionary closure placed under Section C.(2) or Section E.(1) may be immediately lifted; (c) The Authority shall notify the FDA Shellfish Specialist and the ISSC of the determination and provide the supporting basis; (d) The Authority shall document the determination and supporting information in a written record in the central file; and (e) If additional information becomes available (e.g., additional cases, trace-back results, or laboratory results) that implicates shellfish, the Authority shall take the actions required in Sections C. through H. **2. Amend C.(2) by adding new item (d):** A specific growing area placed in a precautionary closed status under this section can be immediately re-opened when one (1) or more of the following conditions are met: (a) ... (b) ... (c) ... (d) The Authority has determined, in accordance with Section B.(3), that the outbreak is not attributable to shellfish. **3. Amend E.(1):** (1) Immediately place the implicated portion(s) of the harvest area(s) in a precautionary closed status. Should the Authorities later determine that the illnesses are related to post-harvest contamination, ~~or~~ mishandling, ~~or~~ harvested from a closed area, or are not attributable to shellfish in accordance with Section B.(3), the suspected growing area can be reopened.
12. Public Health Significance	Section II, Chapter II @.01 establishes the actions an Authority must take once shellfish are epidemiologically associated with an outbreak, and Section E requires a precautionary closure whenever the growing-area determination cannot be completed within 24 hours. The Model Ordinance provides explicit off-ramps for outbreaks traced to post-harvest contamination, mishandling, or illegal harvest (Section D) and for multiple-source outbreaks (Section C.(2)), but it does not explicitly recognize one of the most informative findings in an outbreak investigation: persons in the same illness cluster who became ill but did not eat shellfish. Norovirus and other enteric outbreaks at events, restaurants, and catered functions frequently involve multiple menu items and ill food workers. When shellfish were served, early food histories can create an apparent association with shellfish even though non-consumers in the same cluster became ill with the same agent at the same time. Such cases point to a common source other than shellfish. Without clear authority in the Model Ordinance to act on this evidence, Authorities may feel

	obligated to close growing areas and initiate recalls that do not protect public health, while investigative resources are drawn away from identifying and controlling the actual source. The proposed language gives the Authority discretion, in consultation with the epidemiologist, to conclude that an outbreak is not attributable to shellfish, while building in safeguards that protect public health: Timing of onset: non-consumer cases must be consistent with a common-source exposure and not with secondary person-to-person spread, which commonly follows shellfish-borne norovirus outbreaks and would otherwise be misread as evidence against shellfish. Same agent: cases must share the same etiologic agent, and the same genotype or strain where laboratory data exist. Supporting epidemiology: food histories and attack rates must identify another source or fail to support a shellfish association. Transparency and reversibility: the determination must be documented and reported to the FDA Shellfish Specialist and the ISSC, and the Authority must resume closure and recall actions if new evidence implicates shellfish. This approach is consistent with the epidemiological principles already referenced in @.01 A (International Association for Food Protection, Procedures to Investigate Foodborne Illness) and with the existing reopening criteria in C.(2), which allow reopening based on consumption data and other relevant information provided by state investigators. It aligns shellfish control actions with the weight of the epidemiological evidence, reduces unnecessary closures and recalls, and helps focus outbreak response on the true source of illness.
13. Cost Information	No additional cost to Authorities is anticipated; the proposal uses information already collected during routine outbreak investigations. The proposal is expected to reduce costs to Authorities, harvesters, and dealers by avoiding closures, recalls, and associated sampling and reopening activities in outbreaks not caused by shellfish.
14. Changes Needed to Guidance (if proposed change is adopted)	No changes to the Guidance Documents (Section IV) are required.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Chris Schillaci
3. Affiliation	East Coast Shellfish Growers Association (ECSGA)
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7. Phone	(401) 783-3360
8. Email	chris@ecsga.org
9. Proposal Subject	Risk-Based Response to Shellfish-Related Illnesses Associated with Campylobacter spp.
10. Specific NSSP Guide Reference	NSSP Guide for the Control of Molluscan Shellfish, 2023 Revision. Section II. Model Ordinance, Chapter II. Risk Assessment and Risk Management, @.01 Outbreaks of Shellfish-Related Illness (new paragraph J); new @.08 Shellfish-Related Illnesses Associated with Campylobacter spp.
11. Text of Proposal/ Requested Action	This proposal adds a new Model Ordinance section, Chapter II @.08, that sets out a risk-based, tiered response to shellfish-associated Campylobacter illnesses, patterned on the existing Chapter II @.02 for Vibrio parahaemolyticus. The new section (1) accounts for the time between harvest, illness onset, and case reporting; (2) distinguishes sporadic cases from clusters and outbreaks, using genetic relatedness where available; (3) uses risk per serving together with case counts so that a single sporadic case does not by itself trigger closure of a small production area; and (4) sets closure durations, reopening criteria, and harvest controls based on available purge and survival data. A conforming amendment to Chapter II @.01 routes these illnesses to the new section. New language is shown in red double underline. Deleted language is shown in blue strikethrough. The Executive Office is requested to adjust section and paragraph numbering as needed, including if the companion Salmonella proposal is also adopted. **1. Amend Section II. Model Ordinance, Chapter II. Risk Assessment and Risk Management, @.01 Outbreaks of Shellfish-Related Illness, by adding a new paragraph J:** J. When the investigation outlined in Chapter II @.01 A. indicates that the illness(es) are associated with Campylobacter spp., the Authority shall follow the requirements of Chapter II @.08 in lieu of Chapter II @.01 B. through G., except as referenced in Chapter II @.08. **2. Add the following new section to Section II. Model Ordinance, Chapter II. Risk Assessment and Risk Management:** @.08 Shellfish-Related Illnesses Associated with Campylobacter spp. A. Applicability and Definitions. (1) This section applies when the investigation outlined in Chapter II @.01 A. indicates that the illness(es) are associated with thermotolerant Campylobacter spp. (Campylobacter jejuni, Campylobacter coli, or

	Campylobacter lari) and the consumption of molluscan shellfish. (2) For the purposes of this section: (a) Case means a laboratory-confirmed Campylobacter infection, identified by culture or by a culture-independent diagnostic test (CIDT), that the state or local epidemiologist has determined is epidemiologically associated with the consumption of shellfish from the implicated area. In making this determination the epidemiologist should consider whether the interval between shellfish consumption and illness onset is consistent with the incubation period (typically two (2) to five (5) days) and whether other exposures documented in the food and exposure history (e.g., poultry, unpasteurized dairy products, untreated water, animal contact, or travel) provide a more likely source of infection. (b) Sporadic cases means cases from an implicated area in which no two (2) cases are associated with a single harvest date and clinical isolates, where characterized, are not genetically related. (c) Genetically related means that clinical isolates (and shellfish or environmental isolates, where available) have been determined to be closely related by whole genome sequencing (WGS) or another subtyping method used by PulseNet, as determined by the state public health laboratory or the Centers for Disease Control and Prevention (CDC). (d) Harvest date means the date of harvest shown on the shellstock tag or, where tags are unavailable, the harvest date determined through traceback. (e) Implicated area means the growing area, portion of a growing area, or aquaculture site from which the shellfish associated with the case(s) were harvested. (f) Contributing condition means an identified or suspected source of Campylobacter contamination of the implicated area that the investigation associates with the case(s) or that exceeds conditions normally present in the growing area, including but not limited to congregations of birds and/or mammals on or around aquaculture gear or other structures, failing on-site sewage disposal systems, wastewater discharges, livestock, or agricultural runoff. The presence of birds or other wildlife at levels normally found in the growing area is not by itself a contributing condition. (g) Risk per serving means the number of cases associated with shellfish harvested from the implicated area within a thirty (30) day period, divided by the number of servings harvested from the implicated area during the same period, expressed as illnesses per 100,000 servings. For this calculation one (1) serving equals twelve (12) oysters or hard clams, or the equivalent shucked meat weight, unless the Authority documents an alternative conversion consistent with that used for Vibrio risk-per-serving calculations.

B. Time Between Harvest and Case Reporting.

(1) Cases shall be attributed to the thirty (30) day period in which the implicated shellfish were harvested, regardless of the date the cases are reported to the Authority.

(2) States will not be expected to close growing areas based on cases reported more than thirty (30) days after the last implicated harvest date, unless the investigation in C. indicates that a contributing condition present at the time of harvest persists. When closure is not required under this subsection, the Authority shall complete the investigation in C., require correction of any contributing condition identified, and document its findings.

C. Investigation.

(1) Within seventy-two (72) hours of notification of a case, the Authority shall initiate an investigation to determine:

(a) The extent of the implicated area and the harvest date(s) of the implicated shellfish;

(b) Whether the illness(es) are growing area related or are the result of post-harvest contamination or mishandling, including cross-contamination during storage, preparation, or service (e.g., contact with raw poultry), or illegal harvesting from a closed area;

(c) Whether a contributing condition exists, including a review of bird and/or mammal congregations on or near aquaculture gear and of compliance with any operational plan required under Chapter VI. .04;

(d) Whether the growing area is properly classified, including a review of current water quality data and the sanitary survey; and

(e) The volume of shellfish harvested from the implicated area during the relevant thirty (30) day period, for the purpose of calculating risk per serving.

(2) If the conditions in D. (2) or D. (3) are met and the investigation cannot be completed within seventy-two (72) hours, the Authority shall immediately place the implicated area in the precautionary closed status. Should the Authority later determine that the illnesses are related to post-harvest contamination or mishandling, the implicated area may be immediately reopened.

(3) When the investigation demonstrates that the illnesses are related to post-harvest contamination or mishandling, growing area closure is not required and the Authority shall follow the requirements of Chapter II @.01 D.

(4) When the investigation identifies a human sewage source or an improperly classified growing area, the Authority shall also follow the requirements of Chapter II @.01 G.

D. Risk Management Actions. When the investigation in C. does not indicate post-harvest contamination, mishandling, or illegal harvesting from a closed area, the actions taken by the Authority shall be based on the number of cases,

their relationship to one another, and the span of time, as follows:

(1) Sporadic Cases. When a single case occurs within a thirty (30) day period from the implicated area, or when two (2) or three (3) sporadic cases occur within a thirty (30) day period and the risk does not exceed one (1) illness per 100,000 servings, the Authority shall:

(a) Determine the extent of the implicated area;

(b) Evaluate the implicated area for contributing conditions and, when a contributing condition is identified, require corrective action, which may include development or revision of an operational plan and mitigation or deterrent measures for birds and/or mammals under Chapter VI. .04;

(c) Notify the FDA Shellfish Specialist and the Authorities in receiving States of the case(s) and the actions taken; and

(d) Coordinate with the state epidemiologist to enhance surveillance for additional cases associated with the implicated area.

Closure of the implicated area is not required under this subsection.

(2) Clusters. When (i) two (2) or three (3) cases are associated with a single harvest date; (ii) two (2) or more cases are genetically related; (iii) two (2) or three (3) sporadic cases occur within a thirty (30) day period and the risk exceeds one (1) illness per 100,000 servings; or (iv) four (4) to six (6) cases occur within a thirty (30) day period from the implicated area, the Authority shall:

(a) Determine the extent of the implicated area;

(b) Immediately place the implicated area in the closed status. The closure may be limited to the implicated aquaculture site(s) and/or shellfish species when the investigation identifies a site-specific contributing condition;

(c) As soon as determined by the Authority, transmit to the ISSC, the FDA, and receiving States information identifying the dealers shipping the implicated shellfish;

(d) Determine the appropriateness of a voluntary recall by firms consistent with the Recall Enforcement Policy, Title 21 CFR Part 7. A recall is not required when the implicated product is no longer available on the market or when the Authority determines that a recall would not be effective in preventing additional illnesses; and

(e) Require correction of any contributing condition identified.

(3) Outbreaks. When four (4) or more cases are associated with a single harvest date, or more than six (6) cases occur within a thirty (30) day period from the implicated area, the Authority shall:

(a) Take the actions in D. (2) (a) through (c) and (e);

(b) Promptly initiate a voluntary industry recall consistent with the Recall Enforcement Policy, Title 21 CFR Part 7, unless the Authority determines that the implicated product is no longer available on the

market or that a recall would not be effective in preventing additional illnesses. The recall shall include all implicated products; and

(c) Issue a consumer advisory for the shellfish species implicated in the illnesses.

(4) The ISSC will notify States and FDA Shellfish Specialists of closures and recalls under D. (2) and D. (3), consistent with Chapter II @.01 C. (1) (e).

E. Multiple Implicated Areas. When the investigation does not identify a single implicated area, the Authorities in the States with implicated areas shall evaluate whether the illness(es) should be attributed to one (1) or more of the areas. Evaluations may include but are not limited to:

(1) Contributing conditions in each area at the time of harvest, including bird and/or mammal observations recorded by operators, enforcement staff, or sampling staff;

(2) Comparison with other cases attributed to the areas;

(3) Genetic comparison of clinical, shellfish, and environmental isolates; and

(4) Volume and distribution information.

If the evaluation implicates a single area, the Authority shall take the actions in D. Precautionary closures of multiple implicated areas are not required under D. (1). For D. (2) and D. (3), Chapter II @.01 C. (2) and (3) shall apply.

F. Closure Duration and Reopening.

(1) An area closed under D. (2) shall remain closed for a minimum of seven (7) days, and an area closed under D. (3) for a minimum of fourteen (14) days, from the date the Authority verifies that the contributing condition has been corrected or no longer exists or, when no contributing condition is identified, from the date of closure. When water temperatures in the implicated area are below 10 °C (50 °F), at which shellfish feeding is greatly reduced, the Authority should consider extending the closure period.

(2) Prior to reopening an area closed under this section, the Authority shall:

(a) Verify that the contributing condition has been corrected or no longer exists, including, for aquaculture sites, that required mitigation or deterrent measures are in place and functioning;

(b) Verify that the growing area meets the microbiological standards for its classification under Chapter IV. @.02; and

(c) When determined appropriate by the Authority, collect shellstock samples representative of the implicated area and shellfish types and analyze them for C. jejuni, C. coli, and C. lari using a method acceptable to the Authority until a method is adopted under Chapter III. Isolates recovered shall be submitted for whole genome sequencing or another subtyping method for comparison with the clinical isolates.

	Because Campylobacter spp. may be present in shellfish from growing areas not associated with illness, detection alone shall not be the basis for continued closure. If the outbreak strain is detected, the Authority shall continue the closure and re-evaluate the contributing condition. (3) The Authority shall develop a written report summarizing the findings of the investigation and the actions taken. G. Harvest from Closed Areas and Reconditioning. (1) Shellfish harvesting may occur in an area closed under this section, and shellfish recalled under this section may be reconditioned, when the Authority implements one (1) or more of the following controls: (a) Relaying the shellstock under Chapter V., or placing it in an approved or conditionally approved growing area in the open status where the contributing condition is absent, for not less than seven (7) days, with appropriate controls and documentation determined by the Authority; (b) Depuration in a certified depuration facility using a process the Authority determines, based on appropriate scientific studies, reduces Campylobacter to levels not reasonably likely to cause illness; (c) Post-harvest processing (PHP) using a process validated to reduce Campylobacter to levels not reasonably likely to cause illness; or (d) Other control measures that, based on appropriate scientific studies, are designed to ensure that the risk of Campylobacter illness is no longer reasonably likely to occur, as approved by the Authority.
12. Public Health Significance	**Campylobacter is a common foodborne pathogen but a rare shellfish hazard.** CDC estimates about 1.5 million Campylobacter infections in the United States each year, most of them linked to poultry, unpasteurized dairy, and produce. From 2000 to 2024, CDC outbreak surveillance recorded 11 Campylobacter outbreaks (66 illnesses) linked to oysters, compared with 190 Vibrio and 143 norovirus outbreaks linked to oysters over the same period (ISSC Aquatic Bird Risk Assessment Committee, draft final report). Source attribution using U.S. surveillance isolates attributes only about 3% of human campylobacteriosis to wild birds, compared with about 68% to poultry and 28% to cattle (Pascoe et al. 2024). Most Campylobacter strains, including many carried by birds, are not associated with human illness, and C. jejuni accounts for most human cases. **Background presence in growing areas.** Campylobacter spp. are commonly carried by gulls, waterfowl, and other wildlife and occur in estuarine waters and shellfish from growing areas that are not linked to illness (Carter et al. 1987). Much of what is found in these settings is C. lari or bird-adapted strains that are rarely associated with human illness (Smith et al. 2020). Detection in shellfish or water therefore does not by itself show that a growing area caused a case, and non-detection does not show that an area is free of risk. For this reason the proposal bases actions on case patterns, genetic relatedness, and risk per serving, and ties reopening to correcting the contributing condition rather than to non-detection. **Recent shellfish-associated events and a gap in the Model Ordinance.** Shellfish-associated Campylobacter outbreaks have been linked to birds congregating on

	floating aquaculture gear, including an October 2021 Rhode Island outbreak of C. jejuni (eight ill) (Caron et al. 2023; NSSP Guide Section IV, Chapter VI). In August 2025, Massachusetts closed Katama Bay (Edgartown) to oyster harvest after five confirmed Campylobacter illnesses linked to oysters harvested July 7–17 and eaten July 12–21. The closure took effect August 1, 15 days after the last implicated harvest date, and was to remain in place "until examined by the Division." The Model Ordinance currently addresses these illnesses only through the general outbreak provisions of Chapter II @.01. Under @.01, two illnesses in different households count as an outbreak and require immediate closure and consideration of recall. However, @.01 provides no guidance on sporadic cases, on the lag between harvest and case reporting, on risk relative to production volume, or on closure duration and reopening criteria suited to a bacterial pathogen that shellfish purge quickly. The result can be inconsistent responses between States and open-ended closures of small aquaculture operations. **Time from illness to reporting.** Campylobacter incubation is typically 2–5 days (CDC). For enteric bacterial pathogens, the time from exposure to confirmation that a case is part of an outbreak is typically 2–4 weeks, reflecting care-seeking, laboratory testing, isolate shipping, and subtyping (CDC, Timeline for Reporting Cases of Salmonella Infection). By the time a shellfish Authority is notified, the implicated lots may be beyond their market life, and a short-lived contamination event (e.g., a seasonal bird congregation) may have ended. The 30-day reporting window in the proposed @.08 B. matches the one in @.02 for V.p. It keeps closure authority when a contributing condition persists and still requires investigation and correction in every case. **Sporadic cases vs. outbreaks, and risk per serving.** Because Campylobacter is a common infection with many non-shellfish sources, a single reported case with a history of shellfish consumption does not show that the growing area is the source. The proposal therefore requires investigation and correction of contributing conditions for sporadic cases, and reserves closure for (i) multiple cases from a single harvest date, (ii) genetically related cases, (iii) sporadic cases that exceed the risk-per-serving benchmark already used in @.02 (one illness per 100,000 servings), or (iv) larger case counts. To keep a single case from triggering closure at low-volume sites, the risk-per-serving trigger applies only when two or more cases occur. WGS through PulseNet is now routine and lets investigators tell linked from unrelated cases. **Closure duration and reopening.** Preliminary results from an ISSC-funded study found that Eastern oysters exposed to C. jejuni, C. coli, and C. lari cleared all three species to below molecular (ddPCR) detection in tissue within two days (Richards, Noble and Smith, in prep.). A two-day depuration period was also effective for Sydney rock oysters (Arumugaswamy et al. 1988). Campylobacter can, however, survive in harvested shellfish for up to 22 days at 4 °C (Abeyta et al. 1993; Arumugaswamy et al. 1988), so recall stays relevant when product is still in commerce. The proposed minimum closures (7 and 14 days after the contributing condition is corrected) give a wide margin over observed purge times. The added caution at water temperatures below 10 °C reflects reduced oyster feeding. Fecal indicator bacteria correlate poorly with Campylobacter (Carter et al. 1987; Hellein et al. 2011; Caron et al. 2023). For that reason, reopening is tied to verified correction of the contributing condition, along with the classification standards.

	Optional shellstock testing is used to check whether the outbreak strain persists, not to require non-detection, because these organisms can be present in areas not associated with illness. **Consistency with ISSC work.** The proposal follows the direction of the Aquatic Bird Risk Assessment Committee, which recommends species-specific, risk-based management of C. jejuni, C. coli, C. lari, and Salmonella enterica rather than genus-level responses. It also follows the Committee's recommendation that shellfish Authorities work with epidemiologists to weigh other exposures when attributing illness to shellfish. It builds on the existing requirements for aquaculture sites that attract birds or mammals (Chapter VI. .04) and gives a proportionate, consistent response while research continues. **Supporting References** 1. Abeyta, C., et al. 1993. Campylobacter jejuni in a Washington state shellfish growing bed associated with illness. J Food Prot. 56:323-325. 2. Arumugaswamy, R., R. Proudford, and M. Eyles. 1988. The response of Campylobacter jejuni and Campylobacter coli in the Sydney Rock Oyster during depuration and storage. Int J Food Microbiol. 7:173-183. 3. Caron, G., et al. 2023. Campylobacter jejuni outbreak linked to raw oysters in Rhode Island, 2021. J Food Prot. 86:1-7. 4. Carter, A. M., et al. 1987. Seasonal occurrence of Campylobacter spp. in surface waters and their correlation with standard indicator bacteria. Appl Environ Microbiol. 53:523-526. 5. CDC. Clinical Overview of Campylobacter. https://www.cdc.gov/campylobacter/hcp/clinical-overview/index.html 6. CDC. Timeline for Reporting Cases of Salmonella Infection. https://stacks.cdc.gov/view/cdc/22424 7. CDC. BEAM (Bacteria, Enterics, Ameba, and Mycotics) Dashboard / NORS, 2000–2024, as summarized in the ISSC Aquatic Bird Risk Assessment Committee draft final report. 8. Hellein, K. N., et al. 2011. Culture-based indicators of fecal contamination and molecular microbial indicators rarely correlate with Campylobacter spp. in recreational waters. J Water Health. 9:695-707. 9. ISSC Aquatic Bird Risk Assessment Committee. Final Report: Aquatic Bird Risk Assessment Committee Literature Review (draft). 10. Massachusetts Division of Marine Fisheries. August 1, 2025. Oysters closure due to Campylobacter sp. outbreak in Edgartown (V20). https://www.mass.gov/doc/8125-oysters-closure-due-to-campylobacter-sp-outbreak-in-edgartown-v20/download 11. NSSP Guide for the Control of Molluscan Shellfish, 2023 Revision. Section II, Chapter II @.01–@.02; Section IV, Chapter VI. 12. Pascoe, B., et al. 2024. Machine learning to attribute the source of Campylobacter infections in the United States: A retrospective analysis of national surveillance data. J Infect. 89:1-10.

	13. Richards, G. P., R. Noble, and Smith. In prep. Campylobacter purge rates in Eastern oysters (Appendix 1, ISSC Aquatic Bird Risk Assessment Committee draft report). 14. Smith, O. M., W. E. Snyder, and J. P. Owen. 2020. Are we overestimating risk of enteric pathogen spillover from wild birds to humans? Biol Rev. 95:652-679.
13. Cost Information	No significant new costs are expected. WGS of clinical isolates is already performed routinely through PulseNet. Investigation requirements are similar to those already carried out under Chapter II @.01. Shellstock testing for reopening is optional and at the Authority's discretion. The proposal is expected to reduce economic losses to growers, especially small aquaculture operations, by avoiding closures and recalls that do not reduce risk, while keeping protective actions for clusters and outbreaks.
14. Changes Needed to Guidance (if proposed change is adopted)	No changes to the Guidance Documents (Section IV) are required for adoption. If adopted, the Executive Office is requested to make conforming cross-reference updates to Section IV guidance that refers to Chapter II @.01 outbreak response and to Chapter VI aquaculture bird and mammal requirements, so that it also references new Chapter II @.08.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Chris Schillaci
3. Affiliation	East Coast Shellfish Growers Association (ECSGA)
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5. Address Line 2	
6. City, State, Zip	New Bedford, MA 02740
7. Phone	(401) 783-3360
8. Email	chris@ecsga.org
9. Proposal Subject	Annual Assessment of Shellfish-Related Campylobacter and Salmonella Illnesses
10. Specific NSSP Guide Reference	NSSP Guide for the Control of Molluscan Shellfish, 2023 Revision. Section II. Model Ordinance, Chapter II. Risk Assessment and Risk Management, @.03 Annual Assessment of Vibrio vulnificus and Vibrio parahaemolyticus Illnesses and Shellfish Production, A. and B.; new paragraph C.
11. Text of Proposal/ Requested Action	This proposal amends Chapter II @.03 so that the Authority's annual assessment also covers shellfish-associated Campylobacter spp. and non-typhoidal Salmonella enterica illnesses. It adds the case details needed to evaluate reporting lag, sporadic versus outbreak-associated cases, contributing conditions, and risk per serving. This proposal can stand alone or support the companion Campylobacter proposal, which sets a response framework for that pathogen. For Salmonella, it builds the case data needed before a pathogen-specific response framework is proposed. New language is shown in red double underline. Deleted language is shown in blue strikethrough.

Amend Section II. Model Ordinance, Chapter II. @.03 as follows:

@.03 Annual Assessment of Vibrio vulnificus ~~and~~, Vibrio parahaemolyticus, Campylobacter spp., and Non-typhoidal Salmonella enterica Illnesses and Shellfish Production

A. The Authority shall assess annually Vibrio vulnificus (V.v.) ~~and~~, Vibrio parahaemolyticus (V.p.), Campylobacter spp., and non-typhoidal Salmonella enterica illnesses associated with the consumption of molluscan shellfish. The assessment will include a record of all V.v. ~~and~~, V.p., Campylobacter, and Salmonella shellfish-associated illnesses reported within the State and from receiving States, the numbers of illnesses per event, and actions taken by the Authority in response to the illnesses.

(1) For Campylobacter and Salmonella illnesses, the record shall also include, where available:

(a) The harvest date(s) and implicated growing area or aquaculture site;

(b) The dates of illness onset and of notification to the Authority;

(c) Whether the cases were sporadic or associated with a cluster or outbreak, and any species, serotype, or whole genome sequencing results;

(d) Any contributing condition identified, including bird and/or mammal congregations on or near aquaculture gear; and

	(e) The risk per serving, when calculated. B. The Authority shall collect by month and report annually to the ISSC the volume of shellfish harvested in the State. The report shall include the volume of shellfish harvested for each species. Where available the volume breakdown of the production data will be reported by utilization type (raw, shucked, PHP, etc.). C. The ISSC will compile the Campylobacter and Salmonella information reported under A. and make it available to the appropriate ISSC committee(s) for trend evaluation and for evaluating risk-based management thresholds for these pathogens.
12. Public Health Significance	Shellfish-associated Campylobacter and Salmonella illnesses are rare but have become more visible with the growth of floating and off-bottom aquaculture and documented bird-associated outbreaks (e.g., Rhode Island 2021; Massachusetts 2025). The ISSC Aquatic Bird Risk Assessment Committee found that limited outbreak data and an incomplete picture of underreporting and misattribution are critical knowledge gaps. It recommended developing a risk assessment framework for C. jejuni, C. coli, C. lari, and Salmonella enterica. Without consistent, production-normalized illness data, the Conference cannot evaluate risk per serving, reporting lag, or how well mitigation such as bird deterrents works. The annual Vibrio assessment under @.03 already provides this kind of data for V.v. and V.p. Extending it to Campylobacter and Salmonella builds the dataset needed to set and review science-based thresholds. For Salmonella, shellfish-associated outbreaks are fewer, sources are more varied (including human sewage, livestock, and wildlife), and depuration and survival data are more variable than for Campylobacter. Consistent annual case data are therefore a necessary first step before the Conference evaluates a Salmonella-specific response framework.
13. Cost Information	Minimal. Authorities already record shellfish-associated illnesses and report production volumes under @.03. The added fields are collected during illness investigations and add only modest recordkeeping.
14. Changes Needed to Guidance (if proposed change is adopted)	No changes to the Guidance Documents (Section IV) are required. If the ISSC maintains a reporting template for the annual @.03 assessment, it should be updated to add the Campylobacter and Salmonella fields listed in proposed @.03 A. (1).

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☑ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Jenifer Yeadon and Alissa Dragan
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6. City, State, Zip	Milford, CT, 06460
7. Phone	203-874-0696
8. Email	Jenifer.Yeadon@ct.gov
9. Proposal Subject	Request to add the use of MSC testing for illness investigations when the illness is known to be transmitted person to person.
10. Specific NSSP Guide Reference	Section II, Chapter II, @.01 A (2), B, G (1) (a), and G (2)
11. Text of Proposal/ Requested Action	@.01 A (2) Whether the disease has the potential or is known to be transmitted by shellfish and/or person to person; and @.01 B (3) If the disease is known to be transmitted person to person, the source state may conduct an investigation to determine if the growing area is implicated. The investigation shall include a review as outlined in Chapter II @01. G. and MSC sample data. In the event the producing state's review determines the source of the contamination is not from the growing area, the supporting data used to make the determination shall be forwarded to the Regional FDA Specialist. In coordination with the FDA Specialist, the Authority may reopen the growing area and no recall is required. G (1) (a) i. (a) If the disease is known to be transmitted person to person, the Authority may include MSC analytical sample results from shellfish in the implicated growing area in accordance with the MSC levels established in Chapter IV @.02E (4) or pre-determined MSC levels in shellfish established by the Authority based on studies conducted on regional species under regional conditions not impacted by sewage closures; iv. Examination of water quality during and subsequent to the illness outbreak; v. If the disease is known to be transmitted person to person, the Authority may review the extent of distribution of the implicated product: total harvest amount of the suspected product, total amount in distribution chain, and time frame from harvest to notification. This information will help inform the Authority if the outbreak is isolated to one distribution area, indicating post harvest contamination, or widespread indicating the illness is growing area related. G (2) Keep the area closed until at least 21 days have passed from the last date of harvest of the implicated shellstock if the illness is consistent with viral etiology unless the Authority can collect MSC samples in accordance with Chapter IV @03. A. (5) (d) (iv) a-c; and

12. Public Health Significance	MSC testing is permitted for reopening following a raw sewage discharge prior to 21 days. An Authority implementing MSC background monitoring and sampling of shellstock produces sufficient data to demonstrate that the area is not impacted by viral contaminants. In instances where the producing state can provide sufficient data and make a determination in coordination with their FDA Specialist that a shellfish growing area was unlikely to be the source of the outbreak, the investigation should be directed to other potential sources like person to person transmission.
13. Cost Information	The use of MSC sampling is not a requirement; therefore, if a state does not have the laboratory capability, they do not need to use this strategy.
14. Changes Needed to Guidance (if proposed change is adopted)	Relevant Chapter II guidance.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
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5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Conformance status and scope of technical laboratory evaluations
10. Specific NSSP Guide Reference	Section II Model Ordinance, Chapter 1 Shellfish Sanitation Program Requirements for the Authority, @.03 Evaluation of Shellfish Sanitation Program Elements, B. 1. b.
11. Text of Proposal/ Requested Action	Chapter I @.03 B. Criteria for evaluation of shellfish sanitation program elements shall be as follows: 1. Laboratory a. Requirements for evaluation of shellfish laboratories shall include… b. Laboratory status is determined by the number and types of nonconformities found in the evaluation using NSSP standardized criteria contained in the ~~FDA~~ NSSP Shellfish Laboratory Evaluation Checklists found in Section IV Guidance Documents Chapter II. Growing Areas .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists. i. Quality System Evaluation... ii. Technical Evaluation: Shellfish Laboratory will be technically evaluated and will be assigned the designation of conforms, provisionally conforms or non-conformance for the specific NSSP Approved methods included in the evaluation scope. The criteria used in determining the evaluation designations are included in the NSSP Shellfish Laboratory Evaluation Checklist designated for the specific type of laboratory evaluation being performed.
12. Public Health Significance	The proposed modifications are to clarify that Laboratory Evaluation Checklists are NSSP documents, not FDA documents, and to clarify that NSSP laboratory conformance status is assigned based on the method(s) being evaluated.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
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6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Clarification of proficiency test participation in NSSP laboratories
10. Specific NSSP Guide Reference	Section II Model Ordinance, Chapter III Laboratory, @.01 Quality Assurance, B. (1) (f)
11. Text of Proposal/ Requested Action	Chapter III @.01 A… B. State Program Responsibilities. The Authority shall ensure that all samples are collected, maintained, transported, and analyzed in a manner that assures the validity of the analytical results. Accordingly, the Authority shall: (1) Require laboratories to develop a written quality assurance plan that: (a) Describes the organization and management structure of the laboratory; (b) Describes the laboratory staff training program ensuring that all laboratory personnel are qualified, properly trained, and supervised; (c) Describes all procedures and methods used to analyze samples; (d) Describes quality control measures, their frequency and tolerance limits, for determining equipment performance; (e) Requires maintenance of records of analytical performance, quality control results, and equipment maintenance and calibration; (f) Includes an internal assessment and participation in a recognized annual proficiency test program, as applicable. For microbiology, potential providers include (FDA FoodSHIELD, and Northeast Laboratory Examination Officers and Managers (NELEOM), etc.); and (g) Requires corrective action for any deficiencies found in the laboratory quality assurance program.
12. Public Health Significance	Proficiency test programs are not available for all NSSP Approved methods. The proposed wording provides clarification that participation in annual proficiency test programs is only required when such programs are available.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
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6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	NSSP Sample Collection and Transportation
10. Specific NSSP Guide Reference	Chapter III. @.01 B. (1), Chapter IV. @.02 A., Chapter IV. @.04 A. (2), Chapter IV. @.04 B. (2)
11. Text of Proposal/ Requested Action	Chapter III. @.01 B. State Program Responsibilities. The Authority shall ~~ensure that all samples are collected, maintained, transported, and analyzed in a manner that assures the validity of the analytical results. Accordingly, the Authority shall~~: (1) Ensure all wastewater, seawater and shellfish samples are collected, maintained, transported, and analyzed in accordance with NSSP Laboratory Evaluation Checklist requirements, as applicable. (2~~1~~) Require laboratories to develop a written quality assurance plan that… Chapter IV. @.02 A. General. Either the total coliform or fecal coliform standard shall be applied to a growing area. The Authority may utilize MSC data in conjunction with bacteriological data to evaluate WWSD impacts on shellfish growing areas. The Authority shall ensure requirements of Chapter III @.01. B. (1) are met. Chapter IV. @.04 A. *Contingency Plan*…. (1)… (2) The plan shall…accomplish the following: (a) Ensure the requirements of Chapter III @.01. B. (1) are met; (b~~a~~) Initiate an emergency shellfish sampling program… Chapter IV. @.04 B. *Management Plan* (1)… (2) The plan shall define the administrative procedures and resources necessary to accomplish the following: (a) Ensure the requirements of Chapter III @.01. B. (1) are met;

	(b~~a~~) Maintain….
12. Public Health Significance	The responsibility for ensuring sample integrity and data defensibility is shared among NSSP laboratories and program staff. NSSP Laboratory Evaluation Checklists contain Sample Collection and Transportation requirements that are evaluated by Laboratory Evaluation Officers. The program staff members responsible for sample collection and transportation are sometimes not aware of the requirements. Adding clarifying language to the MO for the Authority the requirements being in the NSSP Guide and not only having them in the NSSP Laboratory Evaluation Checklists will minimize sample rejection at the laboratory, which will maximize collector and laboratory efficiency.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
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9. Proposal Subject	CAMP Reopening Criteria
10. Specific NSSP Guide Reference	Chapter IV. @.03 C. (2) (c)
11. Text of Proposal/ Requested Action	Chapter IV. @.03 C. (2) (c) For management plans based on WWSD function or pollution sources other than WWSD criteria that reliably predict when an area that was placed in the closed status because of failure to comply with its conditional management plan can be returned to the open status. The minimum reopening criteria for conditional management plans are: (i) Performance standards of the plan are fully met; (ii) Sufficient time has elapsed to allow the water quality in the growing area to return to acceptable levels; (iii) Sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. (iv) Shellstock feeding activity is sufficient to achieve pathogen reduction. (v) If (i-iv) are met and if the conditional management plan closure performance standard(s) is(are) based on effects of non-point sources of pollution such as ~~rain events and/or~~ storm water runoff due to rain events, an area may be reopened when the water quality meets classification criteria without a shellstock cleansing study. (vi) If (i-iv) are met and if the ~~For~~ conditional~~ly~~ management~~d~~ plan closure performance standard(s) is(are) ~~areas~~ based on WWSD function~~performance standards~~, the Authority may utilize MSC levels in shellstock to establish that sufficient time has elapsed to allow ~~water quality and~~ shellstock to return to acceptable levels in growing areas adjacent to WWSD. a. Analytical shellstock tissue sample results shall not exceed the MSC levels established in Chapter IV@.02 E.(4); or b. Pre-determined MSC shellstock tissue levels established by the Authority based on studies conducted on regional species under regional conditions. These studies may establish criteria for reopening based on viral levels in the shellfish meats; or c. The area must be in the closed status until the event is over, and ~~twenty one (~~21~~)~~ days have passed.
12. Public Health Significance	Conditional management plans (CAMPs) based on wastewater system

	discharge (WWSD) performance standards present a distinct public health risk profile compared to those based on non-point source pollution. Shellfish harvested from growing areas adjacent to WWSD outfalls may accumulate human enteric pathogens, including norovirus and hepatitis A virus, at levels that cannot be adequately characterized by fecal coliform (FC) water quality indicators alone. Requirements (i) through (iv), must be met for reopening a closed area based on both, (v) non-point pollution source performance standards and (vi) WWSD performance standards. The proposed changes address any confusion, including language requiring that items (i) through (iv) also be met if reopening a closed area based on WWSD performance standards. These revisions make both reopening scenarios consistent and will reduce any confusion, thereby, protecting public health and maintaining consumer confidence in the safety of commercially harvested shellfish.
13. Cost Information	No cost.
14. Changes Needed to Guidance (if proposed change is adopted)	TBD

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Model Ordinance Effectiveness Review Committee
3. Affiliation	ISSC
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6. City, State, Zip	Richmond,VA
7. Phone	804-330-6380
8. Email	issc@issc.org
9. Proposal Subject	Model Ordinance Effectiveness
10. Specific NSSP Guide Reference	Chapter IV. Shellstock Growing Areas
11. Text of Proposal/ Requested Action	**@.01 Sanitary Survey** A. **General.** (1) **The sanitary survey is the written evaluation report of all environmental factors, including actual and potential pollution sources, which have a bearing on water quality in a shellfish growing area. The sanitary survey shall include the data and results of:** (a) **A shoreline survey;** (b) **A survey of the microbiological quality of the water. In growing areas adjacent to waste water system discharge (WWSD)s the Authority may utilize male specific coliphage (MSC) results from analysis of shellfish meat samples and the analysis of the data will be included in the sanitary survey report;** (c) **An evaluation of the effect of any meteorological, hydrographic~~dynamic~~, and geographic characteristics on the growing area; and** (d) **A determination of the appropriate growing area classification.** (2) **The sanitary survey shall be periodically updated through the triennial reevaluation and the annual review in accordance with Section C. to assure that data are current and that conditions are unchanged.** (3) **The documentation supporting each sanitary survey shall be maintained by the Authority. For each growing area, the ~~central~~Authority's file system shall include all data, results, and analyses from:** (a) **The sanitary survey reviewed and signed by the Sanitary Survey Officer;** (b) **The triennial reevaluation; and** (c) **The annual review.**

(4) **The Authority shall maintain a current ~~comprehensive, itemized~~ list of all growing areas, including maps showing the boundaries and classification of each shellstock growing area.**

B. **Sanitary Survey Required.**

(1) **A sanitary survey shall not be required to classify growing areas as prohibited. The findings of a sanitary survey, however, may result in a growing area being classified as prohibited.**

(2) **A sanitary survey, including the triennial reevaluation, when available, of each growing area shall be required prior to:**

(a) **The harvest of shellstock for human consumption; and**

(b) **The classification of a growing area as approved, conditionally approved, restricted, or conditionally restricted.**

C. **Sanitary Survey Performance.**

(1) **A sanitary survey of each growing area shall be performed at least once every twelve (12) years and shall include the components in Section A. (1).**

(2) **When a written sanitary survey report is not completed within the twelve (12) year time frame, the area shall be immediately placed in the closed status.**

(3) **The growing area classification and the supporting data from the sanitary survey shall be reviewed at least every three (3) years.**

(a) **This triennial reevaluation shall include:**

(i) **A water quality review in accordance with Section C. (5)(b)**

(ii) **Documentation of any new pollution sources and an evaluation of their effect on the growing area;**

(iii) **Reevaluation of all pollution sources~~, including the sources previously identified in the sanitary survey, as necessary~~ to ~~fully~~ evaluate any changes in the sanitary conditions of the growing area. The reevaluation may or may not include a site visit;**

(iv) **A comprehensive report which analyzes the sanitary survey data and makes a determination that the existing growing area classification is correct or needs to be revised; and**

(v) **If the triennial reevaluation determines that conditions have changed based on the information and data collected during the triennial review and that the growing area classification is incorrect, immediate action shall be initiated to reclassify the area.**

(b) **When a written triennial reevaluation report is not completed, the Authority shall place the growing area in the closed status.**

(4) **The triennial reevaluation may include:**

	(a) **Inspection of WWSD or collection of additional effluent samples to determine their impact on the growing area;** (b) **Hydrodynamic studies;** (c) **Additional field work to determine the actual impact of pollution sources; and** (d) **Collection of additional water samples.** (5) **On an annual basis, the sanitary survey shall be updated to reflect changes in the conditions in the growing area. The annual reevaluation shall include:** (a) **A field observation of the pollution sources which may include:** (i) **A drive-through survey;** (ii) **Observations made during sample collection; and** (iii) **Information from other sources.** (b) **Review, at a minimum, of the past year's microbiological results by adding the year's sample results to the data base collected in accordance with the requirements for the microbiological standards and sample collection required in Section @.02;** (c) **Review of available inspection reports and effluent samples collected from pollution sources;** (d) **Review of available performance standards for various types of discharges that impact the growing area; and** (e) **A brief report which documents the findings of the annual reevaluation.** (f) **The Authority may use MSC meat sampling data and/or MSC waste water sampling data in the annual reevaluation of (5) (b), (c), and (d) above to evaluate the viral contributions of the performance standards of WWSD impacts on shellfish growing areas. If MSC meat and/or water data are being used, the Authority shall conduct annual sample collection and analysis in determining performance standards.** (6) **If the annual reevaluation determines that conditions have changed based on the information and data collected during the annual review and that the growing area classification is incorrect, immediate action shall be initiated to reclassify the area.** D. **Shoreline Survey Requirements.** (1) **In the shoreline survey for each growing area, the Authority shall:** (a) **Identify and evaluate all actual and potential sources of pollution which may affect the growing area;** (b) **Determine the distance from the pollution sources to the growing area and the impact of each source on the growing area;** (c) **Assess the reliability and effectiveness of sewage or other waste treatment systems.;** (d) **Determine if poisonous or deleterious substances adversely affect the growing area;**

(e) **Consider the presence of domestic, wild animal or resident and migrating bird populations for possible adverse effects on growing areas; and**

(f) Conduct an assessment of pollution sources which may include:

(i) A drive-through survey;

(ii) Observations made during sample collection;

(iii) Other in-field assessments; and/or

(iv) Information from other sources.

(2) **The Authority shall assure that the shoreline survey meets the following minimum requirements:**

(a) **The boundaries, based on the area topography, of each shoreline survey area are determined by an investigation which identifies the properties with the potential to impact the shellfish waters;**

(b) **Each shoreline survey area is identified by a unique designation which results in identification of all data associated with each shoreline survey by the unique designation;**

(c) **Each shoreline survey area is investigated and pollution sources evaluated by qualified, trained personnel;**

(d) **Documentation for each pollution source identified by the Authority as affecting a growing area includes:**

(i) **The location of the site on a comprehensive map of the survey area; and**

(ii) **The determination that the pollution source has a direct or indirect impact on shellfish waters: and**

(e) **A written ~~summary~~ report of the survey findings.**

E. **Sanitary surveys for Federal waters will be the responsibility of FDA. Sanitary surveys will be conducted in accordance with Chapter IV @.01, as applicable.**

@.02 Microbiological Standards

Note: The NSSP allows for a growing area to be classified using either a total or fecal coliform standard. The NSSP further allows the application of either standard to different water bodies within the State. The NSSP also allows for two (2) sample collection strategies for the application of the total or fecal coliform standard: adverse pollution condition and systematic random sampling. The 1992 Task Force II recommended that this portion of the Ordinance be codified in two (2) ways: a total coliform strategy and a fecal coliform strategy so that the State may choose sampling plans on a growing area basis. Within each strategy, provisions would appear for use of both systematic and adverse pollution condition sample collection. The Ordinance has been recodified in this manner. For maximum flexibility, an Authority may wish to adopt the use of both standards and both sampling strategies for

each standard. This codification represents the fecal coliform standards. Additionally, the Authority may choose to use MSC sample data in conjunction with total or fecal coliform data to evaluate areas impacted by WWSD.

A. **General. Either the total coliform or fecal coliform standard shall be applied to a growing area. The Authority may utilize MSC data in conjunction with bacteriological data to evaluate WWSD impacts on shellfish growing areas.**

B. **Water Sample Stations. The Authority shall enassure that the number and location of sampling stations is adequate to effectively evaluate all pollution sources.**

C. **Exceptions.**

(1) **Except for growing areas classified as prohibited, in growing areas where there are pollution sources having an impact on the water quality, a minimum of thirty (30) samples, collected under various environmental conditions, shall be required to classify any growing area not previously classified under Section @.03.**

(2) **Except for growing areas classified as prohibited or when the systematic random sampling standard is applied, in growing areas where there are no pollution sources having an impact on the water quality, a minimum of fifteen (15) samples shall be required to classify any growing area not previously classified under Section @.03.**

(3) **Except for offshore state waters greater than three (3) nautical miles from shore where a sanitary survey shows that there are no pollution sources that will impact the microbiological quality of the water. Offshore state waters greater than three (3) nautical miles from shore may be classified as approved.**

D. **Standard for the Approved Classification of Growing Areas in the Remote Status.**

(1) **Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard below.**

(2) **Fecal Coliform Standard for the Remote Status. The fecal coliform median or geometric mean most probable number (MPN) or membrane filter (MF) (membrane-Thermotolerant *Escherichia coli* [mTEC]) of the water sample results shall not exceed fourteen (14) per 100 ml, and not more than ten (10) percent of the samples shall exceed an MPN or MF (mTEC) of:**

(a) **43 MPN per 100 ml for a five-tube decimal dilution test;**

(b) **49 MPN per 100 ml for a three-tube decimal dilution test;**

(c) **28 MPN per 100 ml for a twelve-tube single dilution test; or**

(d) **31 colony-forming units (CFU) per 100 ml for a MF (mTEC) test.**

(3) **Required Sample Collection.**

(a) **A minimum of two (2) samples shall be collected annually.**

(b) **A minimum of the most recent fifteen (15) samples**

	collected shall be used to calculate the median or geometric mean and percentage to determine compliance with the standard established for the approved classification of remote growing areas. E. **Standard for the Approved Classification of Growing Areas when Evaluated for Adverse Pollution Conditions.** (1) **Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section E. (2).** (2) **Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed fourteen (14) per 100 ml, and not more than ten (10) percent of the samples shall exceed an MPN or MF (mTEC) of:** (a) **43 MPN per 100 ml for a five-tube decimal dilution test;** (b) **49 MPN per 100 ml for a three-tube decimal dilution test;** (c) **28 MPN per 100 ml for a twelve-tube single dilution test; or** (d) **31 CFU per 100 ml for a MF (mTEC) test.** (3) **Required Sample Collection.** (a) **A minimum of five (5) samples shall be collected annually under adverse pollution conditions from each sample station in the growing area.** (b) **A minimum of the most recent fifteen (15) samples collected under adverse pollution conditions from each sample station shall be used to calculate the median or geometric mean and percentage to determine compliance with this standard.** (c) **Sample station locations shall be adjacent to actual or potential sources of pollution.** (4) **For Authorities utilizing MSC data in conjunction with bacteriological data to evaluate WWSD impacts, the MSC level shall not exceed fifty (50) MSC per hundred (100) grams.** F. **Standard for the Approved Classification of Growing Areas when Evaluated for Nonpoint Sources.** (1) **Exception. If the tidal stage increases the fecal coliform concentration, the authority shall use sample results collected during that tidal stage to classify the area.** (2) **Pollution Sources. Growing areas shall be impacted only by randomly occurring, intermittent events.** (3) **Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section E. (2) or Section F. (4).** (4) **Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median (or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed fourteen (14) per 100 ml and the estimated 90th percentile shall not exceed an MPN or MF (mTEC) of:** (a) **43 MPN per 100 ml for a five-tube decimal dilution test;**

	(b) **49 MPN per 100 ml for a three-tube decimal dilution test; or** (c) **31 CFU per 100 ml for a MF (mTEC) test.** (5) **Estimated 90th Percentile. The estimated 90th percentile shall be calculated by:** (a) **Calculating the arithmetic mean and standard deviation of the sample result logarithms (base 10);** (b) **Multiplying the standard deviation in (a) by 1.28;** (c) **Adding the product from (b) to the arithmetic mean;** (d) **Taking the antilog (base 10) of the results in (c) to get the estimated 90th percentile; and** (e) **The MPN values that signify the upper or lower range of sensitivity of the MPN tests in the 90th percentile calculation shall be increased or decreased by one significant number.** (6) **Required Sample Collection.** (a) **Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity and frequency as described in Section E. (3) for application of the standard under Section E. (2).** (b) **Systematic Random Sampling Standard. The requirement for systematic random sample collection shall be met when:** (i) **Sample station locations are adequate to produce the data to effectively evaluate all nonpoint sources of pollution;** (ii) **Sample collection is scheduled sufficiently far in advance to support random collection with respect to environmental conditions. Compliance requires that, prior to implementation, the schedule for random sampling shall be documented in the master file for the growing area, and if conditions at the time of scheduled sample collection are believed to be hazardous to the safety of the individuals assigned to collect samples, sample collection shall be rescheduled at a later date as soon as practical;** (iii) **A minimum of six (6) random samples shall be collected annually from each sample station in the growing area;** (iv) **A minimum of two (2) random samples shall be collected annually from each sample station in the growing area while in the inactive status. The sample collection frequency of six (6) random samples per station per year specified under @.02 F. (6)** (b) **(iii) must resume at least six (6) months before an area is reactivated; and** (v) **A minimum of the thirty (30) most recent randomly collected samples from each sample station shall be used to calculate the median or geometric mean and 90th percentile to determine compliance with this standard.** (c) **Transition from Adverse Pollution Condition Standard**

	to Systematic Random Sampling Standard. If the Authority: (i) **Does not have thirty (30) recent randomly collected sample results from each station, then the previous fifteen (15) samples collected under adverse pollution conditions may be used with the most recent random samples to meet the minimum thirty (30) sample requirement for a transition period not to exceed three (3) years; and** (ii) **Uses the transition period described in (i), as additional random samples are collected; the random samples shall replace chronologically the samples collected under adverse pollution conditions (e.g. sample 31 replaces sample 1).** G. **Standard for the Restricted Classification of Growing Areas when Evaluated for Adverse Pollution Conditions and Used as a Shellstock Source for Shellstock Depuration.** (1) **Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section G. (2).** (2) **Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and not more than ten (10) percent of the samples shall exceed an MPN or MF (mTEC) of:** (a) **260 MPN per 100 ml for a five-tube decimal dilution test;** (b) **300 MPN per 100 ml for a three-tube decimal dilution test;** (c) **173 MPN per 100 ml for a twelve-tube single dilution test; or** (d) **163 CFU per 100 ml for a MF (mTEC) test.** (3) **Required Sample Collection. Samples shall be collected in accordance with Section E. (3).** H. **Standard for the Restricted Classification of Growing Areas when Evaluated for Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration.** (1) **Exception. If the tidal stage increases the fecal coliform concentration, the Authority shall use samples collected under that tidal stage to classify the area.** (2) **Pollution Sources. Growing areas shall meet the requirements in Section F. (2).** (3) **Water Quality. The bacteriological quality of every sample station in the growing area shall meet the fecal coliform standard in Section G. (2) or Section H. (4).** (4) **Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed a MPN or MF (mTEC) of:** (a) **260 MPN per 100 ml for a five-tube decimal dilution test;** (b) **300 MPN per 100 ml for a three-tube decimal dilution test; or** (c) **163 CFU per 100 ml for a MF (mTEC) test.**

(5) **Estimated 90th Percentile. The estimated 90th percentile shall be calculated by the same method described in Section F. (5).**

(6) **Required Sample Collection.**

(a) **Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity and frequency as described in Section E. (3) for application of the standard under Section G. (2).**

(b) **Systematic Random Sampling Standard. The Authority shall collect samples in the same intensity and frequency, and shall apply the sample results in the manner described in Section F. (6) for the application of the standard under Section H. (4).**

@.03 Growing Area Classification

A. **General. Each growing area shall be correctly classified as approved, conditionally approved, restricted, conditionally restricted, or prohibited, as provided by this Ordinance.**

(1) **Emergency Conditions. A growing area or a portion of a growing area (harvest area) shall be placed in the closed status under Section @.03 A. (5) when pollution conditions exist which were not included in the data used to classify the area. If it is determined that an emergency condition or situation exists, then the growing area or harvest area will be immediately (within twenty-four (24) hours) placed in the closed status.**

(2) **Classification of Growing Areas. Each growing area which:**

(a) **Is not subjected to a sanitary survey every twelve (12) years shall be classified as prohibited or, if unclassified, shall be treated as prohibited for NSSP purposes; or;**

(b) **Is subjected to a sanitary survey shall be correctly classified based on the twelve (12) year sanitary survey, and its most recent triennial or annual reevaluation when available, as one (1) or more of the following:**

(i) **Approved;**

(ii) **Conditionally Approved;**

(iii) **Restricted;**

(iv) **Conditionally Restricted; and/or**

(v) **Prohibited.**

(3) **Boundaries. The boundaries of each classified growing area shall be delineated on charts which are:**

(a) **Of sufficient scale and detail so as to adequately describe the boundaries; and**

(b) **Maintained in the central file by the Authority.**

(4) **Revision of Classifications.**

(a) **Any upward revision of a growing area classification shall be supported by an adequate sanitary survey.**

(b) **The appropriate FDA office shall be notified of any revision in growing area classification.**

	(5) **Status of Growing Areas. The status of a growing area is separate and distinct from its classification and may be open, closed, or inactive for the harvesting of shellstock. Supporting information for all changes in the status of growing areas shall be documented by a written record in the central file.** (a) **Open Status. Except for an area in the prohibited classification, any correctly classified growing area is normally open for the purposes of harvesting shellstock, subject to the limitations of its classification.** (b) **Closed Status. Any classified growing area or harvest area may be closed because of:** (i) **An emergency condition or situation;** (ii) **The presence of biotoxins in concentrations of public health significance;** (iii) **Conditions stipulated in the management plan of conditionally approved or conditionally restricted areas;** (iv) **Failure of the Authority to complete a written sanitary survey or triennial review evaluation report; or** (v) **The requirements for biotoxins or conditional area management plans as established in Section @.04 and Section @.03, respectively, are met.** (c) **Controlled Access Status. This status can be applied to allow harvesting in areas with biotoxin concerns where routine monitoring or pre-harvest testing is not practical.** (d) **Reopened Status. A growing area or harvest area temporarily placed in the closed status as provided in (b) above, shall be returned to the open status only when:** (i) **The emergency situation or condition has returned to normal and sufficient time has elapsed to allow the shellstock to reduce pathogens or poisonous or deleterious substances that may be present in the shellstock to acceptable levels.** (ii)**When pathogens are of concern and the area is not impacted by human sewage, studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. Such coliform studies may establish criteria for reopening based on coliform levels in the water.** (iii) **When poisonous or deleterious substances are the concern, sampling shall establish that poisonous or deleterious substances in shellstock do not exceed FDA action levels, tolerances and/or guidance levels and/or levels that are deemed safe through risk evaluation; or** (iv)**For emergency closures of harvest areas caused by the occurrence of raw untreated sewage or partially treated sewage discharged from a sewage collection system or WWSD:**

	a. The MSC analytical sample results in shellfish shall not exceed MSC levels established in Chapter IV @.02 E (4) or **b. Pre-determined MSC levels in shellfish established by the Authority based on studies conducted on regional species under regional conditions from shellfish samples collected no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area potentially impacted or** **c. Until the event is over and twenty-one (21) days have passed; or** (v) **The requirements for biotoxins or conditional area management plans as established in Section @.04 and Section @.03, respectively, are met; and** (vi) **Supporting information is documented by a written record in the central file.** (e) **Inactive Status. The authority may place an approved or restricted growing area affected by non-point sources in the inactive status for up to five (5) years when shellstock harvest is suspended or no longer occurring. Shellstock harvesting shall be closed while an area is in the inactive status. The inactive status must continue for a minimum of one (1) year.** (i) **While in inactive status, the required bacteriological sample collection under @.02 F. (6) (b) (iii) may be reduced to two (2) water samples per station per year collected under the systematic random sample collection strategy. Sanitary survey reports, triennial reevaluations, and annual updates must be completed as required under @.01 C.** (ii) **The sample collection frequency of six (6) random samples per station per year specified under @.02 F. (6) (b) (iii) must resume at least six (6) months before an area is reactivated.** (iii) **Before an area is reactivated, the results of the most recent thirty (30) samples must be reviewed and comply with the requirements under @.02 F.** (f) **Remote Status. A growing area may be placed in the remote status if:** (i) **A sanitary survey determines that the area has no human habitation, and is not impacted by any actual or potential pollution sources; and** (ii) **The area is in the approved classification.** (g) **Seasonally Remote/Approved Status. A growing area may be placed in a seasonally remote/approved status requiring two (2) water samples per year if the following criteria are met:** (i) **The area is initially classified as approved;**

(ii) **The closure time period is defined; and**
(iii) **At least one (1) sample be taken upon reopening the area.**

B. **Approved Classification. Growing areas shall be classified as approved when the following criteria are met.**
(1) **Survey Required. A sanitary survey finds that the area is:**
(a) **Safe for the direct marketing of shellfish;**
(b) **Not subject to contamination from human or animal fecal matter at levels that, in the judgment of the Authority, presents an actual or potential public health hazard; and**
(c) **Not contaminated with:**
(i) **Pathogenic organisms;**
(ii) **Poisonous or deleterious substances;**
(iii) **Marine biotoxins; or**
(iv) **Bacteria concentrations exceeding the bacteriological standards for a growing area in this classification.**
(2) **Water Quality. The water quality in the growing area shall meet the bacteriological standards for an approved classification in Section @.02.**

C. **Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:**
(1) **Survey Required. The sanitary survey meets the following criteria:**
(a) **The factors determining the period the growing area is in open status are known and predictable and are not so complex as to preclude a reasonable management approach as determined by the Authority;**
(b) **Each potential source of pollution that may adversely affect the growing area is evaluated;**
(c) **When conditional management is based at least in part on predicted changes in microbiological water quality, microbiological water quality correlates with environmental conditions or other factors affecting the distribution of pollutants into the growing area; and**
(d) **For Authorities utilizing MSC meat sample data, when conditional management is based at least in part on predicted changes in MSC levels, those data correlate with environmental conditions or other factors affecting the distribution and persistence of viral contaminants into the growing area.**
(2) **Management Plan Required. For each growing area, a written management plan shall be developed and shall include:**
(a) **For management plans based on wastewater treatment plant function, performance standards that include:**
(i) **Peak effluent flow, average flow, and infiltration flow;**
(ii) **Microbiological quality of the effluent;**
(iii) **Physical and chemical quality of the effluent;**
(iv) **Conditions which cause plant failure;**
(v) **Plant or collection system bypasses;**
(vi) **Design, construction, and maintenance to**

	minimize mechanical failure, or overloading; (vii) **Provisions for monitoring and inspecting the waste water treatment plant; and** (viii) **Establishment of an area in the prohibited classification adjacent to a wastewater treatment plant outfall in accordance with Section E. Prohibited Classification;** (b) **For management plans based on pollution sources other than waste water treatment plants:** (i) **Performance standards that reliably predict when criteria for conditional classification are met; and** (ii) **Discussion and data supporting the performance standards.** (c) **For management plans based on WWSD function or pollution sources other than WWSD criteria that reliably predict when an area that was placed in the closed status because of failure to comply with its conditional management plan can be returned to the open status. The minimum reopening criteria for conditional management plans are:** (i) **Performance standards of the plan are fully met;** (ii) **Sufficient time has elapsed to allow the water quality in the growing area to return to acceptable levels;** (iii) **Sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels.** (iv) **Shellstock feeding activity is sufficient to achieve pathogen reduction.** (v) **If (i-iv) are met and if the conditional management plan closure performance standard(s) is(are) based on effects of non-point sources of pollution such as rain events and/or storm water runoff, an area may be reopened when the water quality meets classification criteria without a shellstock cleansing study.** (vi) **For conditionally managed areas based on WWSD performance standards, the Authority may utilize MSC levels in shellstock to establish that sufficient time has elapsed to allow water quality and shellstock to return to acceptable levels in growing areas adjacent to WWSD.** **a. Analytical shellstock tissue sample results shall not exceed the MSC levels established in Chapter IV@.02 E.(4); or** **b. Pre-determined MSC shellstock tissue levels established by the Authority based on studies conducted on regional species under regional conditions. These studies may establish criteria for reopening based on viral levels in the shellfish meats; or** **c. The area must be in the closed status until the**

event is over and twenty-one (21) days have passed.

(d) **For management plans based on a risk assessment made in accordance with Chapter II. Risk Assessment and Risk Management, criteria that reliably determine when the growing area may be placed in the open status and shellfish may be harvested;**

(e) **For management systems based on marine biotoxins, the procedures and criteria that reliably determine when the growing area may be placed in the open status;**

(f) **Procedures for immediate notification to the Authority when performance standards or criteria are not met;**

(g) **Provisions for patrol to prevent illegal harvest; and**

(h) **Procedures to immediately place the growing area in the closed status in twenty-four (24) hours or less when the criteria established in the management plan are not met.**

(3) **Reevaluation of Conditional Classification.**

(a) **The classification shall be reevaluated at least once each year. The reevaluation shall include:**

(i) **Evaluation of compliance with the management plan;**

(ii) **Determination of adequacy of reporting of failure to meet performance standards;**

(iii) **Review of the cooperation of the persons involved;**

(iv) **Evaluation of water quality in the growing area with respect to the bacteriological standards for its classification;**

(v) **Field inspection of critical pollution sources, where necessary; and**

(vi) **Written findings, evaluations and recommendations.**

(b) **Water Sample Collection.**

(i) **When the conditional management plan is based on the absence of pollution from marinas for certain times of the year, monthly water samples are not required when the growing area is in the open status of its conditional classification provided that at least three (3) of the water samples collected to satisfy the bacteriological standard for the open status are collected when the growing area is in the open status.**

(ii) **When the conditional management plan is based on the operation and performance of a WWSD (s); combined sewer overflow(s); or other point sources of pollution, monthly water samples are required when the growing area is in the open status of its conditional classification except when:**

(a) Hydrographic or dilution analysis has been completed to determine the impact of a WWSD performance failure and communication requirements are documented and the WWSD operator provides immediate notification to the Authority during a performance failure; or

(b) Mooring assessment determines the mooring area is not a pollution source.

(iii) **If a monthly sample cannot be collected due to environmental constraints, the monthly sampling requirement will be satisfied if an additional water sampling run is conducted the following month.**

(iv) **When the conditional management plan is based on the effects of non-point sources of pollution, such as rainfall events, storm water runoff, and seasonal variations, a minimum of five (5) sets of water samples (when the Adverse Pollution Condition sampling regimen is used) or six (6) sets of water samples (when the Systematic Random Sampling regimen is used) are required. The samples shall be collected when the growing area is in the open status.**

(v) **When the conditional management plan is based on the effects of non-point sources of pollution, such as rainfall events or storm water runoff, and the area is in the open status for less than six (6) months a minimum of five (5) sets of water samples are required (Adverse Pollution Condition and Systematic Random Sampling). At least one (1) sample shall be collected each month the area is placed in the open status. This sample shall be collected while the area is open. If closed status samples are used to meet the minimum sample requirements only two (2) sets of samples may be utilized and they must have been taken within five (5) days of when the Authority anticipates that the area will be placed in the open status. For growing areas in the open status less than two (2) months, at least one (1) sample must be collected while the area is in the open status. Samples collected during the closed status to meet the minimum five (5) sets of water samples shall be applied to annual and triennial reevaluations of the area.**

(vi) **When the conditional management plan is based on the seasonal opening and closing of the area, and the area is in the open status for a predetermined period of less than six (6) months, a minimum of five (5) sets of water samples are required (Adverse Pollution Condition and Systematic Random Sampling). All samples shall be collected while the area is in the open status unless the Authority has historical water quality data to demonstrate that the area meets open status criteria while in the closed status. If closed status samples are used to meet the minimum sample requirements they must be collected within thirty (30) days prior to the area being placed in the open status.**

(4) **Understanding of and Agreement With the Purpose of the Conditional Classification and Conditions of Its Management**

Plan by All Parties Involved.

(a) **The management plan shall be developed by the Authority in coordination with:**

(i) **The local shellfish industry;**

(ii) **The individuals responsible for the operation of any WWSDs involved; and**

(iii) **Any local or State agencies; and**

(b) **Failure of any one party to agree shall constitute sufficient justification to deny the application of the conditional classification to a growing area.**

(5) **Conditional Area Types. There are two (2) types of conditional areas:**

(a) **Conditionally approved; and**

(b) **Conditionally restricted.**

(6) **Conditionally Approved Classification. Any growing area in the conditionally approved classification shall:**

(a) **Meet the requirements for:**

(i) **An approved area classification when the conditionally approved classification is in the open status; and**

(ii) **A restricted or prohibited classification when the conditionally approved classification is in the closed status; and**

(b) **If the closed status meets the criteria for the restricted classification, designate in its management plan whether the shellstock may be harvested for relaying or depuration.**

(7) **Conditionally Restricted Classification. Any growing area in the conditionally restricted classification shall:**

(a) **Meet the requirements for:**

(i) **A restricted classification when the conditionally restricted classification is in the open status; and**

(ii) **A prohibited classification when the conditionally restricted classification is in the closed status; and**

(b) **Designate in its management plan whether the harvested shellstock are to be relayed or depurated.**

D. **Restricted Classification.**

(1) **General**

(a) **A growing area may be classified as restricted when:**

(i) **A sanitary survey indicates a limited degree of pollution; and**

(ii) **Levels of fecal pollution, human pathogens, or poisonous or deleterious substances are at such levels that shellstock can be made safe for human consumption by either relaying, depuration or low acid-canned food processing.**

(b) **The Authority shall have effective controls to assure that shellfish are harvested from restricted areas only:**

(i) **By special license; and**

(ii) **Under the supervision of the Authority.**

(2) **Water Quality. Water quality in the growing area shall meet**

	the bacteriological standards in Section @.02 for a growing area in the restricted classification if the growing area is used for depuration. (3) **Shellstock Quality Criteria. The Authority shall establish shellstock quality criteria for use in placing an area in the restricted classification. Depending on the treatment process to be applied to the shellstock, the criteria shall be established in accordance with:** (a) **Chapter V. Shellstock Relaying; or** (b) **Chapter XV. Depuration.** E. **Prohibited Classification.** (1) **Exception. The prohibited classification is not required for harvest waters within or adjacent to marinas. The Authority, however, may use the prohibited classification for these waters.** (2) **General. The Authority shall:** (a) **Not permit the harvest of shellstock from any area classified as prohibited, except for the gathering of seed or nursery culture for aquaculture or resource enhancement or the depletion of the areas classified as prohibited; and** (b) **Ensure that shellstock removed from any growing area classified as prohibited is effectively excluded from human consumption unless it complies with the criteria in NSSP Model Ordinance Chapter VI. Shellfish Aquaculture @.02 Seed Shellstock.** (3) **Sanitary Survey. A growing area shall be classified as prohibited if:** (a) **No current sanitary survey exists;** (b) **A sanitary survey determines:** (i) **The growing area is adjacent to a sewage treatment plant outfall or other point source outfall with public health significance;** (ii) **Pollution sources may unpredictably contaminate the growing area;** (iii) **The growing area is contaminated with fecal waste so that the shellfish may be vectors for disease microorganisms;** (iv) **The concentration of biotoxin is sufficient to cause a public health risk as identified in Section @ .04. or** (v) **The area is contaminated with poisonous or deleterious substances causing the shellfish to be adulterated.** (4) **Risk Assessment. A growing area shall be classified as prohibited if a risk assessment performed in accordance with Chapter II. Risk Assessment and Risk Management indicates the shellstock are not safe for human consumption.** (5) **Wastewater Discharges.** (a) **An area classified as prohibited shall be established adjacent to each sewage treatment plant outfall or any other point source outfall of public health significance.**

(b) **The determination of the size of the area to be classified as prohibited adjacent to each outfall shall include the following minimum criteria:**

(i) **The volume flow rate, location of discharge, performance of the wastewater treatment plant and the microbiological quality of the effluent. The Authority may utilize MSC waste water sample data in the determination of the performance of the sewage treatment plant;**

(ii) **The decay rate of the contaminants of public health significance in the wastewater discharged;**

(iii) **The wastewater's dispersion and dilution, and the time of waste transport to the area where shellstock may be harvested; and**

(iv) **The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries.**

F. **FDA is responsible for the classification of growing areas in Federal waters. Federal waters are classified as Approved for shellfish harvesting unless such areas are known to be polluted (i.e., microbiological, chemical, or marine biotoxin hazards) and involve commercial shellfish resources. Should FDA allow harvesting in Federal waters with known marine biotoxin hazards, the FDA will classify the harvest area in a manner equivalent to the requirements of Model Ordinance Chapter IV.**

@.04 Marine Biotoxin Control

A. **Contingency Plan.**

(1) **The Authority shall develop and adopt a marine biotoxin contingency plan for all marine and estuarine shellfish growing areas addressing the management of PSP, ASP, NSP, diarrhetic shellfish poisoning (DSP) and azaspiracid shellfish poisoning (AZP) in the event of the emergence of a toxin-producing phytoplankton that has not historically occurred or an illness outbreak caused by marine biotoxins.**

(2) **The plan shall define the administrative procedures and resources necessary to accomplish the following:**

(a) **Initiate an emergency shellfish sampling program;**

(b) **Close growing areas and embargo shellfish;**

(c) **Prevent harvesting of contaminated species;**

(d) **Provide for product recall;**

(e) **Disseminate information on the occurrences of toxic algal blooms and/or toxicity in shellfish meats to adjacent States and federal partners, shellfish industry, and local health agencies;**

(f) **Coordinate control actions taken by Authorities and Federal agencies; and**

(g) **Establish reopening criteria including the number of**

samples over what period of time.

B. **Marine Biotoxin Management Plan.**
In those areas that have been implicated in an illness outbreak or where toxin-producing phytoplankton have been documented to occur, the toxins are prone to accumulate in shellfish and during times when marine biotoxins are likely to occur, representative samples of water and/or shellfish shall be collected during harvest periods in accordance with one (1) or a combination of the marine biotoxin management strategies listed below in (4). and in accordance with Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans.

(1) The Authority shall develop and adopt a marine biotoxin management plan for all marine and estuarine shellfish growing areas if there is a history of biotoxin closures related to PSP, ASP, NSP, DSP and/or AZP; if toxin-producing phytoplankton have been documented to occur in the growing area; or a reasonable likelihood that biotoxin closures could occur.
(2) The plan shall define the administrative procedures and resources necessary to accomplish the following:
(a) Maintain a toxin-producing phytoplankton and/or shellfish sampling program as described below in (4). It is necessary to recognize that different marine biotoxin management strategies are essential to address specific risks as well as geographic and logistical conditions. Marine biotoxin management strategies must include an appropriate number of samples to adequately address the specific risks. Specific criteria are cited in Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans.
(b) Close growing areas and embargo shellfish;
(c) Prevent harvesting of contaminated species;
(d) Provide for product recall;
(e) Disseminate information on the occurrences of toxic algal blooms and/or toxicity in shellfish meats to adjacent States, shellfish industry, and local health agencies;
(f) Coordinate control actions taken by Authorities and Federal agencies;
(g) Establish reopening criteria; and
(h) Ensure that all shellfish harvested from growing areas or portion(s) of growing areas placed in the controlled access status meets all conditions of

	harvest restrictions prior to being placed in distribution. This would include all sampling, testing or product holds. **(3) The Authority may use precautionary closures based on shellfish toxicity screening or phytoplankton sample results as defined in their marine biotoxin management plan. Precautionary closures may be lifted immediately:** **(a) if confirmatory testing using an approved method shows the level of biotoxin present in shellfish meats is not equal to or above established criteria as described below in C; or** **(b) when shellfish toxicity screening or phytoplankton sample results indicate that the precautionary closure was not necessary.** **(4) Marine biotoxin management strategies are as follows:** (a) Phytoplankton monitoring: this strategy involves a routine program for sampling growing area waters for the presence of phytoplankton species known or suspected to produce marine biotoxins. This is a complementary management strategy that enhances predictive capabilities of anticipating toxicity in shellfish and must be used in combination with other management strategies. Specific criteria are cited in Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans. (b) Routine shellfish toxicity monitoring: this strategy involves a routine program for sampling and testing shellfish meats for the presence of marine biotoxins. Unless species specific shellfish testing is conducted, the highest risk species shall be used. This strategy may be used in combination with other management strategies. Specific criteria are cited in Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans. (c) Pre-harvest shellfish toxicity testing: this strategy involves sampling and testing shellfish meats for the presence of marine biotoxins in the intended harvest area specifically in advance of harvest. This strategy, if used independent of any other strategy, shall permit harvest for a short period of time following testing. This strategy may be used in combination with other management strategies.

Specific criteria are cited in Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans.

(d) Shellfish lot testing: this strategy involves sampling and testing shellfish meats for the presence of marine biotoxins on a lot basis after harvest. This strategy may be combined with a pre-harvest shellfish toxicity testing strategy, the results of which permit harvest. Specific criteria are cited in Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans. Lot testing may also be used on a case by case basis to clear product harvested immediately prior to a biotoxin closure if the Authority determines it is necessary.

(e) Pre-harvest shellfish toxicity screening and lot testing: this strategy requires pre-harvest shellfish toxicity screening of the intended harvest area coupled with shellfish lot testing upon landing. Specific criteria are cited in Section IV. Guidance Documents Chapter IV. Growing Areas @.04 Marine Biotoxin Control .01 Guidance for Developing Marine Biotoxin Plans.

(5) The marine biotoxin management plan shall include agreements or memoranda of understanding, between the Authority and individual shellfish harvesters, individual growers or individual shellfish dealers, to allow harvesting in a growing area that is placed in the controlled access status. Such harvesting shall be conducted with strict assurances of safety and in accordance with the marine biotoxin management strategies listed in (4).

C. **Closed or Controlled Access Status of Growing Areas.**

(1) **A growing area, or portion(s) thereof as provided in Section A.(4), shall be placed in the closed status for the taking of shellstock when the Authority determines that the number of toxin-forming organisms in the growing waters and/or the level of biotoxin present in shellfish meats is sufficient to cause a health risk. The closed status shall be established based on the following criteria:**

(a) **PSP - 80 µg saxitoxin equivalents/100 grams**
(b) **NSP - 20 MU/100 grams (0.8 mg brevetoxin-2 equivalents/kg)**
(c) **AZP - 0.16 mg azaspiracid-1 (AZA-1) equivalents/kg (0.16 ppm)**
(d) **DSP – 0.16 mg okadaic acid (OA) equivalents/kg (0.16 ppm)**
(e) **ASP - 2 mg domoic acid/100 grams (20 ppm)**

	(2) **For any marine biotoxin for which criteria have not been established under this Ordinance, either cell counts of the toxin producing organism in the water column or biotoxin meat concentrations may be used by the Authority as the criteria for not allowing the harvest of shellstock.** (3) **When sufficient data exist to establish that certain shellfish species can be safely exempted , the closed status for harvesting may be applied selectively to some shellfish species and not others.** (4) **The closed status shall remain in effect until the Authority has data to show that the toxin content of the shellfish in the growing area is below the level established for closing the area.** (5) **The determination to return a growing area to the open status shall consider whether toxin levels in the shellfish from adjacent areas are declining.** (6) **The analysis upon which a decision to return a growing area to the open status is based shall be adequately documented.** (7) **A growing area, or portion(s) thereof, shall be placed in the controlled access status for the taking of shellstock when the Authority determines that additional requirements are necessary to ensure the safe harvest of product. Controlled access status is a designation of an approved area or a conditionally approved area in open status. Additional requirements shall be included in harvest permit conditions. All shellstock harvested from growing areas in the controlled access status shall be tagged with Restricted Shellstock tags.** D. **Heat Processing. If heat processing is practiced, a control procedure shall be developed. This procedure shall define the following:** (1) **Toxicity limits for processing;** (2) **Controls for harvesting and transporting the shellstock to processor;** (3) **Special marking for unprocessed shellstock;** (4) **Scheduled processes; and** (5) **End product controls on the processed shellfish.** E. **Records. The Authority shall maintain a copy of all of the following records.** (1) **All information, including monitoring data, relating to the levels of marine biotoxins in the shellfish growing areas;** (2) **Copies of notices placing growing areas in the closed status;** (3) **Evaluation reports; and** (4) **Copies of notices returning growing areas to the open status.** **@.05 Marinas** A. **Marina Proper. The area within any marina which is in or adjacent to a shellstock growing area shall be classified as conditionally approved, conditionally restricted or prohibited.** (1) **Prior to the Authority establishing a classification of conditionally**

approved or conditionally restricted in the marina proper, a pollution assessment supporting the classification will be conducted by the authority.

(2) **The assignment of a prohibited classification within the marina proper does not require a pollution assessment by the Authority.**

B. **Adjacent Waters. Waters adjacent to marina waters classified under Section A. may be impacted by pollution associated with the marina.**

(1) **A dilution analysis shall be used to determine if there is any impact to adjacent waters.**

(2) **The dilution analysis shall be based on the volume of water in the vicinity of the marina.**

(3) **The dilution analysis shall incorporate the following:**

(a) **A slip occupancy rate for the marina;**

(b) **An actual or assumed rate of boats which will discharge untreated waste;**

(c) **An occupancy per boat rate (i.e., number of persons per boat);**

(d) **A fecal coliform discharge rate of 2 x 10^9 fecal coliforms per day; and**

(e) **The assumption that the wastes are completely mixed in the volume of water in and around the marina.**

(f) **Documentation, verification and enforcement of Federal No Discharge Zones and locally well enforced no discharge and occupancy by-laws and regulations.**

(g) **Availability and documented use of pump out boats or facilities.**

(4) **If the dilution analysis predicts a theoretical fecal coliform loading greater than fourteen (14) fecal coliform MPN per 100 ml, the waters adjacent to the marina shall be classified as:**

(a) **Conditionally approved;**

(b) **Restricted;**

(c) **Conditionally restricted; or**

(d) **Prohibited.**

(5) **If the dilution analyses predicts a theoretical fecal coliform loading less than or equal to fourteen (14) fecal coliform MPN per 100 ml, the waters adjacent to the marina may be classified as:**

(a) **Approved; or**

(b) **Conditionally approved.**

(6) **If the Authority chooses not to determine a specific occupancy per boat rate by investigation in specific areas or sites, the Authority shall assume a minimum occupancy rate of two (2) persons per boat.**

@.06 Mooring Areas

A. Mooring Area. The area within any designated mooring area, where there is anchoring of boats, which is in or adjacent to a shellstock growing area shall be classified as conditionally approved, conditionally restricted, restricted or prohibited.

(1) Prior to the Authority establishing a classification of conditionally approved or conditionally restricted or restricted in the mooring area proper, a pollution assessment supporting the classification will be conducted by the authority. The assessment shall include:

	(a) Boat type and usage (b) Density of boats (c) Accessibility to boats which could reduce likelihood of overnight occupancy. (d) Occupancy rates (e) Seasonal Use Pattern (f) An actual or assumed rate of boats which will discharge untreated waste (g) Documentation, verification and enforcement of federal No Discharge Zones, and locally well enforced no discharge and occupancy regulations or by-laws. (h) Availability and documented use of pump out boats. (2) After assessment determines that the mooring area is not a pollution source and it is documented in the Conditional Area Management Plan, the area can be placed in the open status. **(3) The assignment of a prohibited classification within the mooring area proper does not require a pollution assessment by the Authority.** B. Adjacent Waters. Waters adjacent to open water mooring areas classified under Section A. may be impacted by pollution associated with the mooring areas. If determined a pollution source: (1) A dilution analysis shall be used to determine if there is any impact to adjacent waters. (2) The dilution analysis shall be based on the volume of water in the vicinity of the mooring areas. (3) The dilution analysis shall incorporate the following: (a) An occupancy rate for the mooring areas; (b) An actual or assumed rate of boats which will discharge untreated waste; (c) An occupancy per boat rate (i.e., number of persons per boat); (d) A fecal coliform discharge rate of 2×10^9 fecal coliform per day; and (e) The assumption that the wastes are completely mixed in the volume of water in and around the open water mooring areas. (4) If the dilution analysis predicts a theoretical fecal coliform loading greater than fourteen (14) fecal coliform MPN per 100 ml, the waters adjacent to the mooring areas shall be classified as: (a) Conditionally approved; (b) Restricted; (c) Conditionally restricted; or (d) Prohibited. (5) If the dilution analyses predict a theoretical fecal coliform loading less than or equal to fourteen (14) fecal coliform MPN per 100 ml, the waters adjacent to the mooring areas may be classified as: (a) Approved; or (b) Conditionally approved.
12. Public Health Significance	N/A

13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	U.S. Food & Drug Administration (FDA)
3. Affiliation	U.S. Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Documentation requirements for no-discharge zones
10. Specific NSSP Guide Reference	NSSP MO, Section II, Chapter IV. @.05 B. (3) (f) and @.06 A. (1) (g)
11. Text of Proposal/ Requested Action	@.05 B. (3) (f) If the No Discharge Zone designation is utilized in the dilution analysis, provide ~~D~~documentation~~,~~ of ~~verification and~~vessel compliance or non-compliance with and enforcement of ~~Federal~~ statutory No Discharge Zones ~~and~~ including locally well enforced no discharge and occupancy by-laws and regulations. @.06 A. (1) (g) If the No Discharge Zone designation is utilized in the pollution assessment, provide ~~D~~documentation~~,~~ of ~~verification and~~ vessel compliance or non-compliance with and enforcement of ~~Federal~~statutory No Discharge Zones ~~and~~ including locally well enforced no discharge and occupancy by-laws and regulations.
12. Public Health Significance	This is a suggested change to Model Ordinance language to clarify the intent of the requirement to document the NDZ federal regulation (33 CFR 159.7(b)-(c) (PDF)) is being enforced in marinas and mooring areas. The documentation of enforcement is required where the NDZ is being used to justify reducing the impact of waters adjacent to marinas and areas classified as conditionally approved in the open status for mooring areas. The intent of this requirement is to protect public health by ensuring that the Authority provide documentation that the NDZ vessel requirements in marinas and mooring areas are being effectively enforced. NDZ federal regulation (33 CFR 159.7(b)-(c) (PDF)) requirements: Type I or II marine sanitation device (MSD): • Closing the seacock and removing the handle; • Padlocking the seacock in the closed position; • Using a non-releasable wire-tie to hold the seacock in the closed position; or • Locking the door to the space enclosing the toilets with a padlock or door handle key lock. Type III MSDs: • Closing valves leading to overboard discharge and removing the handle; • Padlocking any valves leading to overboard discharge in the closed position; or • Holding overboard discharge valves closed using a non-releasable wire-tie.

	Additionally, the Environmental Protection Agency (EPA) administers the Clean Water Act's (CWA) No Discharge Zone (NDZ) program; however, most existing NDZs were approved by EPA but designated by the state under CWA Section 312(f)(3). Only 4 NDZs are EPA/federal NDZs: the FL Keys National Marine Sanctuary, the Boundary Waters Canoe Area, California Marine Water, and part of the Hudson River in NY. No discharge zones in state waters are not Federal No Discharge Zones. Upon approval of the Environmental Protection Agency (EPA), no discharge zones become state no discharge zones and are subject to state enforcement capabilities. States are responsible for enforcing the statutory requirement and can delegate the responsibility to local entities. Reference: The Clean Water Act: Section 312 (f) (3). https://www.epa.gov/vessels-marinas-and-ports/vessel-sewage-no-discharge-zones
13. Cost Information	No cost.
14. Changes Needed to Guidance (if proposed change is adopted)	TBD

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Matt Richards, Scott Berbells – Washington State Department of Health, David Borkman – Rhode Island Department of Environmental Management, Bryant Lewis – Maine Department of Marine Resources
3. Affiliation	New York State Department of Environmental Conservation
4. Address Line 1	123 Kings Park Blvd
5. Address Line 2	Nissequogue River State Park
6. City, State, Zip	Kings Park, NY 11715
7. Phone	631-444-0491
8. Email	matt.richards@dec.ny.gov
9. Proposal Subject	No Discharge Zone (NDZ) enforcement and verification
10. Specific NSSP Guide Reference	Section II, Chapter IV, @.05 B (3) (f) and @.06 A (1) (g)
11. Text of Proposal/ Requested Action	(f) ~~Documentation, verification and enforcement~~ Documentation of ~~Federal~~ No Discharge Zones and ~~locally well enforced~~local no discharge and occupancy ~~by-laws and regulations.~~regulations or by-laws (g) ~~Documentation, verification and enforcement~~Documentation of ~~federal~~ No Discharge ~~Zones, and locally well enforced~~Zones and local no discharge and occupancy regulations or by-laws.
12. Public Health Significance	No change will occur to public health protection. Established No Discharge Zones (NDZ) and local regulations/by-laws (where used) already prohibit discharges from vessels. Verification of state and local NDZ enforcement efforts by the shellfish authority does not increase the amount of compliance with federal/state/local laws and regulations and will not decrease the amount of potential illicit discharges from vessels. The pollution assessment, dilution analysis, and sizing of necessary closures around marinas and mooring areas when boats are present already account for illicit discharges from vessels. No metrics to measure compliance with this portion of the requirements exist within the NSSP-MO. NDZ enforcement and verification creates unreasonable burdens on the shellfish authority to gather large amounts of information on a regular basis from every marina and mooring facility in the conditionally approved classification. Gathering this information also places additional burdens on state and local authorities by straining resources and relationships, encouraging violation of search and seizure laws, requiring large amounts of time, effort, and personnel by law enforcement agencies, and requiring the cooperation of vessel owners and facility owners/managers to conduct inspections.
13. Cost Information	No cost is associated with this proposal.
14. Changes Needed to Guidance (if proposed change is adopted)	The Marina and Mooring Committee is currently working on proposal 23-121 and proposal 23-124, proposed guidance for marinas and moorings. These proposed changes would need to be incorporated into the work product from that proposal.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Christian Petitpas[1], Matt Camisa[1], Wayne Castonguay[2]
3. Affiliation	Massachusetts Division of Marine Fisheries
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5. Address Line 2	
6. City, State, Zip	New Bedford, MA 02744[1]; Gloucester, MA 01930[2]
7. Phone	(508) 742-9766
8. Email	Christian.Petitpas@mass.gov
9. Proposal Subject	Clarification of re-opening criteria following emergency closure due to discharge of untreated or partially treated sewage
10. Specific NSSP Guide Reference	Section II, Chapter IV, @.03 A. (5) (d) (iv)
11. Text of Proposal/ Requested Action	(iv)For emergency closures of harvest areas caused by the occurrence of raw untreated sewage or partially treated sewage discharged from a sewage collection system or WWSD: a. Shellfish samples collected no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area potentially impacted may be tested for MSC. The MSC analytical sample results in shellfish shall not exceed ~~1.~~ MSC levels established in Chapter IV @.02 E (4) or 1. 2. Pre-determined MSC levels in shellfish established by the Authority based on studies conducted on regional species under regional conditions; ~~from shellfish samples collected no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area potentially impacted~~ or ~~a.~~b. Until the event is over and twenty-one (21) days have passed; or
12. Public Health Significance	The proposed change clarifies that a growing area impacted by raw, untreated or partially treated sewage must be closed a minimum of 7 days prior to MSC testing whether the MSC standard of fifty (50) MSC per hundred (100) grams is used to re-open or a return to pre-determined baseline MSC levels in shellfish. As written in the 2023 MO, it can be interpreted that the 7-day closure only applies if the Authority is using pre-determined baseline MSC levels in shellfish as the standard for re-opening, and that if the Authority is using the MSC standard of 50 PFU/100 g shellfish for re-opening then shellfish could be tested on day 2 or 3 following an untreated sewage discharge and the growing area returned to the open status sooner than 7 days if results do not exceed 50 PFU/100 g. It is believed that the intent of the conference was for the 7-day closure to apply regardless of the MSC level used as the re-opening standard. This is supported by the following language in Guidance (p.312): "The regulatory level of 50 PFU/100 g is a conservative value used for re-opening approved growing areas (after 7 days) after a sewage spill…" AND in Section VIII FDA Manual of Interpretations (p.486): "under emergency conditions…such as a raw sewage discharge from an extreme event, and the SSCA closes the growing area as a result, reopening samples (such

	as assessing the levels of Male Specific Coliphage (MSC) in shellfish meats no sooner than seven (7) days after the event)". The proposed change moves the language "shellfish samples collected no sooner than seven (7) days after contamination has ceased and from representative locations in each growing area potentially impacted" before the two options for MSC levels in shellfish and both options for MSC levels become sub bullets under bullet a. The default 21-day closure option becomes bullet b.
13. Cost Information	No additional costs are associated with the proposed changes.
14. Changes Needed to Guidance (if proposed change is adopted)	No changes to Guidance would be required.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	WWTP minimum dilution zone
10. Specific NSSP Guide Reference	Section II Model Ordinance Chapter IV @.03 E.
11. Text of Proposal/ Requested Action	Chapter IV @.03 E. Prohibited Classification. (1) Exception. The prohibited classification is not required for harvest waters within or adjacent to marinas. The Authority, however, may use the prohibited classification for these waters. (2) General. The Authority shall: (a) Not permit the harvest of shellstock from any area classified as prohibited, except for the gathering of seed or nursery culture for aquaculture or resource enhancement or the depletion of the areas classified as prohibited; and (b) Ensure that shellstock removed from any growing area classified as prohibited is effectively excluded from human consumption unless it complies with the criteria in NSSP Model Ordinance Chapter VI. Shellfish Aquaculture @.02 Seed Shellstock. (3) Sanitary Survey. A growing area shall be classified as prohibited if: (a) No current sanitary survey exists; (b) A sanitary survey determines: (i) The growing area is adjacent to a sewage treatment plant outfall or other point source outfall with public health significance; (ii) Pollution sources may unpredictably contaminate the growing area; (iii) The growing area is contaminated with fecal waste so that the shellfish may be vectors for disease microorganisms; (iv) The concentration of biotoxin is sufficient to cause a public health risk as identified in Section @ .04. or (v) The area is contaminated with poisonous or deleterious substances causing the shellfish to be adulterated. (4) Risk Assessment. A growing area shall be classified as prohibited if a risk assessment performed in accordance with Chapter II. Risk Assessment and Risk Management indicates the shellstock are not safe for human consumption. (5) Wastewater Discharges.

(a) An area classified as prohibited shall be established adjacent to each sewage treatment plant outfall or any other point source outfall of public health significance.
(b) The determination of the size of the area to be classified as prohibited adjacent to each outfall shall include the following minimum criteria:

(i) The volume flow rate~~,~~ location of discharge~~, performance of the wastewater treatment plant and the microbiological quality of the effluent. The Authority may utilize MSC waste water sample data in the determination of the performance of the sewage treatment plant~~;

(ii) The decay rate of the contaminants of public health significance in the wastewater discharged;

(iii) The wastewater's ~~dispersion and~~ dilution, ~~and the time of waste transport to the area where shellstock may be harvested; and~~ performance of the wastewater treatment plant, and the microbiological quality of the effluent prior to disinfection:

a. If wastewater samples are not collected from the WWTP to evaluate the MSC levels and microbiological quality of the effluent to establish the size of a prohibited area around a WWTP outfall, the following minimum dilutions for the boundary line between prohibited and other possible classifications, shall be based on an assumed worst-case fecal coliform value of 1.4×10^6 FC/100 ml:
   (1) Prohibited to Approved: Minimum 100,000:1 dilution that would be equivalent to an MPN of 14 FC/100 ml.
   (2) Prohibited to Conditionally Approved: Minimum 1,000:1 dilution.
   (3) Prohibited to Restricted or Conditionally Restricted (For depuration only**):** Minimum 16,000:1 dilution that would be equivalent to an MPN of 88 FC/100 ml.
   (4) Prohibited to Restricted or Conditionally Restricted (For relay only): Minimum 400:1 dilution. Based on WWTP performance standards.
b. If MSC samples collected from the WWTP to evaluate the microbiological quality of effluent are used to establish the size of a prohibited area around a WWTP outfall, the following minimum requirements shall be met:

	(1) MSC samples are collected from the influent and pre- and post-disinfection effluent from each WWTP to be evaluated: (i) MSC samples are collected to establish effluent quality and effectiveness of disinfection under normal operating conditions for the WWTP; and (ii) MSC samples are collected to establish effluent quality and effectiveness of disinfection under adverse conditions for the WWTP; and (2) The sample collection is conducted to account for environmental and high use period factors such as seasonality, rainfall, and high flows; and (3) The effluent quality and environmental data shall be used to establish the size of the prohibited area. For a prohibited area adjacent to an approved area, worst-case pre-disinfection levels and time of travel must be considered. (4) If (1) through (3) have been completed and the effluent quality and environmental data justifies a reduced prohibited area, the minimum size of the prohibited area shall be based on a minimum dilution of 400:1 using a hydrographic dye study or hydrodynamic dispersion modeling to account for the risk of enteric viruses. Additional sampling shall be required to account for the risk for poisonous or deleterious substances of public health concern present in the wastewater effluent. (iv) The wastewater's dispersion. (v) The wastewater effluent time of travel to the area where shellstock may be harvested; and (~~i~~vi) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries.
12. Public Health	In sizing prohibited areas around a WWTP outfall, broad minimum criteria have been established in the Model Ordinance. Guidance is available that provides

Significance	additional information to assist in meeting the Model Ordinance requirements if microbiological quality samples are not collected. Authorities also have the option to collect microbiological samples to assess the WWTP effluent and justify an alternate prohibited area sizing strategy. Some advanced wastewater treatment plants have become very efficient at treating for and removing fecal coliforms from wastewater effluent. Depending on the disinfection process, fecal coliform may not be the most appropriate indicator of viruses and poisonous or deleterious contaminants of concern as you get closer to the WWTP effluent, creating a false sense of human health protection. This proposal is meant to establish more defined guidelines as well as, a minimum dilution zone requirement around all wastewater treatment plant outfall pipes to ensure protection from viral and other contaminants (poisonous or deleterious substances) that fecal coliforms may not represent. In addition, Section II. Chapter XV. .03 K. already sets precedence for a minimum prohibited area for depuration: "K. Supplemental Requirements for Depuration using MSC Viral Controls for Shellstock Harvested from Conditionally Restricted Growing Areas Impacted by Wastewater System Discharge (WWSD) outside of a 300:1 dilution or the EPA Toxic Dilution Zone (whichever is greater) and within an area determined to be impacted by wastewater treatment system discharge. These requirements would allow harvesting in areas that would otherwise be classified as prohibited due to viral pollution concerns. The harvest area that could be considered would include the area between 300:1 dilution or the EPA Toxic Dilution Zone (whichever is greater) and the established boundary for depuration (Chapter IV @.02 G.)."
13. Cost Information	No cost.
14. Changes Needed to Guidance (if proposed change is adopted)	TBD

pProposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Model Ordinance Effectiveness Review Committee
3. Affiliation	ISSC
4. Address Line 1	4801 Hermitage Rd
5. Address Line 2	
6. City, State, Zip	Richmond,VA
7. Phone	804-330-6380
8. Email	issc@issc.org
9. Proposal Subject	Model Ordinance Effectiveness
10. Specific NSSP Guide Reference	Chapter VI. Shellfish Aquaculture
11. Text of Proposal/ Requested Action	**@ .01 General** A. Aquaculture activities which may pose a ~~significant~~ public health concern and are regulated in this Chapter include, but are not limited to: (1) Seed production in waters classified as Prohibited or Unclassified; (2) Aquaculture structures that attract birds or mammals; and (3) Land based aquaculture. B. The Authority shall: (1) Approve the written operational plan for operations as outlined in @.01A above; (2) Inspect operations outlined in @.01A above at least annually; and (3) At a minimum inspect operator records to verify that appropriate permits are up to date and operational plans required in @ .01 A (1). are being implemented. (4) Consistent with Chapter IV @ .01 D(1)(e) when aquaculture as defined in the Model Ordinance attracts birds or mammals, their presence should be considered for possible adverse effects on growing area water quality. **@ .02 Seed Shellstock** A. The Authority shall establish the maximum seed size for each species of shellfish that can be produced in prohibited waters. In determining the maximum seed size Authorities shall establish sizes that require a minimum of 120 days of growing to reach market size. B. The Authority shall establish appropriate corrective actions for when seed exceeds the maximum seed size when it has been produced in waters classified as prohibited. C. All sources of seed produced or collected in prohibited waters shall be sanctioned by the Authority.

@.03 Aquaculture in Federal Waters

A. Federal Agency Responsibilities. Once the ~~appropriate~~ permits for the construction of the aquaculture facility have been obtained,
   (1) NOAA is responsible for establishing a contract, in consultation with FDA, with the aquaculture facility describing requirements of the NSSP including:
      (a) the frequency with which NOAA will audit the aquaculture facility and vessels;
      (b) testing requirements of the aquaculture facility; and
      (c) the generation of product identification for traceability (i.e., tag numbers); and
   (2) FDA is responsible for reviewing the aquaculture facility operational plan prior to the start of operations, as well as the annual inspection of records, to ensure adherence to NSSP requirements. FDA is also responsible for the classification of the growing area(s) associated with the aquaculture facility.

Requirements for the Harvester/Dealer

.01 Exceptions

Hatcheries and nurseries rearing larvae and/or seed that are located in:
   A. Approved or conditionally approved growing areas are exempt from these requirements; or
   B. Restricted or Conditionally Restricted (in the open status) would be exempt from these requirements but subject to relay requirements in Ch V. or depuration requirements in Chapter XV for seed that exceeds the maximum seed size established by the Authority.

.02 General

   A. Any person who performs aquaculture as defined in the Model Ordinance or operates an aquaculture facility to raise shellfish for human consumption shall obtain:
      (1) A permit from the Authority for the activity and functioning of his facility;
      (2) A harvester's license; and
      (3) Certification as a dealer, where necessary.
   B. Shellfish aquaculture as defined in the Model Ordinance shall be practiced ~~only~~ in ~~strict~~ compliance with the provisions of the permit issued by the Authority for the aquaculture activity. Authorization shall be based on the operator's written operational plan.
   C. Prior to beginning his activity, an operator shall obtain the permission of the Authority for use of his facility.

D. Any shellfish seed raised in aquaculture that exceeds the maximum seed size established by the Authority shall be subjected to relaying or depuration prior to direct marketing if the culture area or facility is located in or using water which is in:
   (1) The closed status of the conditionally approved classification;
   (2) The restricted classification; or
   (3) The open status of the conditionally restricted classification.

E. Only drugs sanctioned by the FDA shall be used for shellfish treatment.

F. Harvesting, processing, storage, and shipping requirements for shellfish raised in a land-based aquaculture facility or a seed rearing facility or system that exceeds the maximum seed size established by the Authority shall be the same as the requirements for shellfish specified in Chapters V., VII., VIII., IX., X., XI., XII., XIII. and XIV.

G. Complete and accurate records shall be maintained for at least two (2) years by the operator of the aquaculture facility and shall include the:
   (1) Source of shellfish, including seed if the seed is from growing areas which are not in the approved or conditionally approved classification; and
   (2) Water source, its treatment method, if necessary, and its quality in land based systems.

.03 Seed Production in Water Classified as Prohibited or Unclassified

Seed may come from any growing area, or from any growing area in any classification, provided that:

A. The source of the seed, if from waters classified as prohibited or unclassified, is sanctioned by the Authority; and

B. Operational Plan. Each aquaculture site that cultures seed in waters classified as prohibited or unclassified shall have a written operational plan. The plan shall be approved by the Authority prior to its implementation and shall include:
   (1) A description of the design and activities of the culture facility;
   (2) The specific site and boundaries in which shellfish aquaculture activities will be conducted;
   (3) The types and locations of any structures, including rafts, pens, cages, nets, or floats which will be placed in the waters;
   (4) The species of shellfish to be cultured and harvested;
   (5) Procedures to assure that no poisonous or deleterious substances are introduced from the seed production activities; and
   (6) Corrective actions for addressing seed exceeding the maximum seed size as defined by the Authority.

.04 Aquaculture That Attracts Birds or Mammals

A. Operational Plan. Each aquaculture site that the Authority determines may attract sufficient birds and/or mammalswhose ~~that their~~ waste presents a human health risk shall have a written operational plan. The plan shall be approved by the Authority prior to its implementation and shall include:

(1) A description of the design and activities of the culture facility;
(2) The specific site and boundaries in which shellfish aquaculture activities will be conducted;
(3) The types and locations of any structures, including rafts, pens, cages, nets, or floats which will be placed in the waters;
(4) The species of shellfish to be cultured and harvested;
(5) Procedures to assure that no poisonous or deleterious substances are introduced from the aquaculture activities;
(6) A description of the mitigation or deterrent measures to minimize the potential pollution impact of birds and/or mammals; and
(7) Maintenance of the required records.

.05 Land Based Aquaculture

A. Operational Plan. Each facility shall have a written operational plan. The facility must obtain approval from the Authority prior to its implementation and shall include:
(1) A description of the design and activities of the culture facility;
(2) The specific site and boundaries in which shellfish culture activities will be conducted;
(3) The types and locations of any structures, including rafts, pens, cages, nets, tanks, ponds, or floats which will be placed in the waters;
(4) The species of shellfish to be cultured and harvested;
~~(5) Procedures to assure that no poisonous or deleterious substances are introduced into the activities;~~
~~(6)~~(5) A program of sanitation, maintenance, and supervision to prevent contamination or adulteration of the shellfish products;
~~(7)~~(6) A description of the water source, including the details of any water treatment process or method;
~~(8)~~(7) A program to maintain water quality, which includes collection of microbial water samples and their method of analysis and routine temperature and salinity monitoring (the bacterial indicator monitored shall be the same as used for monitoring growing areas);
~~(9)~~(8) If applicable, collection of data concerning the quality of food production (algae or other) used in the artificial harvest system; and
~~(10)~~(9) Maintenance of the required records.
B. Each land-based facility conducting aquaculture as defined by the Model Ordinance shall maintain the following records while the aquaculture activity continues:
(1) Construction and remodeling plans for any permitted aquaculture facility;
(2) Aquaculture operational plans; and
(3) Aquaculture permits.
C. Water Systems.
If the land-based aquaculture system is of continuous flow through design, water from a growing area classified as approved, or in the

open status of the conditionally approved classification at all times shellfish are held, may be used without treatment.

D. Water Quality.

(1) Shellstock cultured in a closed or recirculating system that exceeds the maximum seed size and meets the requirements for water quality and testing in Chapter VII C. .04 (3) (a), (b), (c), and (d) may be used in direct marketing.

(2) Shellstock cultured in a closed or recirculating system that exceeds the maximum seed size and does not meet the requirements for water quality and testing in Chapter VII C. .04 (3) (a), (b), (c), and (d) shall be relayed or depurated consistent with Chapters ~~I~~V and XV prior to direct marketing.

.06 Polyculture Systems

A polyculture system shall:

A. Meet all requirements in Section .05 Land Based Systems;

B. Provide information concerning all sources and species of all organisms to be cultivated, cultured, and harvested; and

C. Include in its operational plan requirements to:

(1) Monitor for human pathogens, unacceptable levels of animal drugs, and other poisonous or deleterious substances that might be associated with polyculture activities; and

(2) Subject all harvested shellstock to relaying or depuration if human pathogens, unacceptable levels of animal drugs, and other poisonous or deleterious substances exist at levels of public health significance.

.07 Requirements for the Harvester in Aquaculture in Federal Waters

A. Prior to beginning any aquaculture activities, the person who performs aquaculture or operates an aquaculture facility to raise shellfish in Federal waters for human consumption shall obtain the required ~~appropriate~~ permission(s) from Federal agencies as described in @.03.

B. Operational Plan. Each aquaculture facility shall have a written operational plan as described for Land Based Aquaculture in Section II Chapter VI .05(A). The operational plan shall also include:

(1) Description of harvest, tagging, handling, storage, transportation, and landing procedures;

(2) Description of a marine biotoxin management and contingency plan (Section II Chapter IV @.04) to include marine biotoxin sampling consistent with Section II Chapter IV @.04(B)(6) and ensure product segregation and control until biotoxin results confirm the shellfish do not contain biotoxins equal to or exceeding criteria established in Section IV Chapter II .08.;

	(3) Description of a contingency in the event of an emergency situation or condition (e.g., sewage or oil spills); and **(4) Procedures for implementing product recalls.** **Each aquaculture facility shall obtain a review from the FDA to ensure adherence to NSSP requirements prior to its implementation. If the aquaculture facility makes changes to the operational plan, they shall obtain a new review from the FDA to ensure adherence to the NSSP requirements.**
12. Public Health Significance	N/A
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☑ Administrative
2. Submitter	Meredith L. Zahara, Leanne J. Flewelling
3. Affiliation	Florida Fish & Wildlife Conservation Commission
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6. City, State, Zip	St. Petersburg, FL 33701
7. Phone	727-896-8626
8. Email	Meredith.Zahara@myfwc.com; Leanne.Flewelling@myfwc.com;
9. Proposal Subject	NSSP PSP Bioassay Checklist: Part II 2.4 Calculation of toxicity for MBA
10. Specific NSSP Guide Reference	NSSP 2023 Revision: Section IV Guidance Documents – Chapter III Laboratory
11. Text of Proposal/ Requested Action	This proposal requests a revision to NSSP PSP Bioassay Checklist linked in the NSSP 2023 Revision: Part II 2.4 Calculation of toxicity for MBA. Current text: *Part II 2.4.5 The toxicity of each sample is calculated as follows:* *µg STX eq/100 g of sample = MCMU x CF x DF x 200 except when less than 100 grams of sample is used for analysis.* *In this case an adjustment for sample weight must be made such that the formula for calculating sample toxicity becomes:* *µg STX eq/100 grams of sample = MCMU x CF x DF x 200/Adjusted weight of the acidified sample x 200.* *Where:* *MCMU=Median Corrected Mouse Unit for the sample* *CF=Laboratory Conversion Factor* *DF=Dilution Factor (if additional dilution is required)* The calculation provided for cases when less than 100 grams of sample is used for analysis is incorrect. The PSP Bioassay Checklist requires a strict 1:1 ratio between sample weight (g) and acid volume (mL) added (2.2.3). The final weight (g) is adjusted as needed due to evaporative losses to the pre-boiling weight (2.2.10). In the primary equation, when 100 g of sample is used for analysis: µg STX eq/100 g of sample = MCMU x CF x DF x 200 • MCMU x CF x DF generates the µg STX eq. in a 1 mL injection (volume injected into each mouse). • The 200 multiplier extrapolates the STX eq in 1 mL (0.5g eq) of extract to 200 g (100g eq). When this proportional scaling is maintained, as required, the final calculation remains unchanged because the total extract weight divided by sample mass **will always reduce to 2.**

	However, to explicitly reflect using <100 g in the calculation, the formula could be corrected to µg STX eq/100 g of sample = MCMU x CF x DF x (Total Extract Weight (g) / Sample Mass (g)) × 100 grams
12. Public Health Significance	The calculation in its current form substantially overestimates the toxin level in samples and can prevent harvest. Although this does not pose a public health risk, it can cause significant negative impacts to the aquaculture industry and economic harm to local communities.
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	Proposed revision to the PSP Bioassay Checklist: *Part II 2.4.5 The toxicity of each sample is calculated as follows:* *µg STX eq/100 g of sample = MCMU x CF x DF x 200* *except when less than 100 grams of sample is used for analysis.* ~~*In this case an adjustment for sample weight must be made such that the formula for calculating sample toxicity becomes:*~~ ~~*µg STX eq/100 grams of sample = MCMU x CF x DF x 200/Adjusted weight of the acidified sample x 200.*~~ *To document the use of less than 100 g, use the following calculation:* *µg STX eq/100 g of sample = MCMU x CF x DF x (Total Extract Weight (g) / Sample Mass (g)) × 100 grams* *Where:* *MCMU=Median Corrected Mouse Unit for the sample* *CF=Laboratory Conversion Factor* *DF=Dilution Factor (e.g. 1:1 dilution, DF=2)*

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative

Submitter	Shelley Lankford,
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Proposal Subject	Matrix Extensions for the Receptor Binding Assay for detecting Paralytic Shellfish Poisoning [SLV (2023;Proposal13-114)]
Specific NSSP Guide Reference	Section IV Guidance Documents – Chapter IV. Growing Areas Paralytic Shellfish Poisoning (PSP) Shellfish Lab Methods page 351
Text of Proposal/ Requested Action	This submission presents the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination for consideration to upgrade its current limited use status to an NSSP Fully Approved Use Method from mussels (*Mytilus species*) to include Pacific geoduck clam (*Panopea generosa*) gutball, whole Pacific oysters (*Crassostrea gigas*), northern razor clam (*Siliqua patula*) meat only, and whole hard clams, comprising three species: butter clams (*Saxidomus gigantea*), Pacific littleneck clams (*Protothaca staminea*), and Japanese littleneck clams (also known as Manila clams; *Venerupis philippinarum).* The mouse bioassay (MBA) remains the most widely accepted method for detecting saxitoxins worldwide and has been demonstrated to adequately protect public health. The receptor binding assay (RBA) is a competitive assay in which radiolabeled saxitoxin competes with unlabeled saxitoxin for a finite number of available receptor sites. The resulting measurement provides an estimate of overall PSP toxicity in a shellfish sample (Van Dolah et al., 2009). The RBA was approved for use with mussels and approved for limited use with clams and scallops in 2014. To support full implementation of the RBA, Washington State conducted matrix extension studies to evaluate and expand the use of the method to additional species of molluscan shellfish. The validation and matrix extension study report is included with this proposal submission. The requested action is to expand the NSSP approved use of the RBA to include mussels, clams, geoduck clams (gutball), razor clam (meat), and oysters as a fully approved use method for PSP toxicity determination. **Paralytic Shellfish Poisoning (PSP) Toxins**

Shellfish Lab Methods	The mouse bioassay is still the most widely accepted detection method for the saxitoxins around the world and has been shown to adequately protect the public's health. In 2009, the Interstate Shellfish Sanitation Conference approved a post-column oxidation HPLC- PCOX method, making it the newest regulatory method available for PSP toxins in the U.S. The receptor binding assay (RBA), a competition assay whereby radiolabeled saxitoxin competes with unlabeled saxitoxin for a finite number of available receptor sites, provides a measure of

		overall PSP toxicity in a sample (Van Dolah et al., 2009). The RBA was approved for mussels in 2014, Pacific geoduck gutball, whole Pacific oysters, northern razor clam (meat only), and whole hard clams, comprising three species: butter clams, Pacific littleneck clams and Japanese littleneck clams in 2026 and approved limited use for ~~clams~~ and scallops in 2014.
Public Health Significance	**Public Health Significance:** Paralytic shellfish poisoning (PSP) is a serious public health concern because exposure to paralytic shellfish toxins can cause severe illness and, in some cases, death. The receptor binding assay (RBA) provides a more sensitive and efficient approach for detecting saxitoxin and related toxins in shellfish. The RBA can detect toxins at approximately five times lower concentrations than the mouse bioassay, providing an earlier warning of toxin accumulation. This increased sensitivity supports more timely public health actions, including increased sampling, harvest closures, and reopening decisions, helping reduce the potential for contaminated shellfish to reach consumers and recreational harvesters. The implementation of the RBA strengthens Washington's ability to protect public health while supporting efficient, sustainable shellfish monitoring.	
Cost Information	The estimated cost per 96-well plate assay is ~$300. Including standards and samples with triplicate measurements (as well as three dilutions per sample [ranging from 3.5-600 µg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$40. If running multiple plates or in screening mode (single dilutions), sample costs could be reduced significantly. A microplate scintillation counter is needed as a one-time purchase, and the cost is ~$70-250K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$13K. In 2025, the Washington State Public Health Laboratories (WA PHL) analyzed approximately 3,000 paralytic shellfish poisoning (PSP) samples. Replacing the more costly Mouse Bioassay ($145 per sample) with the less expensive Receptor Binding Assay (RBA) ($40 per sample) could have resulted in an estimated savings of approximately $315,000 in a single year. Expanding the use of the RBA offers significant potential for long term cost savings while maintaining the capacity needed to support ongoing public health surveillance and shellfish safety testing.	

ISSC Method Application Format for Biotoxin Methods Matrix Extension

The purpose of laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that methods under consideration for adoption by the NSSP are fit for their intended use in the Program. This document provides a detailed outline of the types of information and data the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Committee (LC) requests from submitters for extension of current NSSP methods to cover additional matrixes (i.e., molluscan shellfish species). These recommendations are intended for methods which have already undergone a single laboratory validation (SLV) and are being considered for use with a new matrix. Included are the method performance criteria that should be examined for inclusion in the validation package, along with LC recommendations for each criterion. Data generated for the more robust performance criteria may be used to satisfy multiple criteria, if applicable.

Method Overview
Method Title: Application of Receptor Binding Assay to Multispecies Monitoring for Paralytic Shellfish Poison
Method Submitter(s) and Contact Information: Shelley Lankford, Washington State Department of Health Public Health Laboratories, Biotoxin Laboratory and WA DOH Shellfish Program Tod Leighfield & Kelly Cusick, NOAA Charleston Lab Sitka Tribe of Alaska Environmental Research Laboratory
Intended or Target Use: (approved, approved limited use, or emergency use) The receptor binding assay (RBA) is intended for use as an NSSP Approved Method for the quantitative assessment of paralytic shellfish poison (PSP) in multiple species. The application of this project is specifically intended for use in regulatory laboratory settings. The RBA serves as an alternative to the mouse bioassay (MBA) in these applications, offering a measure of integrated toxicity with high throughput and the elimination of live animal testing.
Rationale for this Method in the NSSP: The overall goal of this project is to fully validate the Receptor Binding Assay for use in testing paralytic shellfish poison in multiple shellfish species. This project will take on the necessary work to make the RBA assay a viable regulatory method for PSP monitoring. It will address NOAA's Preventative, Control, and Mitigations of Harmful Algal Blooms (PCMHAB) program's goal of translating a method into a product that is widely available. By putting the method through conditions that simulate regulatory screening we will attempt to demonstrate that the assay is equivalent to the mouse bioassay. To date, only mussels have been fully approved for RBA testing in the NSSP, yet a number of matrices can be analyzed using limited use criteria. A significant effort is needed to expand the validations for the limited-use approved and other non-validated shellfish species using the RBA method for its utility to be realized by

PSP testing programs.

Method Principle/Basis:(receptor binding assay, immunoassay, LC-MS, etc.)
This PSP receptor binding assay is based on the interaction between the toxins and their native receptor, the voltage-gated sodium channel. All paralytic shellfish toxins (PSTs) bind to site 1 of the voltage-gated sodium channels relative to their affinity to the channel (receptor). These affinities are a primary driver of in vivo toxicity and the read-out of the RBA, results in a measure of integrated potency (independent of knowing which toxin congeners are present) similar to mouse intraperitoneal potency. In the RBA, tritiated saxitoxin (3H-STX) competes with unlabeled PSTs in a shellfish extract for a finite number of available receptor sites in a rat brain membrane preparation. After a binding equilibrium is reached, unbound 3H-STX is removed by filtration and the remaining 3H-STX is measured with a scintillation counter (measured as counts per minute or CPM). The amount of 3H-STX present is indirectly related to the quantity of unlabeled PSTs in the sample. This output is compared to a standard curve prepared from saxitoxin dihydrochloride and is reported at saxitoxin dihydrochloride equivalents agnostic to the specific PSP congener composition of the extract.

Target Matrix/Matrices:
(list shellfish species by common and scientific names)
The target matrices include Pacific geoduck clam (*Panopea generosa*) gutball, whole Pacific oysters (*Crassostrea gigas*), northern razor clam (*Siliqua patula*) meat only, and whole hard clams, comprising three species: butter clams (*Saxidomus gigantea*), Pacific littleneck clams (*Protothaca staminea*), and Japanese littleneck clams (also known as Manila clams; *Venerupis philippinarum).*

Target Toxin(s):
The target toxin class are paralytic shellfish toxins (PSTs), with NIST Saxitoxin Dihydrochloride (RM 8642a) serving as the certified reference standard for generating calibration curves and spikes for shellfish samples.

Existing Certification(s) of the Method:
(AOAC, etc.)
The RBA has successfully undergone AOAC single laboratory validation (Van Dolah et al. 2009 – Appendix II) and a full collaborative study (Van Dolah et al. 2012 – Appendix III). The RBA is now considered an AOAC Official Method of Analysis (OMA 2011.27 – Appendix IV) and was adopted in the FDA NSSP in the 2015 edition for analysis of mussels as an alternative to the mouse bioassay and as a limited use method for screening and precautionary closure. The current proposal provides data to seek consideration for the RBA to be a NSSP Approved Method for these additional matrices: geoduck gutball, whole oyster, whole clams, and razor clam meat.

Equipment Required:
(Provide a list of specialized equipment needed to perform the method.)
The following list identifies the equipment and supplies needed for conducting the RBA.

<u>For the assay:</u>

(a) Microplate Scintillation counter suitable for measuring low counts of tritium (<1000dpm) (e.g. MicroBeta from Revvity)
(b) An 8-channel pipettor (5-200 µl variable volume and disposable tips)
(c) Micropipettors (1-1000 µl variable volumes and disposable tips)
(d) 96-well microtitre filter plate (1 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (MilliporeSigma; Cat. No. MSFB N6B 50)
(e) MultiScreen vacuum manifold (MilliporeSigma; Cat. No. NSVMHTS00)
(f) Vacuum pump
(g) Centrifuge tubes (15 and 50 ml, conical, plastic)
(h) Mini dilution tubes in 96-tube array
(i) Reagent reservoirs
(j) Ice bucket and ice
(k) Vortex mixer
(l) Sealing tape (Millipore; Cat. No. MATA HCL00)
(m) Volumetric flask or graduated beaker (1 L) (for buffer preparation)
(n) -80 °C freezer
(o) Refrigerator
(p) pH meter (for buffer preparation)

For sample extraction:
(q) Blender or homogenizer for sample homogenization
(r) Pipets
(s) Centrifuge tubes (15 ml, conical, plastic)
(t) pH meter or pH paper
(u) Hot plate or water bath
(v) Graduated centrifuge tubes (15 ml)
(w) Centrifuge and rotor for 15 ml tubes

For rat brain isolation:
(x) Teflon/glass homogenizer (Motorized tapered Teflon pestle and glass tune (15 ml)
(y) Motorized tissue homogenizer (Polytron or small handheld blender)
(z) High-speed centrifuge and fixed angle rotor (20,000 xg rcf)
(aa) Centrifuge tubes (12-15 ml, rated for 20,000 xg)
(bb) plastic cryovials (2 ml)
(cc) Graduated beaker (300 or 500 ml)
(dd) Pipets (5-10 ml, disposable)
(ee) Forceps
(ff) Ice bucket and ice
(gg) top loading balance

Reagents Required:
(Provide a list of specialized chemicals, reagents, etc. needed to perform the method.)
For the assay:
(a) STX diHCl standards (NIST RM 8642a; National Institute of Standards and Technology; www.nist.gov) [This is the same standard used for the MBA]
(b) 3H-STX (0.05 mCi per ml, ≥10 Ci per mmol, ≥90% radiochemical purity; American

Radiolabeled Chemicals, St. Louis, MO)
(c) 3-Morpholinopropanesulfonic acid (MOPS; Sigma; St. Louis, MO; Cat. No. M3183-500G [or equivalent])
(d) Choline chloride (Sigma; Cat. No. C7527-500G [or equivalent])
(e) ultrapure water (MilliQ or equivalent)

For microplate counter only:
(f) Ultima Gold liquid scintillation cocktail (Revvity.; Waltham, MA; Cat. No. 6013321 [or equivalent])

For sample extraction:
(g) Hydrochloric acid (HCl; 1.0 and 0.1 N)
(h) Sodium hydroxide (0.1 N)
(i) Water (distilled or deionized [18 μΩ])

For rat brain isolation:
(j) 20 rat brains (male, 6-week-old Sprague-Dawley; available through Hilltop Lab Animals, Inc., Scottdale, PA; www.hilltoplabs.com [or equivalent])
(k) MOPS, pH 7.4 (Sigma; Cat. No. M3183-500G [or equivalent])
(l) Choline chloride (100 mM; Sigma; Cat. No. C7527-500G [or equivalent])
(m) Phenyl methylsulfonyl fluoride (PMSF; Sigma: Cat. No. P7626)
(n) Isopropanol
(o) Micro bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL)

Proprietary Aspects:
(Provide any aspects of the method that are proprietary or trade secret.)
None. All reagents can be prepared or purchased.

Safety Requirements:
(Describe the safety measures, beyond those of routine laboratory practices, required to perform the method, including personal protective equipment, fume hoods, etc.)
General safety requirements (e.g., personal protective equipment including gloves, safety glasses, and laboratory coat) for working with toxins, biological reagents, and radioactive material must be followed. Users must be trained in and follow all in-house safety procedures for working with toxins and radiolabeled materials. Although low levels of radiation are used for this assay, sometimes under an NRC exemption clause for the ARC sourced 3H-STX users must follow all local, state and federal laws and procedures regarding the receipt, use, and disposal of radioisotopes. Please see Appendix C for further safety requirements.

Method Cost:
(Provide an estimate of cost per analysis, including start-up costs for specialized equipment, personnel, etc.)
The estimated cost per 96-well plate assay is ~$240. Including standards and samples with triplicate measurements (as well as three dilutions per sample [ranging from 3.5-600 μg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$35.50. If running multiple plates or in screening mode, sample costs

would be reduced. A microplate scintillation counter is needed, and the cost is ~$70-250K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$13K.

Sample Throughput:
(Provide a description of how many samples can be analyzed by this method in a given time frame; please specify under what conditions this throughput can be achieved.)
Microplate scintillation counting provides the ability to test multiple samples simultaneously with a turnaround time for data in approximately 3 hours. Up to four plates per analyst are possible in one day, yielding a throughput of at least 36 samples per day.

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability -** The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – The minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – The minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – The range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – The closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – The measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – The measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System -** The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which

appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.

15. **Recovery –** The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** – The ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – The ability of a method to measure only what it is intended to measure.[1]
18. **Working Range –** The range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
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Background:

Proposal 13-114 was approved at the 2015 ISSC meeting for the analysis of paralytic shellfish toxins (PSTs) in mussels using the receptor binding assay (RBA) and was granted a limited-use designation for application in clams and scallops.

Successful implementation of the RBA method by the Sitka Tribe of Alaska and by countries supported by the International Atomic Energy Agency (IAEA) has generated continued interest in the adoption of RBA within laboratories operating under the National Shellfish Sanitation Program (NSSP). However, broader adoption has just begun, as the method is currently approved as an MBA replacement for mussel samples. The four target matrices under evaluation include Pacific geoduck clam (*Panopea generosa*) gutball, whole Pacific oysters (*Crassostrea gigas*), Northern razor clam (*Siliqua patula*) meat only, and whole hard clams, comprising three species: butter clams (*Saxidomus gigantea*), Pacific littleneck clams (*Protothaca staminea*), and Japanese littleneck (also known as Manila) clams (*Venerupis philippinarum).*

Organizations Involved:

- NOAA National Ocean Service**,** Charleston, SC (NOAA)
- Washington State Department of Health (WADoH)
- Sitka Tribe of Alaska Environmental Research Laboratory (STAERL)
- Alaska Department of Environmental Conservation (AKDEC)

A total of 1,572 samples were collected for this project, including both samples with little to no detectable PSP-toxins and with PSP-toxins detected using the mouse bioassay (MBA). However, sourcing suitable 'blank' material proved challenging. The 'blank' materials used in the study contained very low, but still detectable, PSP-like activity, ranging from less than 2 µg STX/100 g of shellfish tissue to approximately 10 µg STX/100 g of shellfish tissue. PSP-free samples are rare, which made the execution of this study difficult and may present challenges for obtaining suitable 'blank' materials in future PSP method evaluations. Initial screening using the MBA (limit of detection ~40 µg STX/100g of shellfish tissue) identified negative samples, which were subsequently re-screened using a modified RBA with a lower limit of detection of ~2 µg STX/100 g. To achieve the required quantity for the validation study, the identified 'blank' materials were combined to produce the 15 homogenates with 100 sub-aliquots needed for spikes at 1/4x, 1/2x 1x, 2x, and 4x the regulatory limit (80 ug STX/100 g of shellfish tissue) for each shellfish matrix as shown in the study design (Figure 1). The 'blank' sample for each homogenate served as a reference blank to be subtracted from the spiked sub-aliquots after performing RBA to ensure accuracy in measured spiked samples (RBA-Blank)

Negative samples
Naturally incurred samples
Recovery
Repeatability
Linear range, LOD, & LOQ
Comparability
Uncertainty
≥40 µg STX/ 100 g shellfish

Figure 1. Matrix Extension study design showing allocation of 126 samples per shellfish matrix type to meet five validation data criteria: recovery (yellow), repeatability (blue), linear range, LOD and LOQ (red), comparability (green), and uncertainty (purple).

In total, 952 PSP samples were below the MBA's limit of detection from both Washington state and Alaska (STAERL uses RBA for screening), and 598 Washington state samples were re-screened using a modified extraction procedure (0.5 g sample + 0.5 mL 0.1 N HCl). A total of 159 Pacific geoduck clam gutball MBA negative samples were re-screened using a modified RBA to identify 'blank' samples, and of these were selected for inclusion in the study. Of the 44 whole Pacific oyster samples, 35 were re-screened and used in the study. Similarly, 199 hard clam samples were re-screened, with 34 included in the study. Among 103 northern razor clam meat samples re-screened, 20 were used for further analysis (Figure 2).

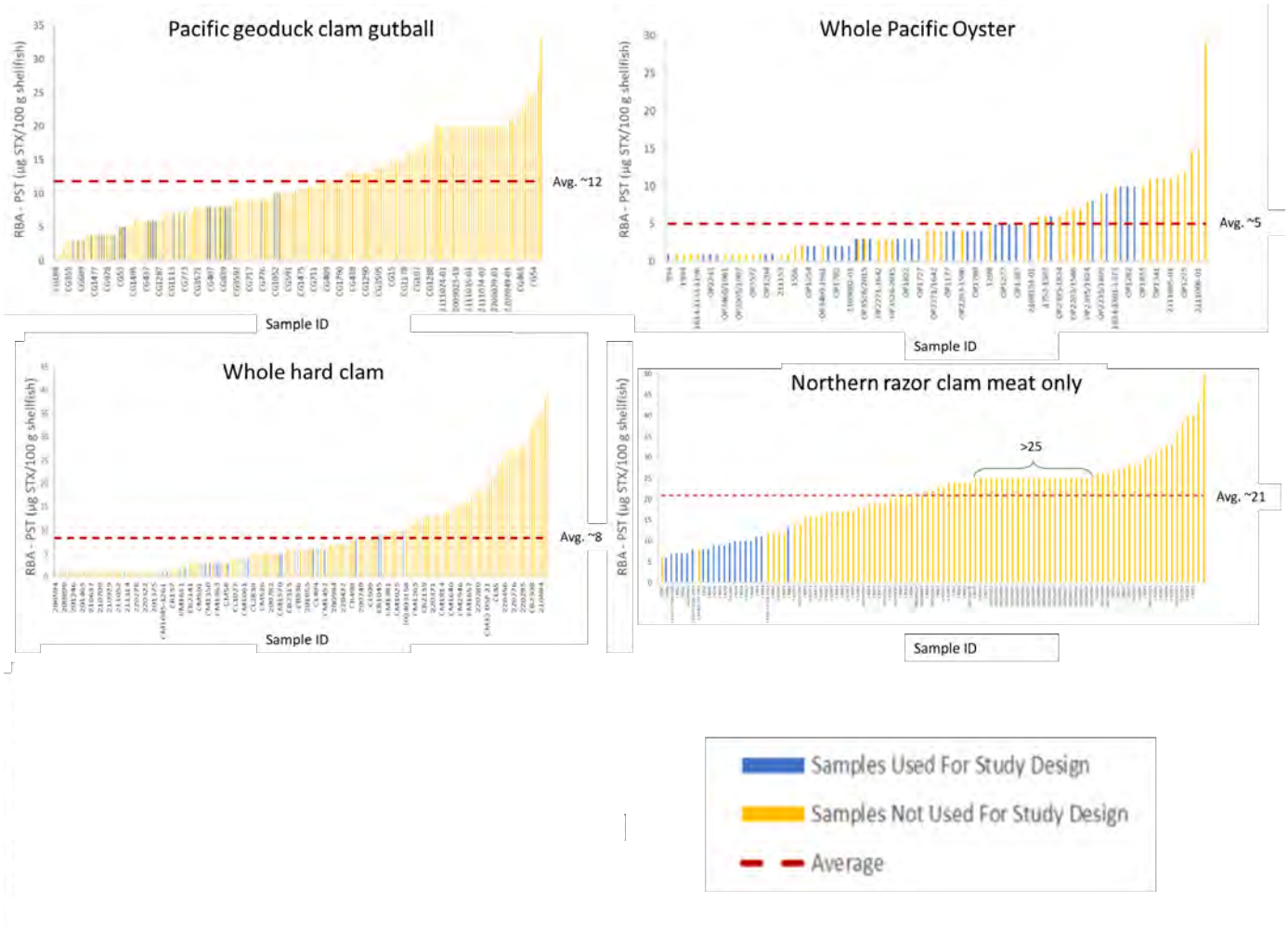


Figure 2. Samples from five shellfish matrices that tested negative for toxins using the MBA were re-screened with a modified RBA to identify "blank" samples for spiking in homogenates for the Matrix Extension study. Average PST values (indicated by the red dashed line) for Pacific geoduck clam gutball, whole Pacific oyster, northern razor clam meat, and whole hard clams are 12, 5, 21, and 8 µg STX/100 g shellfish tissue, respectively.

Samples were collected along the U.S. West Coast, specifically from Washington and Alaska, across various harvest days, locations, and calendar seasons. The seasons were defined as follows: Spring (March 20–June 20), Summer (June 21–September 21), Fall (September 22–December 20), and Winter (December 21–March 19). To meet the required tissue weight for each homogenate, multiple individual samples were combined prior to spiking. This approach ensured sufficient sample mass while maintaining temporal and seasonal consistency across collections.

Table 1. Summary of shellfish tissue samples (Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam, and razor clam) collected across Washington and Alaska sites between 2020 and 2025, categorized by group, location, and season.

Group Letter	Shellfish Matrix	Location	Collection Date	Season
A	geoduck	Kingston, Kitsap - WA	3/2024	Winter
B	geoduck	Sequim Bay, Clallam - WA	5-6/2024	Spring
C	geoduck	Agate Passage, Kitsap - WA	7/2024	Summer
D	geoduck	South King County, King - WA	5-6/2024	Spring
E	geoduck	Admiralty Inlet, King - WA	4/2024	Spring
F	geoduck	East Bainbridge, Kitsap - WA	6-7/2022	Summer
G	geoduck	Port Madison, Kitsap - WA	7/11/2022	Summer
H	geoduck	Sequim Bay, Clallam - WA	6/7/2022	Spring
I	geoduck	Agate Passage, Kitsap - WA	5-6/2024	Spring
J	geoduck	Agate Passage, Kitsap - WA	3/4/2024	Winter
K	geoduck	Georgia Straits South, Whatcom - WA	4-5/2024	Spring
L	geoduck	Sequim Bay, Clallam - WA	3/4/2024	Winter
M	geoduck	Hale Passage, Pierce WA	6/21/2024	Summer
N	geoduck	Port Madison, Kitsap - WA	4/2024	Spring
O	geoduck	Sequim Bay, Clallam - WA	6/25/2024	Summer
A	oyster	Grays Harbor Coast, Grays Harbor - WA	7/2024	Summer
B	oyster	Willapa Bay, Pacific - WA	7/2024	Summer
C	oyster	Sequim Bay, Clallam - WA	7/2024	Summer
D	oyster	Willapa Bay, Pacific - WA	6/2024	Spring
E	oyster	Grays Harbor Coast, Grays Harbor - WA	6/2024	Spring
F	oyster	Canoe Lagoon, Blashke Islands - AK	10/30/2021	Fall
G	oyster	Peterson Bay, Kachemak Bay - AK	8/30/2021	Summer
H	oyster	Betton Island East, Ketchikan - AK	8/30/2021	Summer
I	oyster	Samish Bay, Skagit - WA	6/14/2022	Spring
J	oyster	East Passage, Kitsap - WA	6/4/2024	Spring
K	oyster	Shikat Bay, Sea Otter Sound East - AK	8/4/2021	Summer
L	oyster	Dabob Bay, Jefferson - WA	10/3/2022	Fall
M	oyster	Sequim Bay, Clallam - WA	6/6/2024	Spring
N	oyster	Port Madison, Kitsap - WA	7/9/2024	Summer
O	oyster	Grays Harbor Coast, Grays Harbor - WA	09-10/2022	Fall
A	butter clam	Fidalgo Bay, Skagit - WA	5/2024	Spring
B	manila clam	Haro Strait, San Juan - WA	07-08/2024	Summer
C	manila clam	Willapa Bay, Pacific - WA	06/07/2024	Summer
D	manila clam	Willapa Bay, Pacific - WA	05-06/2024	Spring
E	manila clam	Padilla Bay, Skagit - WA	06-07/2024	Summer
F	butter clam	Lone Tree Point, Skagit - WA	1/18/2023	Winter
G	littleneck clam	Sequim Bay, Clallam - WA	6/25/2024	Summer

H	butter clam	Skagit Bay, Skagit - WA	5/7/2024	Spring
I	manila clam	Sequim Bay, Clallam - WA	5/9/2024	Spring
J	butter clam	North King County, King - WA	5/24/2024	Spring
K	littleneck clam	Starrigavan North, Sitka - AK	6/24/2020	Summer
L	littleneck clam	Puget Cove, Yakutat - AK	12/15/2020	Fall
M	littleneck clam	False Island Boat Launch, Craig - AK	10/15/2021	Fall
N	manila clam	Drayton Harbor, Whatcom - WA	8/11/2022	Summer
O	littleneck clam	Tongass Beach, Ketchikan - AK	9/9/2020	Summer
A	razor clam	Pacific County Coast, Pacific - WA	1-2/2024	Winter
B	razor clam	Grays Harbor Coast, Grays Harbor - WA	2/13/2024	Winter
C	razor clam	Grays Harbor Coast, Grays Harbor - WA	4/16/2024	Spring
D	razor clam	Grays Harbor Coast, Grays Harbor - WA	5/2/2024	Spring
E	razor clam	Grays Harbor Coast, Grays Harbor - WA	3/19/2024	Winter
F	razor clam	Pacific County Coast, Pacific - WA	9/25/2023	Fall
G	razor clam	Grays Harbor Coast, Grays Harbor - WA	1/30/2024	Winter
H	razor clam	Grays Harbor Coast, Grays Harbor - WA	1/2/2024	Winter
I	razor clam	Grays Harbor Coast, Grays Harbor - WA	2/27/2024	Winter
J	razor clam	Grays Harbor Coast, Grays Harbor - WA	4/2/2024	Spring
K	razor clam	Willapa Bay, Pacific - WA	4/14/2024	Spring
L	razor clam	Pacific County Coast, Pacific - WA	1/2/2024	Winter
M	razor clam	Grays Harbor Coast, Grays Harbor - WA	11/30/2023	Fall
N	razor clam	Grays Harbor Coast, Grays Harbor - WA	3/12/2024	Winter
O	razor clam	Grays Harbor Coast, Grays Harbor - WA	3/21/2024	Spring

Validation Data

1. **Recovery:** Recovery is the fraction or percentage of an analyte recovered following sample analysis. To determine method accuracy/trueness/recovery, the concentration of the target analyte as measured by the analytical method under study is compared to a true value or accepted reference concentration. Consider using certified reference materials (if available).

Suggested procedure**:** Use shellfish free of target analyte(s); analyze intended blank matrix tissue for background interferents. For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. Take four aliquots of the sample homogenate appropriately sized for the work and spike one with the target analyte(s) at half the action level. Spike a second aliquot with the target analyte(s) at the action level. Spike the third aliquot with the target analyte(s) at twice the action level. Do not spike the fourth aliquot; this is the sample blank. Process each aliquot to determine the concentration for the target analyte(s). Repeat this process with a minimum of five samples for each shellfish type of interest collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots. Additional samples may be required to examine the effects of seasonal and/or geographical differences in shellfish matrix components or analyte profiles on the method performance.

Data:
Working range/Range of interest: 40 µg saxitoxin diHCl equiv/100g shellfish tissue to 160 µg saxitoxin diHCl equiv /100g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oysters, whole hard clams (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

Data Handling

Recovery

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.
4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.
5. Perform a one-way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Concentration	2		
Error	27		

Total 29

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. Use log transformed data for microbiological methods.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.
4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.
5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.

Data Summary:

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant?

  - Pacific geoduck clam gutball: Not significant (F: 0.33 ≤ Fcrit: 3.2)
  - Whole Pacific oyster: Not significant (F: 1.84 < Fcrit: 3.2)
  - Whole hard clam: Not significant (F: 0.69 < Fcrit: 3.2)
  - Razor clam meat: Not significant (F: 0.43 < Fcrit: 3.2)

- Is the one-way analysis of variance to determine the consistency of recovery of the method under study significant?

  - Pacific geoduck clam gutball: Not significant (p = 0.72)
  - Whole Pacific oyster: Not significant (p = 0.17)
  - Whole hard clam: Not significant (p = 0.50)
  - Razor clam meat: Not significant (p = 0.65)

- At what concentrations is the one-way analysis of variance significant?
    - Pacific geoduck clam gutball: N/A
    - Whole Pacific oyster: N/A
    - Whole hard clam: N/A
    - Razor clam meat: N/A

- What is the overall percent recovery of the method under study?
    - Pacific geoduck clam gutball: 102.7%
    - Whole Pacific oyster: 103.6%
    - Whole hard clam: 102.1%
    - Razor clam meat: 95.00%

The data for each species were log-transformed to follow the ISSC's protocol for data handling prior to statistical analysis. A one-way ANOVA, conducted as described above, produced F-test results for each matrix type that were not statistically significant at the 95% confidence level for all species. These findings indicate that the method's recovery, as implemented by the laboratory, is consistent across the concentration range relevant to the method's intended application

Table 1.1. One-way ANOVA results for each shellfish matrix type.

Pacific geoduck clam gutball

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.003	2	0.002	0.33	0.72	3.22
Error	0.19	42	0.005			
Total	0.20	44				

Whole Pacific oyster

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.03	2	0.014	1.85	0.170	3.22
Error	0.33	42	0.008			
Total	0.358	44				

Whole hard clam

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.01	2	0.003	0.69	0.50	3.22
Error	0.21	42	0.005			
Total	0.21	44				

Razor clam meat

Source of Variation	*Sum of Squares*	*df*	*Mean Square*	*F*	*P-value*	*F crit*
Concentration	0.01	2	0.00	0.43	0.65	3.22
Error	0.29	42	0.01			
Total	0.29	44				

The method's overall percent recovery was 102.7% in Pacific geoduck clam gutball, 103.6% in whole Pacific oyster, 102.1% in whole hard clam, 95.0% in razor clam meat.

Full Data:

Sample ID	Shellfish Matrix	Spike Level	Spike Concentration (ug/100g)	RBA (ug/100g)	Blank Value	RBA - Blank	Percent Recovery (%)
CGa-16	geoduck	Blank	0	7	7	0	0.000
CGa-06	geoduck	1/2x	40	52	7	45	112.5
CGa-20	geoduck	1/2x	40	66	7	59	147.5
CGa-21	geoduck	1/2x	40	45	7	38	95.00
CGa-07	geoduck	1x	80	92	7	85	106.3
CGa-08	geoduck	1x	80	99.5	7	92.5	115.6
CGa-09	geoduck	1x	80	94	7	87	108.8
CGa-10	geoduck	2x	160	135	7	128	80.00
CGa-11	geoduck	2x	160	178	7	171	106.9
CGa-12	geoduck	2x	160	163	7	156	97.50
CGb-00	geoduck	Blank	0	6	6	0	0.000
CGb-20	geoduck	1/2x	40	52	6	46	115.0
CGb-21	geoduck	1/2x	40	39	6	33	82.50
CGb-05	geoduck	1/2x	40	46	6	40	100.0
CGb-07	geoduck	1x	80	86	6	80	100.0
CGb-08	geoduck	1x	80	85	6	79	98.75
CGb-09	geoduck	1x	80	77	6	71	88.75
CGb-10	geoduck	2x	160	192	6	186	116.3
CGb-11	geoduck	2x	160	138	6	132	82.50
CGb-12	geoduck	2x	160	167	6	161	100.6
CGc-16	geoduck	Blank	0	10	10	0	0.000
CGc-04	geoduck	1/2x	40	55	10	45	112.5
CGc-05	geoduck	1/2x	40	50	10	40	100.0
CGc-06	geoduck	1/2x	40	40	10	30	75.00
CGc-07	geoduck	1x	80	110	10	100	125.0
CGc-08	geoduck	1x	80	115	10	105	131.3
CGc-09	geoduck	1x	80	84	10	74	92.50
CGc-10	geoduck	2x	160	230	10	220	137.5
CGc-11	geoduck	2x	160	135	10	125	78.13
CGc-12	geoduck	2x	160	167	10	157	98.13
CGd-16	geoduck	Blank	0	11	11	0	0.000
CGd-20	geoduck	1/2x	40	49	11	38	95.00
CGd-21	geoduck	1/2x	40	49	11	38	95.00
CGd-22	geoduck	1/2x	40	46	11	35	87.50
CGd-07	geoduck	1x	80	103	11	92	115.0
CGd-08	geoduck	1x	80	84	11	73	91.25
CGd-09	geoduck	1x	80	80	11	69	86.25
CGd-10	geoduck	2x	160	199	11	188	117.5

CGd-11	geoduck	2x	160	197	11	186	116.3
CGd-12	geoduck	2x	160	157	11	146	91.25
CGe-00	geoduck	Blank	0	6	6	0	0.000
CGe-04	geoduck	1/2x	40	47	6	41	102.5
CGe-05	geoduck	1/2x	40	44	6	38	95.00
CGe-06	geoduck	1/2x	40	46	6	40	100.0
CGe-07	geoduck	1x	80	88	6	82	102.5
CGe-08	geoduck	1x	80	72	6	66	82.50
CGe-09	geoduck	1x	80	115	6	109	136.3
CGe-10	geoduck	2x	160	156	6	150	93.75
CGe-11	geoduck	2x	160	175	6	169	105.6
CGe-12	geoduck	2x	160	172	6	166	103.8
OPa-00	oyster	Blank	0	0	0	0	0.000
OPa-04	oyster	1/2x	40	47	0	47	117.5
OPa-21	oyster	1/2x	40	35	0	35	87.50
OPa-06	oyster	1/2x	40	46	0	46	115.0
OPa-07	oyster	1x	80	87	0	87	108.8
OPa-08	oyster	1x	80	102	0	102	127.5
OPa-09	oyster	1x	80	85	0	85	106.3
OPa-10	oyster	2x	160	186	0	186	116.3
OPa-11	oyster	2x	160	119	0	119	74.38
OPa-12	oyster	2x	160	159	0	159	99.38
OPb-00	oyster	Blank	0	6	6	0	0.000
OPb-04	oyster	1/2x	40	32	6	26	65.00
OPb-05	oyster	1/2x	40	41	6	35	87.50
OPb-06	oyster	1/2x	40	42	6	36	90.00
OPb-07	oyster	1x	80	101	6	95	118.8
OPb-08	oyster	1x	80	74	6	68	85.00
OPb-09	oyster	1x	80	85	6	79	98.75
OPb-10	oyster	2x	160	168	6	162	101.3
OPb-11	oyster	2x	160	142	6	136	85.00
OPb-12	oyster	2x	160	126	6	120	75.00
OPc-00	oyster	Blank	0	0	0	0	0.000
OPc-20	oyster	1/2x	40	28	0	28	70.00
OPc-21	oyster	1/2x	40	40	0	40	100.0
OPc-06	oyster	1/2x	40	42	0	42	105.0
OPc-07	oyster	1x	80	80	0	80	100.0
OPc-08	oyster	1x	80	75	0	75	93.75
OPc-09	oyster	1x	80	91	0	91	113.8
OPc-10	oyster	2x	160	128	0	128	80.00
OPc-11	oyster	2x	160	192	0	192	120.0
OPc-12	oyster	2x	160	91	0	91	56.88

OPd-16	oyster	Blank	0	5	5	0	0.000
OPd-04	oyster	1/2x	40	46	5	41	102.5
OPd-05	oyster	1/2x	40	45	5	40	100.0
OPd-22	oyster	1/2x	40	44	5	39	97.50
OPd-07	oyster	1x	80	79	5	74	92.50
OPd-08	oyster	1x	80	115	5	110	137.5
OPd-09	oyster	1x	80	100	5	95	118.8
OPd-10	oyster	2x	160	168	5	163	101.9
OPd-11	oyster	2x	160	167	5	162	101.3
OPd-12	oyster	2x	160	181	5	176	110.0
OPe-16	oyster	Blank	0	5	5	0	0.000
OPe-20	oyster	1/2x	40	48	5	43	107.5
OPe-21	oyster	1/2x	40	57	5	52	130.0
OPe-04	oyster	1/2x	40	66	5	61	152.5
OPe-07	oyster	1x	80	115	5	110	137.5
OPe-08	oyster	1x	80	116	5	111	138.8
OPe-09	oyster	1x	80	77	5	72	90.00
OPe-10	oyster	2x	160	202	5	197	123.1
OPe-11	oyster	2x	160	210	5	205	128.1
OPe-12	oyster	2x	160	153	5	148	92.50
CBa-00	butter clam	Blank	0	9	9	0	0.000
CBa-04	butter clam	1/2x	40	42	9	33	82.50
CBa-05	butter clam	1/2x	40	40	9	31	77.50
CBa-06	butter clam	1/2x	40	39	9	30	75.00
CBa-07	butter clam	1x	80	77	9	68	85.00
CBa-08	butter clam	1x	80	71	9	62	77.50
CBa-09	butter clam	1x	80	97	9	88	110.0
CBa-10	butter clam	2x	160	175	9	166	103.8
CBa-11	butter clam	2x	160	151	9	142	88.75
CBa-12	butter clam	2x	160	177	9	168	105.0
CMb-28	manila clam	Blank	0	0	0	0	0.000
CMb-04	manila clam	1/2x	40	42	0	42	105.0
CMb-05	manila clam	1/2x	40	45	0	45	112.5
CMb-06	manila clam	1/2x	40	47	0	47	117.5
CMb-07	manila clam	1x	80	87	0	87	108.8
CMb-08	manila clam	1x	80	85	0	85	106.3
CMb-09	manila clam	1x	80	70	0	70	87.50
CMb-10	manila clam	2x	160	159	0	159	99.40
CMb-11	manila clam	2x	160	153	0	153	95.60
CMb-12	manila clam	2x	160	132	0	132	82.50
CMc-00	manila clam	Blank	0	0	0	0	0.000
CMc-04	manila clam	1/2x	40	42	0	42	105.0

CMc-05	manila clam	1/2x	40	50	0	50	125.0
CMc-06	manila clam	1/2x	40	47	0	47	117.5
CMc-07	manila clam	1x	80	113	0	113	141.3
CMc-08	manila clam	1x	80	72	0	72	90.00
CMc-09	manila clam	1x	80	92	0	92	115.0
CMc-10	manila clam	2x	160	189	0	189	118.1
CMc-11	manila clam	2x	160	146	0	146	91.30
CMc-12	manila clam	2x	160	197	0	197	123.1
CMd-00	manila clam	Blank	0	6	6	0	0.000
CMd-04	manila clam	1/2x	40	48	6	42	105.0
CMd-05	manila clam	1/2x	40	38	6	32	80.00
CMd-06	manila clam	1/2x	40	37	6	31	77.50
CMd-07	manila clam	1x	80	99	6	93	116.3
CMd-08	manila clam	1x	80	74	6	68	85.00
CMd-09	manila clam	1x	80	108	6	102	127.5
CMd-10	manila clam	2x	160	196	6	190	118.8
CMd-11	manila clam	2x	160	152	6	146	91.30
CMd-12	manila clam	2x	160	188	6	182	113.8
CMe-00	manila clam	Blank	0	4	4	0	0.000
CMe-04	manila clam	1/2x	40	45	4	41	102.5
CMe-05	manila clam	1/2x	40	48	4	44	110.0
CMe-06	manila clam	1/2x	40	37	4	33	82.50
CMe-07	manila clam	1x	80	91	4	87	108.8
CMe-08	manila clam	1x	80	94	4	90	112.5
CMe-09	manila clam	1x	80	76	4	72	90.00
CMe-10	manila clam	2x	160	197	4	193	120.6
CMe-11	manila clam	2x	160	180	4	176	110.0
CMe-12	manila clam	2x	160	156	4	152	95.00
CRa-00	razor clam	Blank	0	10	10	0	0.000
CRa-04	razor clam	1/2x	40	46	10	36	90.00
CRa-05	razor clam	1/2x	40	48	10	38	95.00
CRa-06	razor clam	1/2x	40	47	10	37	92.50
CRa-07	razor clam	1x	80	64	10	54	67.50
CRa-08	razor clam	1x	80	78	10	68	85.00
CRa-09	razor clam	1x	80	99	10	89	111.3
CRa-10	razor clam	2x	160	140	10	130	81.25
CRa-11	razor clam	2x	160	139	10	129	80.63
CRa-12	razor clam	2x	160	119	10	109	68.13
CRb-00	razor clam	Blank	0	10	10	0	0.000
CRb-04	razor clam	1/2x	40	66	10	56	140.0
CRb-05	razor clam	1/2x	40	39	10	29	72.50
CRb-20	razor clam	1/2x	40	53	10	43	107.5

CRb-07	razor clam	1x	80	69	10	59	73.75
CRb-08	razor clam	1x	80	104	10	94	117.5
CRb-09	razor clam	1x	80	81	10	71	88.75
CRb-10	razor clam	2x	160	144	10	134	83.75
CRb-11	razor clam	2x	160	165	10	155	96.88
CRb-12	razor clam	2x	160	212	10	202	126.3
CRc-00	razor clam	Blank	0	14	14	0	0.000
CRc-04	razor clam	1/2x	40	54	14	40	100.0
CRc-05	razor clam	1/2x	40	53	14	39	97.50
CRc-06	razor clam	1/2x	40	56	14	42	105.0
CRc-07	razor clam	1x	80	72	14	58	72.50
CRc-08	razor clam	1x	80	109	14	95	118.8
CRc-09	razor clam	1x	80	115	14	101	126.3
CRc-10	razor clam	2x	160	139	14	125	78.13
CRc-11	razor clam	2x	160	160	14	146	91.25
CRc-12	razor clam	2x	160	183	14	169	105.6
CRd-00	razor clam	Blank	0	11	11	0	0.000
CRd-04	razor clam	1/2x	40	50	11	39	97.50
CRd-05	razor clam	1/2x	40	45	11	34	85.00
CRd-06	razor clam	1/2x	40	37	11	26	65.00
CRd-07	razor clam	1x	80	95	11	84	105.0
CRd-08	razor clam	1x	80	84	11	73	91.25
CRd-09	razor clam	1x	80	105	11	94	117.5
CRd-10	razor clam	2x	160	117	11	106	66.25
CRd-11	razor clam	2x	160	169	11	158	98.75
CRd-12	razor clam	2x	160	168	11	157	98.13
CRe-00	razor clam	Blank	0	15	15	0	0.000
CRe-04	razor clam	1/2x	40	50	15	35	87.50
CRe-05	razor clam	1/2x	40	61	15	46	115.0
CRe-06	razor clam	1/2x	40	57	15	42	105.0
CRe-07	razor clam	1x	80	101	15	86	107.5
CRe-08	razor clam	1x	80	76	15	61	76.25
CRe-09	razor clam	1x	80	87	15	72	90.00
CRe-10	razor clam	2x	160	201	15	186	116.3
CRe-11	razor clam	2x	160	152	15	137	85.63
CRe-12	razor clam	2x	160	165	15	150	93.75

2. **Repeatability:** Repeatability is the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.

Suggested procedure**:** Use shellfish free of target analyte(s). For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. Take four aliquots of the sample homogenate appropriately sized for the work and spike one with the target analyte(s) at half the action level. Spike a second aliquot with the target analyte(s) at the action level. Spike the third aliquot with the target analyte(s) at twice the action level. Do not spike the fourth aliquot; this is the sample blank. For each aliquot, excluding the sample blank, prepare three sub-aliquots for analysis. Process each sub-aliquot, including the sample blank, to determine the method concentration of the target analyte(s). Repeat this process for each shellfish type of interest with a minimum of five samples for each shellfish type of interest collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots.

When available, shellfish with naturally incurred target analyte(s) should be included. Use a minimum of 10-12 animals per sample and prepare as a homogenate. For each shellfish type of interest, use three samples at a range of concentrations bracketing the action level (below, at or near, and above). For each sample homogenate prepare a minimum of three aliquots for analysis. Process each aliquot to determine the method concentration of the target analyte(s).

Data Handling

Response:
A doubly-nested ANOVA was selected as the most robust model to isolate within-run repeatability (technical replicates) from the variance introduced by the hierarchical spike levels and individual tissue homogenates.

Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

Precision
To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Samples	9		
Concentrations in samples	20		
Determinations within concentrations	30		
Total	59		

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations.

If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA, variance component, Total.
3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and,
4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

Data:

Working range/Range of interest: 40 µg saxitoxin diHCl equiv/100g of shellfish tissue to 160 µg saxitoxin diHCl equiv /100g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

All data were log-transformed for the statistical analyses.

Nested ANOVA results for Pacific geoduck clam gutball:

	Degrees of freedom	Sum of Squares	Mean Squared Error (MSE)	F value	p-value
Homogenate	4	0.008	0.002	0.439	0.779
Homogenate:SpikeLevels	5	2.768	0.554	116.3	$< 2 \times 10^{-16}$
Residuals	35	0.167	0.005		

Following the data handling procedure and utilizing log-transformed data, the initial F-test confirmed significant variance components for Pacific geoduck clam gutball. To accurately model this variance, a doubly nested ANOVA was employed to match the hierarchical nature of the experimental design. In this model, the three technical replicates are nested within each spike level, which are in turn nested within the five individual homogenates. This doubly nested approach ensures that the analysis accounts for internal correlation within samples and provides a valid test of the effects of concentration across the species.

For all boxplots shown for each species below, the solid line inside the box represents the median, and the box edges (top and bottom lines of the box) represent the first and third quartiles. The whiskers are the lines that extend from the box and represent the maximum and minimum values within 1.5 times the interquartile range.

Doubly-nested ANOVA model - results:

- Pacific geoduck clam gutball:
  - F value: 0.0075
  - P-value: 0.9999

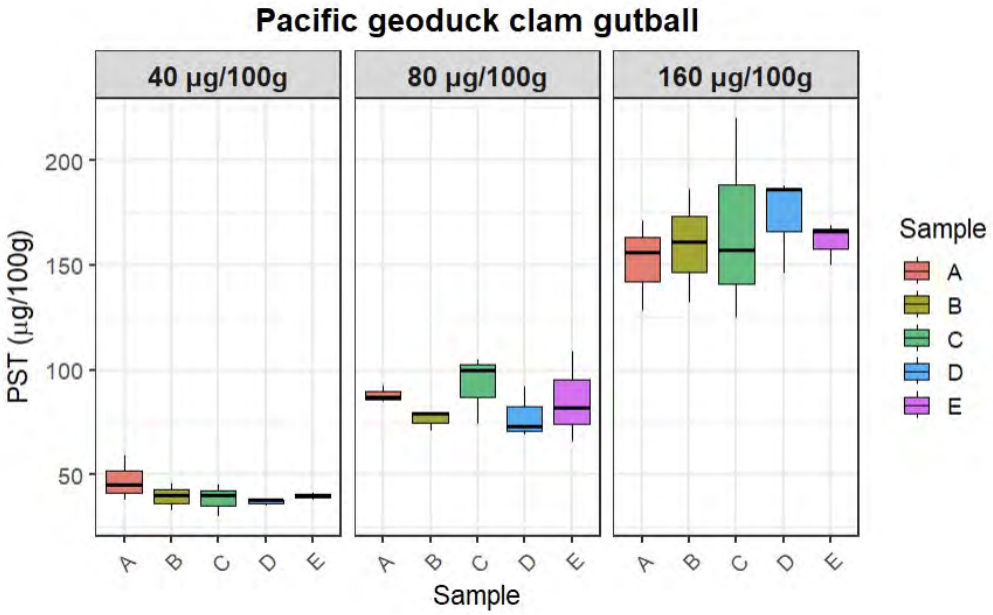


Figure 2.1. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five Pacific geoduck clam gutball samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

- Whole Pacific oyster:
  - F value: 0.1033
  - P-value: 0.9788

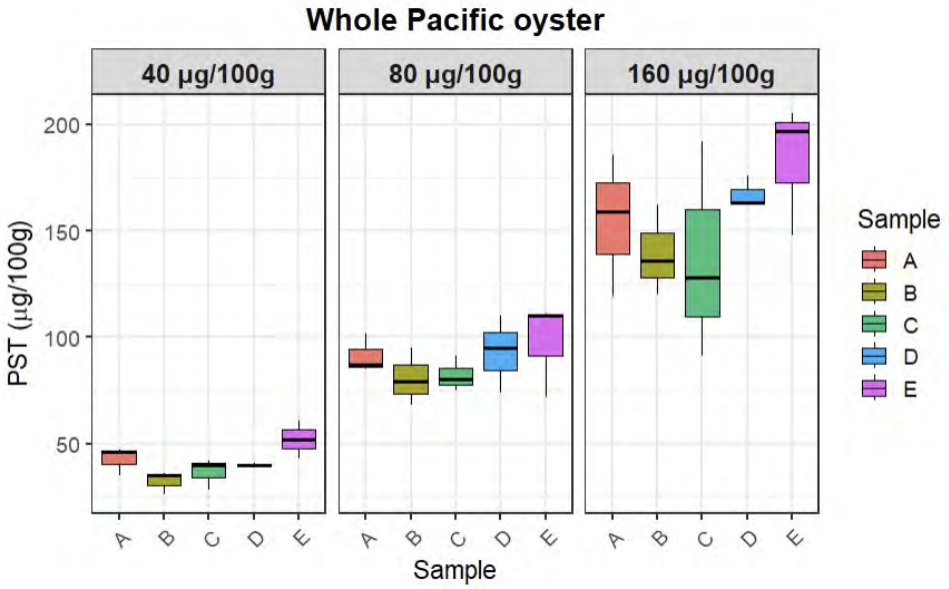


Figure 2.2. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five whole Pacific oyster samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

- Whole hard clam:
  - F value: 0.0425
  - P-value: 0.996

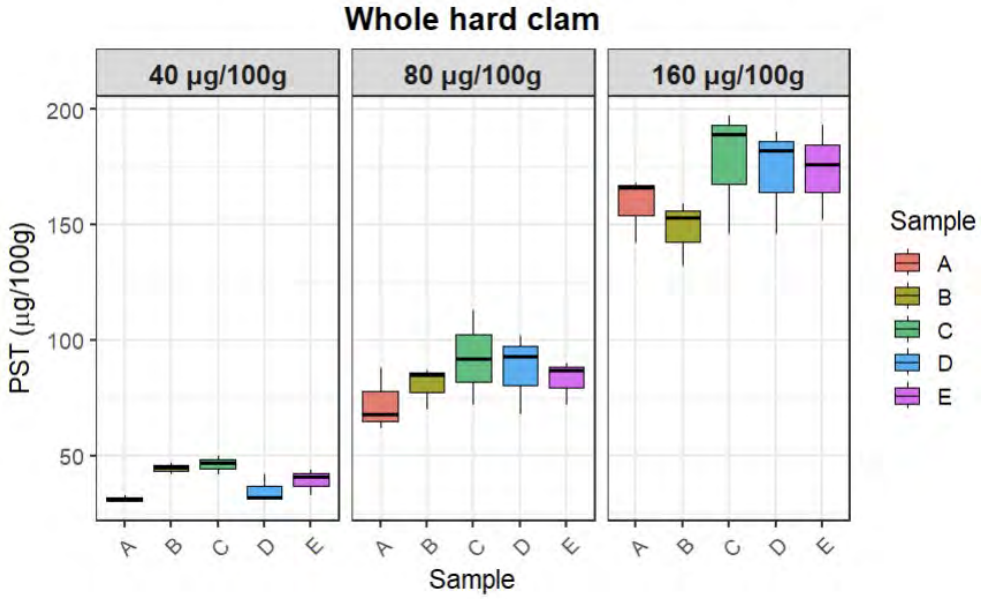


Figure 2.3. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five whole hard clam samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

- Razor clam meat:
  - F value: 0.0284
  - P-value: 0.9982

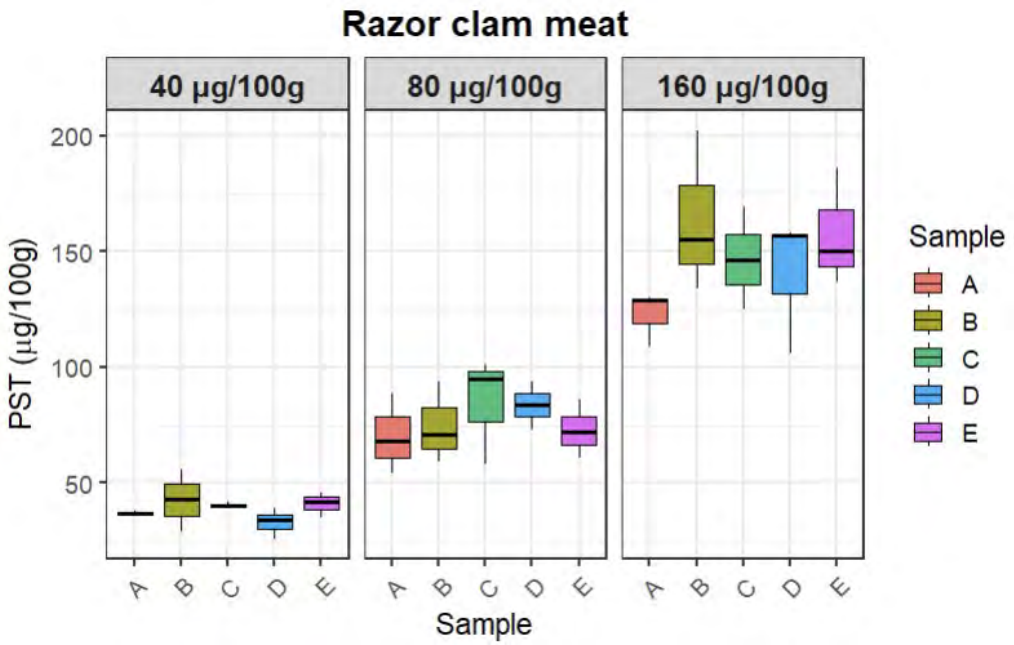


Figure 2.5. Box plots displaying RBA concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in five razor clam meat samples at three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue), each analyzed in triplicate.

• Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant?

- Pacific geoduck clam gutball: No
- Whole Pacific oysters: No
- Whole hard clams: No
- Razor clam meat: No

The resulting small F-value for the variance components indicates a high degree of consistency across the different spike levels. Rather than reflecting a lack of sensitivity, this tiny variance ratio demonstrates that the method's performance is stable and reproducible across the tested concentrations. This confirms that the subdivision of the five primary samples into nested spike levels and replicates did not introduce significant experimental error, highlighting the robustness of the data handling procedure for all shellfish species.

• If the variability of the method as implemented by the laboratory is consistent over the range in

concentrations important to its intended applications, what is the coefficient of variation? **See below.**

Table 2.1. Summary of RBA concentration results for Pacific geoduck clam gutball, whole Pacific oyster, and whole hard clam across three spike levels (40, 80, and 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Shown are the number of replicates (n), average measured concentration (µg saxitoxin diHCl equiv /100 g shellfish tissue), standard deviation, and percent coefficient of variation (%CV).

Pacific geoduck clam gutball

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	40.4	6.7	16.7
Medium (80 µg/100 g)	15	84.3	13.2	15.7
High (160 µg/100 g)	15	162.7	25.7	15.8

Whole Pacific oyster

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	40.7	8.8	21.6
Medium (80 µg/100 g)	15	88.9	14.5	16.4
High (160 µg/100 g)	15	156.3	32.7	20.9

Whole hard clam

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	39.3	6.9	17.6
Medium (80 µg/100 g)	15	83.3	14.3	17.1
High (160 µg/100 g)	15	166.1	20.9	12.6

Razor clam meat

	n	*Average*	*Standard Deviation*	*%CV*
Low (40 µg/100 g)	15	38.8	7.1	18.2
Medium (80 µg/100 g)	15	77.3	15.5	20.1
High (160 µg/100 g)	15	146.2	26.5	18.1

Percent coefficient of variation (%CV) was calculated separately for each spike level to accurately assess precision and repeatability. Overall, the variability of the method is consistent over the range of concentrations for all shellfish species.

Full Data: The full data used for Repeatability can be found under the Recovery section.

Repeatability for Naturally Incurring Samples:

In this section, each sample was subdivided into three 5-gram tissue aliquots, each assigned to a specific concentration range: low (<60 µg saxitoxin diHCl equiv /100 g shellfish tissue), medium (60-120 µg saxitoxin diHCl equiv /100 g shellfish tissue), and high (>120 µg saxitoxin diHCl equiv /100 g shellfish tissue). Because these levels are unique to each sample and do not overlap across the experimental groups, a collective statistical analysis was not performed. This lack of shared reference levels precludes a direct comparison of main effects, as any observed variance could be attributed to either the group identity or the specific concentration range. Consequently, these two factors are statistically confounded and cannot be decoupled for a unified analysis.

Note: The naturally incurring northern razor clam meat was analyzed using post-column oxidation (PCOX) method rather than the traditional mouse bioassay.

Pacific geoduck clam gutball:

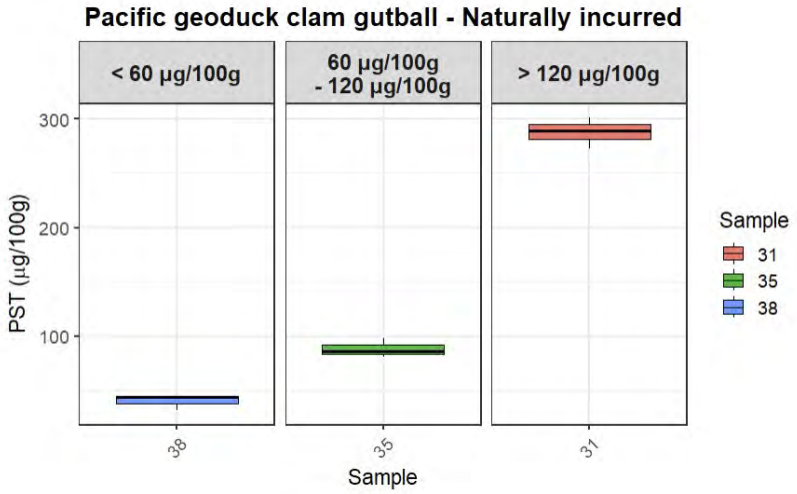


Figure 2.3. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred Pacific geoduck clam gutball samples, each sample representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g shellfish tissue, 60–120 µg saxitoxin diHCl equiv /100 g shellfish tissue, and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Each sample was analyzed in triplicate.

Whole Pacific oyster:

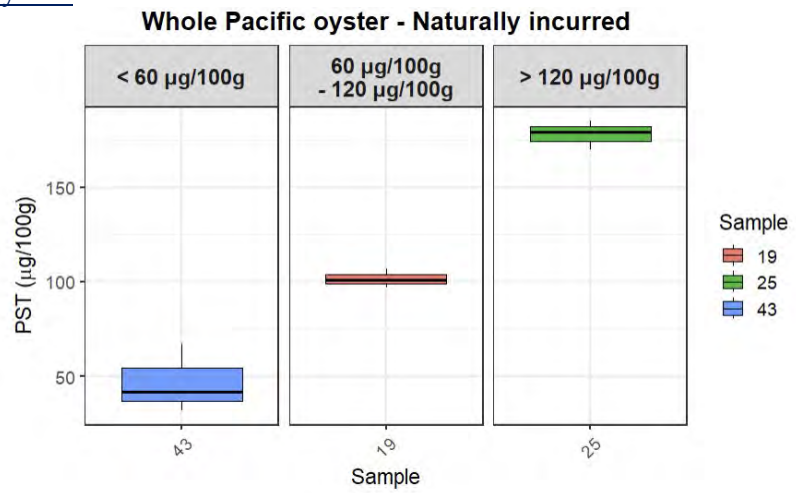


Figure 2.4. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred whole Pacific oyster samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g shellfish tissue, 60–120 µg saxitoxin diHCl equiv /100 g shellfish tissue, and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Each sample was analyzed in triplicate.

Whole hard clam:

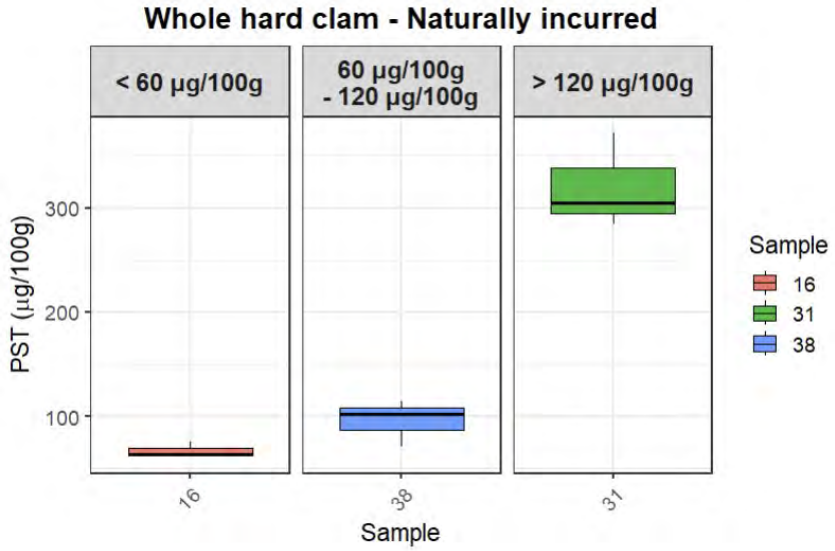


Figure 2.5. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred whole hard clam samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g shellfish tissue 60–120 µg saxitoxin diHCl equiv /100 g shellfish tissue, and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Each sample was analyzed in triplicate.

Northern razor clam meat:

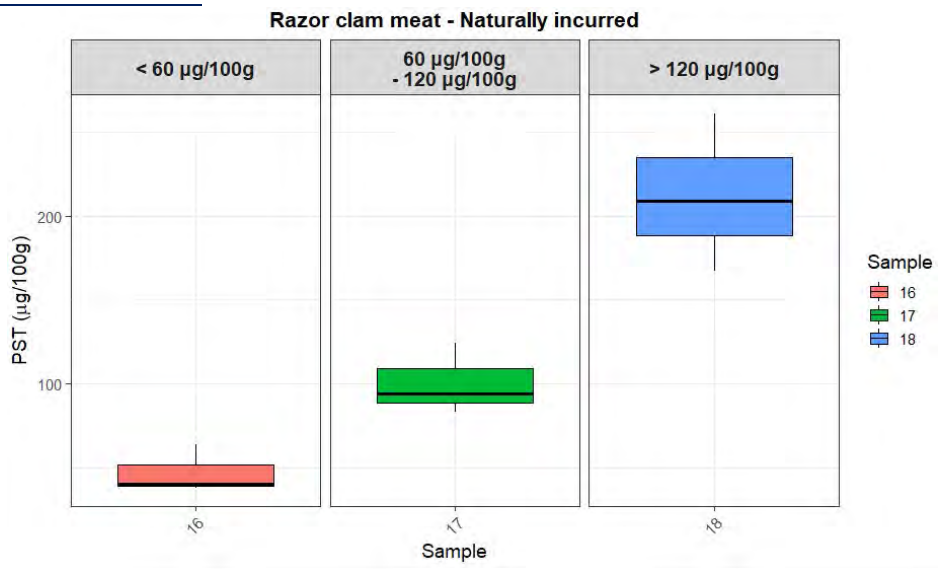


Figure 2.6. Box plots showing PST concentrations (µg saxitoxin diHCl equiv /100 g shellfish tissue) in three naturally incurred northern razor clam meat samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv /100 g; 60 to120 µg saxitoxin diHCl equiv /100 g; and > 160 µg saxitoxin diHCl equiv /100 g). Each sample was analyzed in triplicate.

• If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **See below.**

Table 2.2. Summary of PST concentration results for naturally incurred shellfish samples, each representing a different concentration range (< 60 µg saxitoxin diHCl equiv/100 g shellfish tissue; 60 to 120 µg saxitoxin diHCl equiv /100 g shellfish tissue; and > 160 µg saxitoxin diHCl equiv /100 g shellfish tissue). Shown are the number of replicates (n), MBA concentration, and average measured concentration (µg saxitoxin diHCl equiv /100 g shellfish tissue), standard deviation, and percent coefficient of variation (%CV) for RBA PST values.

Pacific geoduck clam gutball

	n	*MBA (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (<60 ug/100 g)	3	50.00	40.33	7.234	17.94
Medium (60 - 120 ug/100 g)	3	87.00	88.33	8.737	9.891
High (>120 ug/100 g)	3	254.0	287.7	14.05	4.883

Whole Pacific oyster

	n	*MBA (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (40 µg/100 g)	3	40.00	47.00	18.03	38.86
Medium (80 µg/100 g)	3	87.00	101.7	5.033	4.951
High (160 µg/100 g)	3	235.0	178.0	7.550	4.241

Whole hard clam

	n	*MBA (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (40 µg/100 g)	3	40.00	66.67	8.145	12.22
Medium (80 µg/100 g)	3	95.00	95.67	22.19	23.19
High (160 µg/100 g)	3	236.00	320.33	45.96	14.35

Razor clam meat

	n	*PCOX (µg/100g)*	*Average RBA (µg/100g)*	*Standard Deviation (RBA)*	*%CV (RBA)*
Low (40 µg/100 g)	3	37.00	47.33	14.47	30.57
Medium (80 µg/100 g)	3	99.00	100.3	21.22	21.15
High (160 µg/100 g)	3	184.00	212.3	47.09	22.18

Full Data:

Pacific geoduck clam gutball

Action Level	*Sample ID*	*MBA (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	38-1	50	32
Low (< 60 µg/100g)	38-2	50	44
Low (< 60 µg/100g)	38-3	50	45
Medium (60-120 µg/100g)	35-1	87	81
Medium (60-120 µg/100g)	35-2	87	98
Medium (60-120 µg/100g)	35-3	87	86
High (> 120 µg/100g)	31-1	254	289
High (> 120 µg/100g)	31-2	254	301
High (> 120 µg/100g)	31-3	254	273

Whole Pacific oyster

Action Level	*Sample ID*	*MBA (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	43-1	40	32
Low (< 60 µg/100g)	43-2	40	67
Low (< 60 µg/100g)	43-3	40	42
Medium (60-120 µg/100g)	19-3	87	101
Medium (60-120 µg/100g)	19-4	87	97
Medium (60-120 µg/100g)	19-5	87	107
High (> 120 µg/100g)	25-1	235	179
High (> 120 µg/100g)	25-2	235	170
High (> 120 µg/100g)	25-3	235	185

Whole hard clam

Action Level	*Sample ID*	*MBA (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	16-1	40	63
Low (< 60 µg/100g)	16-2	40	61
Low (< 60 µg/100g)	16-3	40	76
Medium (60-120 µg/100g)	38-1	95	102
Medium (60-120 µg/100g)	38-2	95	71
Medium (60-120 µg/100g)	38-3	95	114
High (> 120 µg/100g)	31-1	236	284
High (> 120 µg/100g)	31-2	236	305
High (> 120 µg/100g)	31-3	236	372

Razor clam meat

Action Level	*Sample ID*	*PCOX (µg/100g)*	*RBA (µg/100g)*
Low (< 60 µg/100g)	16-1	37	38
Low (< 60 µg/100g)	16-2	37	64
Low (< 60 µg/100g)	16-3	37	40
Medium (60-120 µg/100g)	17-1	99	94
Medium (60-120 µg/100g)	17-2	99	124
Medium (60-120 µg/100g)	17-3	99	83
High (> 120 µg/100g)	18-1	184	167
High (> 120 µg/100g)	18-2	184	261
High (> 120 µg/100g)	18-3	184	209

3. Linear Range, Limit of Detection, and Limit of Quantification: Linear range is the range within the working range where the results are proportional to the concentration of the analyte present in the sample. The limit of detection is the minimum concentration at which the analyte can be identified. Limit of detection is matrix and analyte dependent. The limit of quantification is the minimum concentration of the analyte that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Suggested procedure**:** Use samples free of target analyte(s); analyze intended blank matrix tissue for background interferents. For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. For each sample take a minimum of six aliquots of the homogenate appropriately sized for the work and spike five of the six aliquots with five different concentrations of the target analyte(s), spanning beyond the desired working range and including levels half, at, and twice the action level. Do not spike the sixth aliquot; this is the sample blank. Process each aliquot, including the sample blank to determine concentration for the target analyte(s). For each aliquot, excluding the sample blank, sub-aliquot for three replicate analyses. Repeat this process for each shellfish type of interest with a minimum of five samples collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots. Use the same spike levels for each of the samples analyzed.

Data:
Working range/Range of interest: 20 to 320 µg saxitoxin diHCl equiv /100 g shellfish tissue
Range in spiking levels used: 20, 40, 80, 160, & 320 µg saxitoxin diHCl equiv /100 g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oysters, whole hard clams (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

Data Handling

Linear Range
To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to

it.

6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the

analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response

7. Multiply the value obtained in step 5 by 0.95 and 1.05.
8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects

either of the lines obtained above.

10. The linear range of the method as implemented by the laboratory is comprised of the range in

concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organism of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of “blank + 3σ” and “blank + 10σ” generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly, the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

Data Summary

Data was produced according to the outlined procedure (ten samples spiked at five concentration levels, each analyzed in triplicate along with a blank) for every matrix type evaluated. However, the resulting data could not be processed using the methods specified in the data handling section of

this SOP. The majority of this data was not utilized for determining linearity or LOQ/LOD, it is included at the end of this data summary for reference.

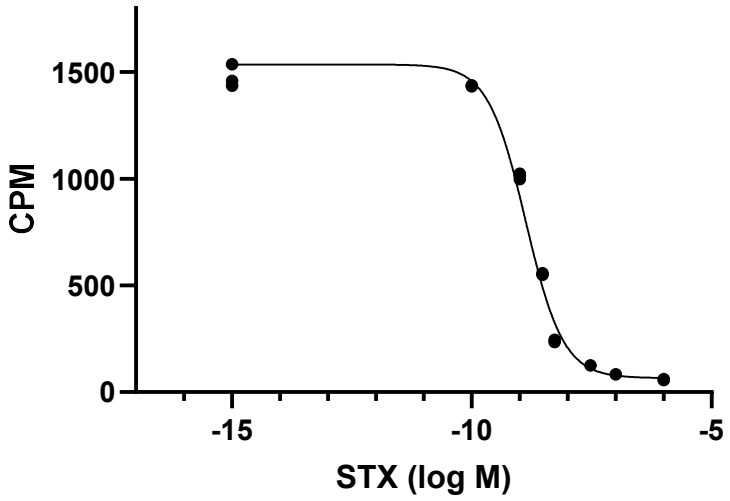
STX Standard Curve: RM 8642a FDA Saxitoxin Dihydrochloride Solution reference standard (100 µg/mL or 268.8 µM) used to prepare a bulk standard curve made up in advance and stored at 4°C for up to 1 month. The stock standard curve was made consisted of eight concentrations of STX in 0.003 M HCl [6 x 10^{-6}, 6 x 10^{-7}, 1.8 x 10^{-7}, 6 x 10^{-8}, 1.8 x 10^{-8}, 6 x 10^{-9}, 6 x 10^{-10}, 6 x10^{-11}, and 0.003 M only HCl (reference)], which when diluted 1:6 in the assay, resulted in a standard curve of 0.01 nM–1000 nM STX. The reference provided a measure of total 3H-STX binding in the absence of unlabeled STX.

For a given sample, if none of the sample dilutions falls within the linear range of 0.2 to 0.7 (i.e., the concentration is too high, b/bo < 0.2), further dilutions must be made, and the sample reanalyzed if a quantitative value is desired. If the sample concentration is too low to be quantified (i.e., b/bo > 0.7) at sample dilution 1:10, the sample must be reported as below the LOQ.

1) Linear Range of Assay

To evaluate the linear range of the assay, 8-point STX standard curves (ranging from 0 to 1000 nM) from 80 RBA plates run in this validation were generated using the sigmoidal dose-response with variable slope, using a four-parameter logistic to perform curve fitting in Prism 10 (GraphPad Software). Based from the AOAC Official Method 2011.27, sample quantification should be done only on dilutions that on the linear part of the curve [b/bo = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and Bo is the maximum CPM)].

(a)



(b)

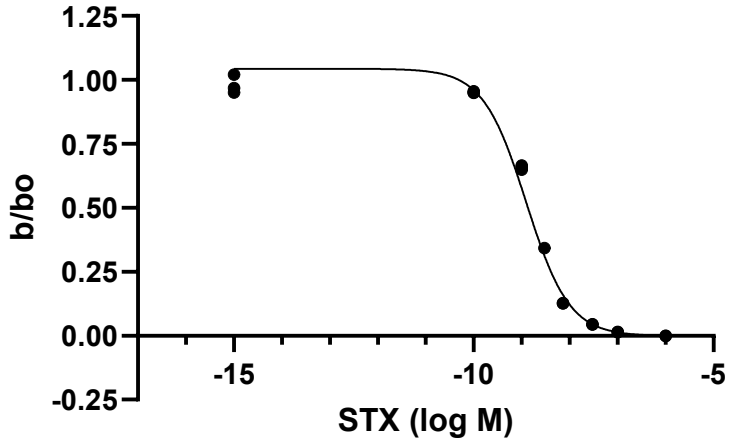
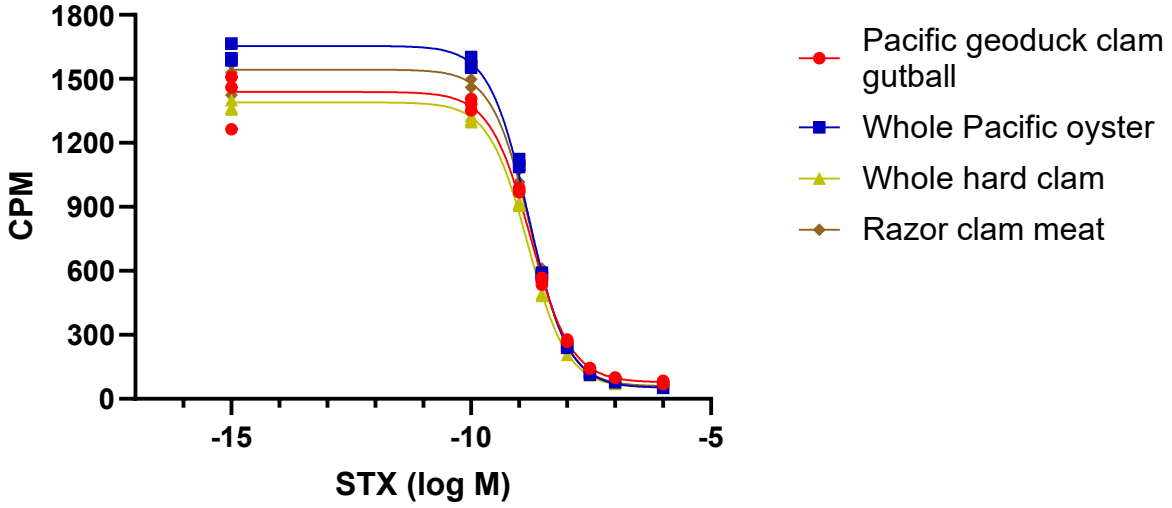


Figure 3.1. Average calibration curves derived from 80 independent assays performed by a single laboratory during the study period. The average IC_{50} was 1.65 ± 0.09 nM, with an average slope of 1.06 ± 0.024. Dots represent the average of each replicate. (a) Binding activity is measured in counts per minute (CPM). (b) Binding activity is measured in b/bo, where b represents the amount of radiolabeled ligand currently bound to the receptors and bo represents the total number of available receptor sites in the sample.

(a)



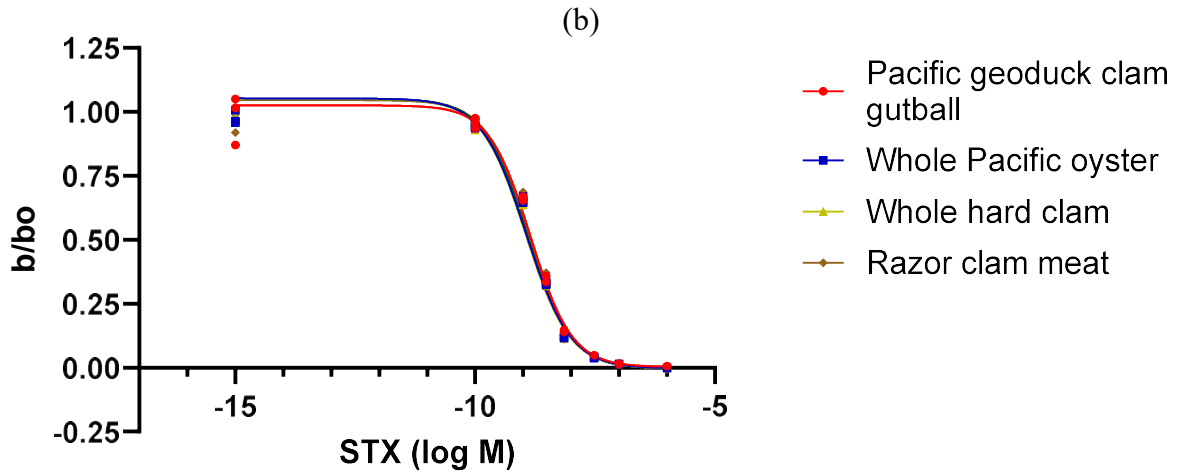


Figure 3.2. Competitive binding inhibition of saxitoxin (STX) in various shellfish tissues. Curves represent the displace of radiolabeled ligand by unlabeled STX (log M) across five shellfish matrices: Pacific geoduck clam gutball (n = 20; red), whole Pacific oyster (n = 19; blue), whole hard clam (n = 19; yellow), and razor clam meat (n = 19; brown). (a) Binding activity is measured in counts per minute (CPM). (b) Binding activity is measured in b/bo, where b represents the amount of radiolabeled ligand currently bound to the receptors and bo represents the total number of available receptor sites in the sample.

Table 3.1. Average and standard deviation of IC_{50} and slope values for each shellfish matrix type.

	Average IC_{50} (nM)	*Standard deviation IC_{50} (nM)*	*Average slope*	*Standard deviation slope*
All matrix types	1.65	0.20	1.068	0.123
Pacific geoduck clam gutball	1.74	0.15	1.034	0.089
Whole Pacific oyster	1.59	0.21	1.065	0.071
Whole hard clam	1.55	0.19	1.078	0.075
Razor clam meat	1.73	0.26	1.095	0.258

Linearity within the range defined by the bend points was evaluated using serial dilutions of shellfish samples spiked with STX at five concentrations (20, 40, 80, 160, and 320 µg STX diHCl equiv./100 g shellfish tissue). For each matrix type, five samples were prepared, and three replicate extractions were performed and analyzed per sample. According to the method protocol, the extraction procedure produces a twofold dilution (5 g of shellfish extracted to a final volume of 5 mL). A standardized dilution series (1:10, 1:50, and 1:200) was applied to each sample, ensuring that at least one dilution would fall within the linear region of the competition curve for shellfish containing approximately 5–1500 µg STX diHCl equiv./100g shellfish tissue. Each extract was subsequently diluted six times in MOPS/choline chloride assay buffer (pH 7.4) on the assay plate, yielding a total of five dilution levels per sample.

The expected and mean measured values are listed in Table 3.2. Figure 3.3 displayed the linear regression with the slope and r^2 value for each shellfish matrix.

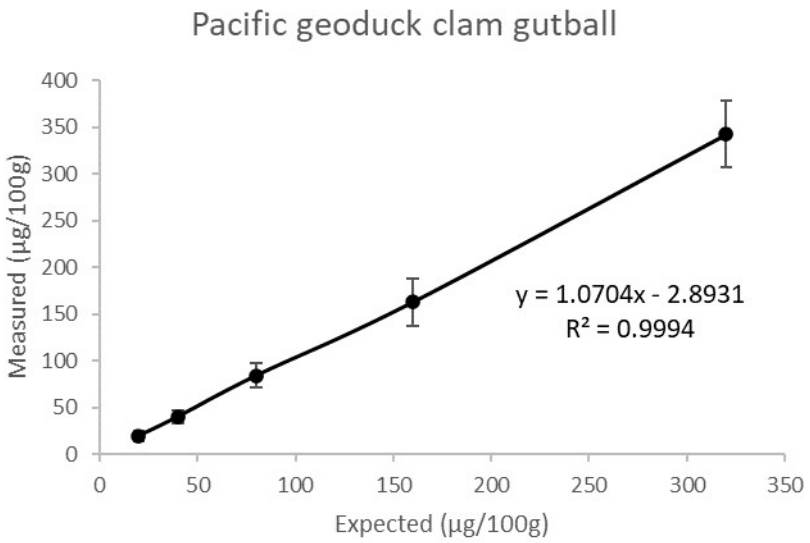
Table 3.2. Expected concentration and average measured concentrations measured by RBA (µg saxitoxin diHCl equiv /100 g shellfish tissue). Shellfish were spiked to five different concentrations with STX, and three replicates each of five samples per matrix were analyzed (n=20).

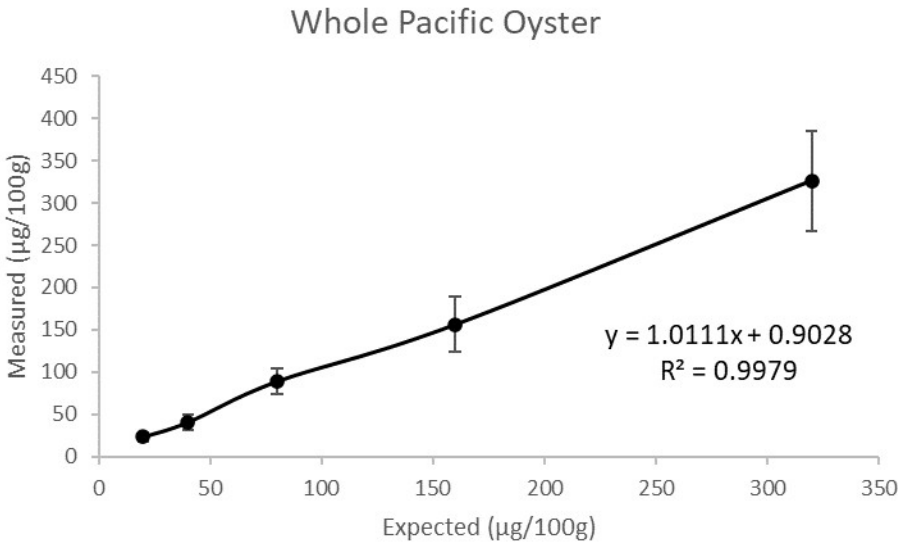
	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Pacific geoduck clam gutball	20	19.8	5.3	26.9
	40	40.4	6.7	16.7
	80	84.3	13.2	15.7
	160	162.7	25.7	15.8
	320	341.9	35.6	10.4

	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Whole Pacific oyster	20	23.5	5.7	24.4
	40	40.7	8.8	21.6
	80	88.9	14.5	16.4
	160	156.3	32.7	20.9
	320	326.3	58.9	18.0

	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Whole hard clam	20	20.1	4.2	21.0
	40	39.3	6.9	17.6
	80	83.3	14.3	17.1
	160	166.1	20.9	12.6
	320	316.1	39.1	12.4

	Expected Concentration (µg/100g)	*Average measured concentration (µg/100g)*	*Standard Deviation*	*%CV*
Razor clam meat	20	18.9	6.8	35.8
	40	38.8	7.1	18.2
	80	77.3	15.5	20.1
	160	146.2	26.5	18.1
	320	309.5	59.1	19.1


Pacific geoduck clam gutball
Measured (µg/100g)
Expected (µg/100g)
y = 1.0704x - 2.8931
R² = 0.9994
0
50
100
150
200
250
300
350
400


Whole Pacific Oyster
Measured (µg/100g)
Expected (µg/100g)
y = 1.0111x + 0.9028
R² = 0.9979
0
50
100
150
200
250
300
350
400
450

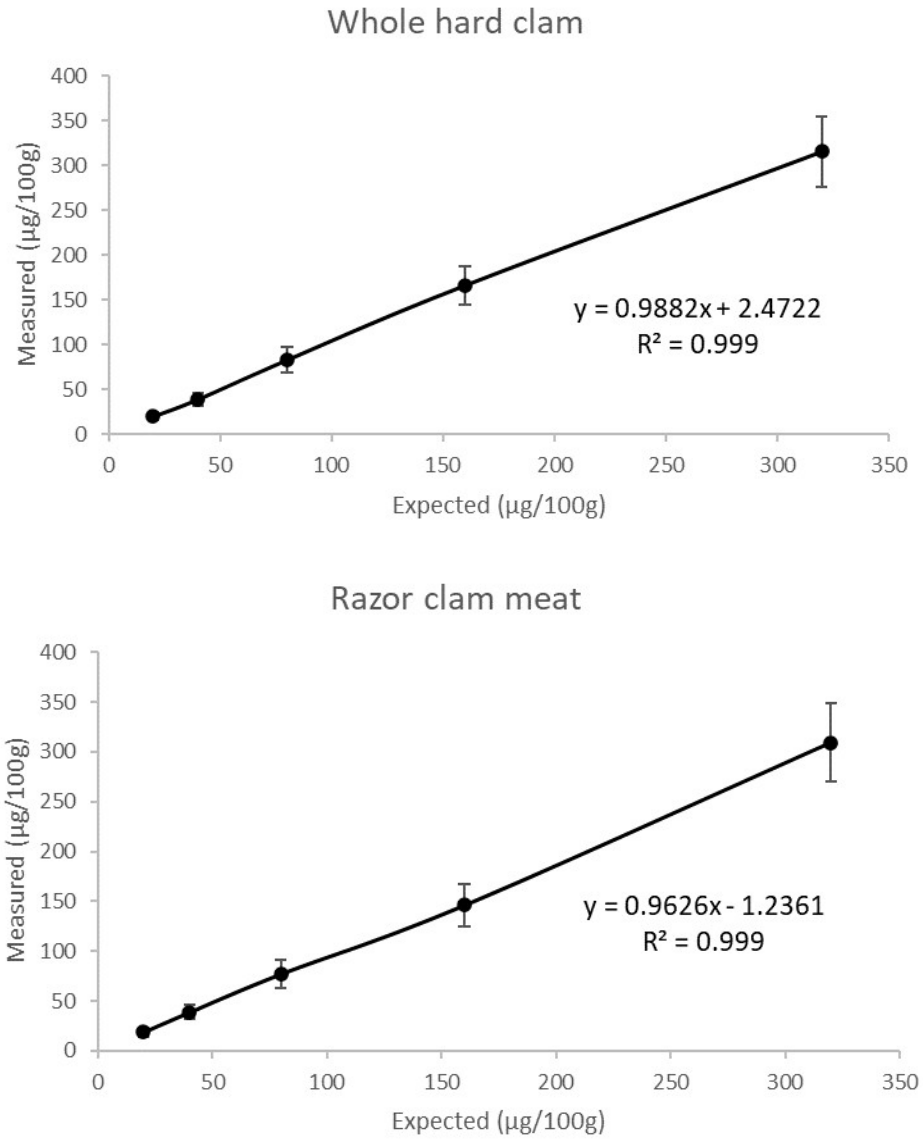


Figure 3.3. Expected concentrations vs. average concentrations measured by RBA (µg STX diHCl equiv./100g shellfish tissue). Five spike levels: 20, 40, 80, 160, and 320 µg STX diHCl equiv./100g shellfish tissue were used for five samples with three replicates per spike level. Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat were spiked with RM 8642a FDA Saxitoxin Dihydrochloride Solution and analyzed (n=60). Error bars represent standard deviation.

Calibration standard (QC check): A reference standard containing 1.8 x 10^{-8} M STX standard (3.0 x 10^{-9} M STX in assay) was prepared in 0.003 M hydrochloric acid, aliquoted in 1 mL volumes, and stored at 4°C for routine use (stable up to 1 month). On the day of the assay, 200 µL of each standard were pipetted into mini-dilution tubes for ease of pipetting into the microplate using an eight-channel pipettor. The QC check standard should fall within ±30% of the stated value (3.0 nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.

In addition to evaluating the linear range, a calibration standard (QC check) was included to assess assay accuracy and consistency. At the WADoH laboratory, two QC checks were performed at the beginning and end of each assay plate. Across all shellfish matrices, a total of 160 QC checks were conducted, yielding an average concentration of 3.17 ± 0.48 nM.

2. Limit of Detection and Limit of Quantitation

The limits of detection (LOD) and quantitation (LOQ) were determined based on the average LOD values obtained from assay plates for each shellfish matrix. These values were derived from the IC_{80} data using the equations below, assuming a 1:10 sample dilution.

(nM STX eq) x (10 (sample dilution)) x (210 µL total volume/35 µL sample) = mM STX eq in extract

(nM STX diHCl eq in extract) x (1L/1000 mL) x (372 ng/nmol) x (1 µg/1000 ng) = µg STX diHCl eq/mL

µg STX diHCl equiv./mL x (mL extract/g shellfish) x (100 g/g)
= µg STX diHCl equiv./100g shellfish tissue

Table 3.3. The average limit of detection (LOD) and limit of quantitation (LOQ) for each shellfish matrix in µg STX /100g shellfish tissue

.

	LOD	*LOQ*
All matrix types	2.01	3.31
Pacific geoduck clam gutball	2.04	3.42
Whole Pacific oyster	1.91	3.13
Whole hard clam	1.89	3.12
Razor clam meat	2.19	3.57

Full Data:

Sample ID	Shellfish Matrix	Spike Level	Spike (µg/100g)	RBA (µg/100g)	Blank value (µg/100g)	RBA-Blank (µg/100g)	Dilution
CGa-16	geoduck	Blank	0	7	7	0	10
CGa-01	geoduck	1/4x	20	32	7	25	50
CGa-03	geoduck	1/4x	20	33	7	26	50
CGa-18	geoduck	1/4x	20	23.5	7	16.5	Avg. of 10 & 50
CGa-06	geoduck	1/2x	40	52	7	45	50
CGa-20	geoduck	1/2x	40	66	7	59	50
CGa-21	geoduck	1/2x	40	45	7	38	50
CGa-07	geoduck	1x	80	92	7	85	50
CGa-08	geoduck	1x	80	99.5	7	92.5	Avg. of 10 & 50
CGa-09	geoduck	1x	80	94	7	87	50
CGa-10	geoduck	2x	160	135	7	128	200
CGa-11	geoduck	2x	160	178	7	171	200
CGa-12	geoduck	2x	160	163	7	156	200
CGa-13	geoduck	4x	320	316	7	309	200
CGa-14	geoduck	4x	320	361	7	354	200
CGa-15	geoduck	4x	320	316	7	309	200
CGb-00	geoduck	Blank	0	6	6	0	10
CGb-02	geoduck	1/4x	20	29.5	6	23.5	Avg. of 10 & 50
CGb-03	geoduck	1/4x	20	23.5	6	17.5	Avg. of 10 & 50
CGb-17	geoduck	1/4x	20	26	6	20	50
CGb-20	geoduck	1/2x	40	52	6	46	50
CGb-21	geoduck	1/2x	40	39	6	33	50
CGb-05	geoduck	1/2x	40	46	6	40	50
CGb-07	geoduck	1x	80	86	6	80	50
CGb-08	geoduck	1x	80	85	6	79	50
CGb-09	geoduck	1x	80	77	6	71	50
CGb-10	geoduck	2x	160	192	6	186	200
CGb-11	geoduck	2x	160	138	6	132	50
CGb-12	geoduck	2x	160	167	6	161	200
CGb-13	geoduck	4x	320	304	6	298	200
CGb-14	geoduck	4x	320	309	6	303	200
CGb-15	geoduck	4x	320	380	6	374	200
CGc-16	geoduck	Blank	0	10	10	0	10
CGc-19	geoduck	1/4x	20	29.5	10	19.5	Avg. of 10 & 50
CGc-01	geoduck	1/4x	20	24	10	14	10
CGc-02	geoduck	1/4x	20	21	10	11	50
CGc-04	geoduck	1/2x	40	55	10	45	50
CGc-05	geoduck	1/2x	40	50	10	40	50

CGc-06	geoduck	1/2x	40	40	10	30	50
CGc-07	geoduck	1x	80	110	10	100	50
CGc-08	geoduck	1x	80	115	10	105	50
CGc-09	geoduck	1x	80	84	10	74	50
CGc-10	geoduck	2x	160	230	10	220	200
CGc-11	geoduck	2x	160	135	10	125	200
CGc-12	geoduck	2x	160	167	10	157	200
CGc-13	geoduck	4x	320	343	10	333	200
CGc-14	geoduck	4x	320	354	10	344	200
CGc-15	geoduck	4x	320	442	10	432	200
CGd-16	geoduck	Blank	0	11	11	0	10
CGd-17	geoduck	1/4x	20	24	11	13	10
CGd-18	geoduck	1/4x	20	26	11	15	50
CGd-19	geoduck	1/4x	20	33	11	22	50
CGd-20	geoduck	1/2x	40	49	11	38	50
CGd-21	geoduck	1/2x	40	49	11	38	50
CGd-22	geoduck	1/2x	40	46	11	35	50
CGd-07	geoduck	1x	80	103	11	92	50
CGd-08	geoduck	1x	80	84	11	73	50
CGd-09	geoduck	1x	80	80	11	69	50
CGd-10	geoduck	2x	160	199	11	188	200
CGd-11	geoduck	2x	160	197	11	186	200
CGd-12	geoduck	2x	160	157	11	146	200
CGd-13	geoduck	4x	320	357	11	346	200
CGd-14	geoduck	4x	320	381	11	370	200
CGd-15	geoduck	4x	320	334	11	323	200
CGe-00	geoduck	Blank	0	6	6	0	10
CGe-01	geoduck	1/4x	20	25.5	6	19.5	Avg. of 10 & 50
CGe-02	geoduck	1/4x	20	33	6	27	50
CGe-03	geoduck	1/4x	20	34	6	28	50
CGe-04	geoduck	1/2x	40	47	6	41	50
CGe-05	geoduck	1/2x	40	44	6	38	50
CGe-06	geoduck	1/2x	40	46	6	40	50
CGe-07	geoduck	1x	80	88	6	82	50
CGe-08	geoduck	1x	80	72	6	66	50
CGe-09	geoduck	1x	80	115	6	109	50
CGe-10	geoduck	2x	160	156	6	150	200
CGe-11	geoduck	2x	160	175	6	169	200
CGe-12	geoduck	2x	160	172	6	166	200
CGe-13	geoduck	4x	320	323	6	317	200
CGe-14	geoduck	4x	320	379	6	373	200
CGe-15	geoduck	4x	320	350	6	344	200

OPa-00	oyster	Blank	0	0	0	0	10
OPa-32	oyster	1/4x	20	24	0	24	Avg. of 10 & 50
OPa-33	oyster	1/4x	20	23.5	0	23.5	Avg. of 10 & 50
OPa-34	oyster	1/4x	20	24.5	0	24.5	Avg. of 10 & 50
OPa-04	oyster	1/2x	40	47	0	47	50
OPa-21	oyster	1/2x	40	35	0	35	50
OPa-06	oyster	1/2x	40	46	0	46	50
OPa-07	oyster	1x	80	87	0	87	50
OPa-08	oyster	1x	80	102	0	102	50
OPa-09	oyster	1x	80	85	0	85	50
OPa-10	oyster	2x	160	186	0	186	200
OPa-11	oyster	2x	160	119	0	119	200
OPa-12	oyster	2x	160	159	0	159	200
OPa-13	oyster	4x	320	230	0	230	200
OPa-14	oyster	4x	320	319	0	319	200
OPa-15	oyster	4x	320	303	0	303	200
OPb-00	oyster	Blank	0	6	6	0	10
OPb-01	oyster	1/4x	20	29	6	23	50
OPb-02	oyster	1/4x	20	27	6	21	50
OPb-03	oyster	1/4x	20	48	6	42	50
OPb-04	oyster	1/2x	40	32	6	26	50
OPb-05	oyster	1/2x	40	41	6	35	50
OPb-06	oyster	1/2x	40	42	6	36	50
OPb-07	oyster	1x	80	101	6	95	50
OPb-08	oyster	1x	80	74	6	68	50
OPb-09	oyster	1x	80	85	6	79	50
OPb-10	oyster	2x	160	168	6	162	200
OPb-11	oyster	2x	160	142	6	136	200
OPb-12	oyster	2x	160	126	6	120	200
OPb-13	oyster	4x	320	357	6	351	200
OPb-14	oyster	4x	320	269	6	263	200
OPb-15	oyster	4x	320	394	6	388	200
OPc-00	oyster	Blank	0	0	0	0	10
OPc-01	oyster	1/4x	20	23.5	0	23.5	Avg. of 10 & 50
OPc-18	oyster	1/4x	20	24	0	24	Avg. of 10 & 50
OPc-19	oyster	1/4x	20	20.5	0	20.5	Avg. of 10 & 50
OPc-20	oyster	1/2x	40	28	0	28	50
OPc-21	oyster	1/2x	40	40	0	40	50
OPc-06	oyster	1/2x	40	42	0	42	50
OPc-07	oyster	1x	80	80	0	80	50
OPc-08	oyster	1x	80	75	0	75	50
OPc-09	oyster	1x	80	91	0	91	50

OPc-10	oyster	2x	160	128	0	128	200
OPc-11	oyster	2x	160	192	0	192	200
OPc-12	oyster	2x	160	91	0	91	200
OPc-13	oyster	4x	320	268	0	268	200
OPc-14	oyster	4x	320	324	0	324	200
OPc-15	oyster	4x	320	295	0	295	200
OPd-16	oyster	Blank	0	5	5	0	10
OPd-01	oyster	1/4x	20	30	5	25	Avg. of 10 & 50
OPd-02	oyster	1/4x	20	24	5	19	50
OPd-19	oyster	1/4x	20	28	5	23	50
OPd-04	oyster	1/2x	40	46	5	41	50
OPd-05	oyster	1/2x	40	45	5	40	50
OPd-22	oyster	1/2x	40	44	5	39	50
OPd-07	oyster	1x	80	79	5	74	50
OPd-08	oyster	1x	80	115	5	110	50
OPd-09	oyster	1x	80	100	5	95	50
OPd-10	oyster	2x	160	168	5	163	200
OPd-11	oyster	2x	160	167	5	162	200
OPd-12	oyster	2x	160	181	5	176	200
OPd-13	oyster	4x	320	400	5	395	200
OPd-14	oyster	4x	320	311	5	306	200
OPd-15	oyster	4x	320	375	5	370	200
OPe-16	oyster	Blank	0	5	5	0	10
OPe-01	oyster	1/4x	20	22	5	17	Avg. of 10 & 50
OPe-18	oyster	1/4x	20	24	5	19	Avg. of 10 & 50
OPe-19	oyster	1/4x	20	25.5	5	20.5	Avg. of 10 & 50
OPe-20	oyster	1/2x	40	48	5	43	50
OPe-21	oyster	1/2x	40	57	5	52	50
OPe-0	oyster	1/2x	40	66	5	61	50
OPe-07	oyster	1x	80	115	5	110	50
OPe-08	oyster	1x	80	116	5	111	50
OPe-09	oyster	1x	80	77	5	72	50
OPe-10	oyster	2x	160	202	5	197	200
OPe-11	oyster	2x	160	210	5	205	200
OPe-12	oyster	2x	160	153	5	148	200
OPe-13	oyster	4x	320	374	5	369	200
OPe-14	oyster	4x	320	449	5	444	200
OPe-15	oyster	4x	320	275	5	270	200
CBa-00	butter clam	Blank	0	9	9	0	10
CBa-01	butter clam	1/4x	20	26	9	17	50
CBa-02	butter clam	1/4x	20	23	9	14	50
CBa-03	butter clam	1/4x	20	20	9	11	50

CBa-04	butter clam	1/2x	40	42	9	33	50
CBa-05	butter clam	1/2x	40	40	9	31	50
CBa-06	butter clam	1/2x	40	39	9	30	50
CBa-07	butter clam	1x	80	77	9	68	50
CBa-08	butter clam	1x	80	71	9	62	50
CBa-09	butter clam	1x	80	97	9	88	50
CBa-10	butter clam	2x	160	175	9	166	200
CBa-11	butter clam	2x	160	151	9	142	200
CBa-12	butter clam	2x	160	177	9	168	200
CBa-13	butter clam	4x	320	330	9	321	200
CBa-14	butter clam	4x	320	265	9	256	200
CBa-15	butter clam	4x	320	307	9	298	200
CMb-28	manila clam	Blank	0	0	0	0	10
CMb-01	manila clam	1/4x	20	23	0	23	10
CMb-02	manila clam	1/4x	20	17	0	17	50
CMb-03	manila clam	1/4x	20	17.5	0	17.5	Avg. of 10 & 50
CMb-04	manila clam	1/2x	40	42	0	42	50
CMb-05	manila clam	1/2x	40	45	0	45	50
CMb-06	manila clam	1/2x	40	47	0	47	50
CMb-07	manila clam	1x	80	87	0	87	50
CMb-08	manila clam	1x	80	85	0	85	50
CMb-09	manila clam	1x	80	70	0	70	50
CMb-10	manila clam	2x	160	159	0	159	200
CMb-11	manila clam	2x	160	153	0	153	200
CMb-12	manila clam	2x	160	132	0	132	200
CMb-13	manila clam	4x	320	280	0	280	200
CMb-14	manila clam	4x	320	310	0	310	200
CMb-15	manila clam	4x	320	258	0	258	200
CMc-00	manila clam	Blank	0	0	0	0	10
CMc-01	manila clam	1/4x	20	23	0	23	Avg. of 10 & 50
CMc-02	manila clam	1/4x	20	23	0	23	50
CMc-03	manila clam	1/4x	20	21	0	21	50
CMc-04	manila clam	1/2x	40	42	0	42	50
CMc-05	manila clam	1/2x	40	50	0	50	50
CMc-06	manila clam	1/2x	40	47	0	47	50
CMc-07	manila clam	1x	80	113	0	113	50
CMc-08	manila clam	1x	80	72	0	72	50
CMc-09	manila clam	1x	80	92	0	92	50
CMc-10	manila clam	2x	160	189	0	189	200
CMc-11	manila clam	2x	160	146	0	146	200
CMc-12	manila clam	2x	160	197	0	197	200
CMc-13	manila clam	4x	320	342	0	342	200

CMc-14	manila clam	4x	320	342	0	342	200
CMc-15	manila clam	4x	320	372	0	372	200
CMd-00	manila clam	Blank	0	6	6	0	10
CMd-01	manila clam	1/4x	20	33	6	27	50
CMd-02	manila clam	1/4x	20	29.5	6	23.5	Avg. of 10 & 50
CMd-03	manila clam	1/4x	20	25	6	19	10
CMd-04	manila clam	1/2x	40	48	6	42	50
CMd-05	manila clam	1/2x	40	38	6	32	50
CMd-06	manila clam	1/2x	40	37	6	31	50
CMd-07	manila clam	1x	80	99	6	93	50
CMd-08	manila clam	1x	80	74	6	68	50
CMd-09	manila clam	1x	80	108	6	102	50
CMd-10	manila clam	2x	160	196	6	190	200
CMd-11	manila clam	2x	160	152	6	146	200
CMd-12	manila clam	2x	160	188	6	182	200
CMd-13	manila clam	4x	320	329	6	323	200
CMd-14	manila clam	4x	320	310	6	304	200
CMd-15	manila clam	4x	320	408	6	402	200
CMe-00	manila clam	Blank	0	4	4	0	10
CMe-01	manila clam	1/4x	20	25	4	21	50
CMe-02	manila clam	1/4x	20	25	4	21	50
CMe-03	manila clam	1/4x	20	28	4	24	10
CMe-04	manila clam	1/2x	40	45	4	41	50
CMe-05	manila clam	1/2x	40	48	4	44	50
CMe-06	manila clam	1/2x	40	37	4	33	50
CMe-07	manila clam	1x	80	91	4	87	50
CMe-08	manila clam	1x	80	94	4	90	50
CMe-09	manila clam	1x	80	76	4	72	50
CMe-10	manila clam	2x	160	197	4	193	200
CMe-11	manila clam	2x	160	180	4	176	200
CMe-12	manila clam	2x	160	156	4	152	200
CMe-13	manila clam	4x	320	329	4	325	200
CMe-14	manila clam	4x	320	321	4	317	200
CMe-15	manila clam	4x	320	296	4	292	200
CRa-00	razor clam	Blank	0	10	10	0	10
CRa-01	razor clam	1/4x	20	25	10	15	Avg. 10 & 50
CRa-02	razor clam	1/4x	20	43	10	33	50
CRa-03	razor clam	1/4x	20	30	10	20	10
CRa-04	razor clam	1/2x	40	46	10	36	50
CRa-05	razor clam	1/2x	40	48	10	38	50
CRa-06	razor clam	1/2x	40	47	10	37	50
CRa-07	razor clam	1x	80	64	10	54	50

CRa-08	razor clam	1x	80	78	10	68	50
CRa-09	razor clam	1x	80	99	10	89	50
CRa-10	razor clam	2x	160	140	10	130	50
CRa-11	razor clam	2x	160	139	10	129	200
CRa-12	razor clam	2x	160	119	10	109	200
CRa-13	razor clam	4x	320	310	10	300	200
CRa-14	razor clam	4x	320	281	10	271	200
CRa-15	razor clam	4x	320	285	10	275	200
CRb-00	razor clam	Blank	0	10	10	0	10
CRb-01	razor clam	1/4x	20	27	10	17	50
CRb-02	razor clam	1/4x	20	38	10	28	50
CRb-03	razor clam	1/4x	20	35	10	25	50
CRb-04	razor clam	1/2x	40	66	10	56	50
CRb-05	razor clam	1/2x	40	39	10	29	50
CRb-20	razor clam	1/2x	40	53	10	43	50
CRb-07	razor clam	1x	80	69	10	59	50
CRb-08	razor clam	1x	80	104	10	94	50
CRb-09	razor clam	1x	80	81	10	71	50
CRb-10	razor clam	2x	160	144	10	134	200
CRb-11	razor clam	2x	160	165	10	155	200
CRb-12	razor clam	2x	160	212	10	202	200
CRb-13	razor clam	4x	320	317	10	307	200
CRb-14	razor clam	4x	320	360	10	350	200
CRb-15	razor clam	4x	320	261	10	251	200
CRc-00	razor clam	Blank	0	14	14	0	10
CRc-01	razor clam	1/4x	20	28	14	14	50
CRc-02	razor clam	1/4x	20	32	14	18	50
CRc-03	razor clam	1/4x	20	32	14	18	50
CRc-04	razor clam	1/2x	40	54	14	40	50
CRc-05	razor clam	1/2x	40	53	14	39	50
CRc-06	razor clam	1/2x	40	56	14	42	50
CRc-07	razor clam	1x	80	72	14	58	50
CRc-08	razor clam	1x	80	109	14	95	50
CRc-09	razor clam	1x	80	115	14	101	50
CRc-10	razor clam	2x	160	139	14	125	200
CRc-11	razor clam	2x	160	160	14	146	200
CRc-12	razor clam	2x	160	183	14	169	200
CRc-13	razor clam	4x	320	352	14	338	200
CRc-14	razor clam	4x	320	297	14	283	200
CRc-15	razor clam	4x	320	235	14	221	200
CRd-00	razor clam	Blank	0	11	11	0	10
CRd-01	razor clam	1/4x	20	37	11	26	50

CRd-02	razor clam	1/4x	20	21	11	10	Avg. 10 & 50
CRd-03	razor clam	1/4x	20	30.5	11	19.5	Avg. 10 & 50
CRd-04	razor clam	1/2x	40	50	11	39	50
CRd-05	razor clam	1/2x	40	45	11	34	50
CRd-06	razor clam	1/2x	40	37	11	26	50
CRd-07	razor clam	1x	80	95	11	84	50
CRd-08	razor clam	1x	80	84	11	73	50
CRd-09	razor clam	1x	80	105	11	94	50
CRd-10	razor clam	2x	160	117	11	106	50
CRd-11	razor clam	2x	160	169	11	158	200
CRd-12	razor clam	2x	160	168	11	157	200
CRd-13	razor clam	4x	320	268	11	257	200
CRd-14	razor clam	4x	320	452	11	441	200
CRd-15	razor clam	4x	320	407	11	396	200
CRe-00	razor clam	Blank	0	15	15	0	10
CRe-01	razor clam	1/4x	20	34	15	19	50
CRe-02	razor clam	1/4x	20	27	15	12	50
CRe-03	razor clam	1/4x	20	24	15	9	Avg. 10 & 50
CRe-04	razor clam	1/2x	40	50	15	35	50
CRe-05	razor clam	1/2x	40	61	15	46	50
CRe-06	razor clam	1/2x	40	57	15	42	50
CRe-07	razor clam	1x	80	101	15	86	50
CRe-08	razor clam	1x	80	76	15	61	50
CRe-09	razor clam	1x	80	87	15	72	50
CRe-10	razor clam	2x	160	201	15	186	200
CRe-11	razor clam	2x	160	152	15	137	200
CRe-12	razor clam	2x	160	165	15	150	200
CRe-13	razor clam	4x	320	286	15	271	200
CRe-14	razor clam	4x	320	376	15	361	200
CRe-15	razor clam	4x	320	335	15	320	200

4. **Measurement uncertainty:** Measurement uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including overall precision of the complete method, the method and laboratory bias and matrix effects.

Suggested procedure: Use shellfish free of the target analyte(s). For each shellfish type of interest use a minimum of 10-12 animals per sample and prepare as a homogenate. Take two aliquots of the sample homogenate appropriately sized for the work and spike one with the target analyte(s) at the action level. Do not spike the second aliquot as this is the sample blank. Process each aliquot to determine the concentration for the target analyte(s). Repeat this process with a minimum of 15 samples for each shellfish type of interest collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots.

Data:

Working range/Range of interest: 80 µg saxitoxin diHCl equiv/100 g shellfish tissue
Range in spiking levels used: 80 µg saxitoxin diHCl equiv/100 g shellfish tissue
Sample Type: Pacific geoduck clam gutball, whole Pacific oyster, whole hard clam (e.g., butter clam, Pacific littleneck clam, manila clam), and razor clam meat
Agar used to determine spike concentration: N/A
~~Organism~~ (Toxin) used for spiking: RM 8642a FDA Saxitoxin Dihydrochloride Solution

Data Handling

Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

Data Summary

All data were log-transformed to measure uncertainty.

Calculate measurement uncertainty:

- Pacific geoduck clam gutball: 6.83×10^{-5} – 0.047
- Whole Pacific oyster: 0.017 – 0.068
- Whole hard clam: -2.90×10^{-4} – 0.056
- Razor clam meat: -0.074 – -0.006

Full Data:

Group	Sample ID	Shellfish Matrix	Spike (µg/100g)	Blank value (µg/100g)	RBA-Blank (µg/100g)
A	CGa-07	geoduck	80	7	85
A	CGa-08	geoduck	80	7	92.5
A	CGa-09	geoduck	80	7	87
B	CGb-07	geoduck	80	6	80
B	CGb-08	geoduck	80	6	79
B	CGb-09	geoduck	80	6	71
C	CGc-07	geoduck	80	10	100
C	CGc-08	geoduck	80	10	105
C	CGc-09	geoduck	80	10	74
D	CGd-07	geoduck	80	11	92
D	CGd-08	geoduck	80	11	73
D	CGd-09	geoduck	80	11	69
E	CGe-07	geoduck	80	6	82
E	CGe-08	geoduck	80	6	66
E	CGe-09	geoduck	80	6	109
F	CGf-01	geoduck	80	5	84
G	CGg-02	geoduck	80	5	99
H	CGh-01	geoduck	80	5	86
I	CGi-01	geoduck	80	11	78
J	CGj-01	geoduck	80	8	77
K	CGk-01	geoduck	80	0	88
L	CGL-01	geoduck	80	8	81
M	CGm-01	geoduck	80	4	86
N	CGn-01	geoduck	80	9	85
O	CGo-01	geoduck	80	4	100
A	OPa-07	oyster	80	0	87
A	OPa-08	oyster	80	0	102
A	OPa-09	oyster	80	0	85
B	OPb-07	oyster	80	6	95
B	OPb-08	oyster	80	6	68
B	OPb-09	oyster	80	6	79
C	OPc-07	oyster	80	0	80
C	OPc-08	oyster	80	0	75
C	OPc-09	oyster	80	0	91
D	OPd-07	oyster	80	5	74
D	OPd-08	oyster	80	5	110
D	OPd-09	oyster	80	5	95
E	OPe-07	oyster	80	5	110

E	OPe-08	oyster	80	5	111
E	OPe-09	oyster	80	5	72
F	OPf-01	oyster	80	0	100
G	OPg-01	oyster	80	0	101
H	OPh-01	oyster	80	0	87
I	OPi-01	oyster	80	0	82
J	OPj-01	oyster	80	0	105
K	OPk-01	oyster	80	7	74
L	OPL-01	oyster	80	0	88
M	OPm-03	oyster	80	0	83
N	OPn-01	oyster	80	0	89
O	OPo-01	oyster	80	0	85
A	CBa-07	butter clam	80	9	68
A	CBa-08	butter clam	80	9	62
A	CBa-09	butter clam	80	9	88
B	CMb-07	manila clam	80	0	87
B	CMb-08	manila clam	80	0	85
B	CMb-09	manila clam	80	0	70
C	CMc-07	manila clam	80	0	113
C	CMc-08	manila clam	80	0	72
C	CMc-09	manila clam	80	0	92
D	CMd-07	manila clam	80	6	93
D	CMd-08	manila clam	80	6	68
D	CMd-09	manila clam	80	6	102
E	CMe-07	manila clam	80	4	87
E	CMe-08	manila clam	80	4	90
E	CMe-09	manila clam	80	4	72
F	CBf-01	butter clam	80	0	78
G	CLg-01	littleneck clam	80	9.5	103.5
H	CBh-01	butter clam	80	7	87
I	CMi-03	manila clam	80	11	106
J	CBj-01	butter clam	80	9	85
K	CLk-01	littleneck clam	80	0	101
L	CLL-01	littleneck clam	80	0	84
M	CLm-01	littleneck clam	80	4	76
N	CMn-01	manila clam	80	3	100
O	CLo-01	littleneck clam	80	4	88
A	CRa-07	razor clam	80	10	54
A	CRa-08	razor clam	80	10	68
A	CRa-09	razor clam	80	10	89
B	CRb-07	razor clam	80	10	59
B	CRb-08	razor clam	80	10	94

B	CRb-09	razor clam	80	10	71
C	CRc-07	razor clam	80	14	58
C	CRc-08	razor clam	80	14	95
C	CRc-09	razor clam	80	14	101
D	CRd-07	razor clam	80	11	84
D	CRd-08	razor clam	80	11	73
D	CRd-09	razor clam	80	11	94
E	CRe-07	razor clam	80	15	86
E	CRe-08	razor clam	80	15	61
E	CRe-09	razor clam	80	15	72
F	CRf-01	razor clam	80	18	67
G	CRg-01	razor clam	80	10	74
H	CRh-01	razor clam	80	7	88
I	CRi-01	razor clam	80	9	80
J	CRj-01	razor clam	80	11	54
K	CRk-01	razor clam	80	10	72
L	CRL-01	razor clam	80	17	63
M	CRm-01	razor clam	80	8	58
N	CRn-01	razor clam	80	9	77
O	CRo-01	razor clam	80	8	62

5. **Comparability** is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:
New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method:
This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data Handling:

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.

2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.

3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.

4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.

5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.

- If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.

- If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.

- If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.

- If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:

Value for the test of symmetry for the distribution of the data generated by the officially recognized method – **Mouse Bioassay for all shellfish species except for northern razor clam meat, which was analyzed by post-column oxidation method.**

- Pacific geoduck clam gutball: 0.09
- Whole Pacific oyster: 0.99
- Whole hard clam: -0.04
- Razor clam meat: -0.30

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method – **Receptor Binding Assay**

- Pacific geoduck clam gutball: -0.01
- Whole Pacific oyster: 0.23
- Whole hard clam: -0.19
- Razor clam meat: -0.15

Variance of the data generated from the officially recognized analytical method– **Mouse Bioassay for all shellfish species except for northern razor clam meat, which was analyzed by post-column oxidation method.**

- Pacific geoduck clam gutball: 0.128
- Whole Pacific oyster: 0.110
- Whole hard clam: 0.136
- Razor clam meat: 0.050

Variance of the data generated from the proposed alternative analytical method– **Receptor Binding Assay**

- Pacific geoduck clam gutball: 0.161
- Whole Pacific oyster: 0.085

- Whole hard clam: 0.105
- Razor clam meat: 0.049

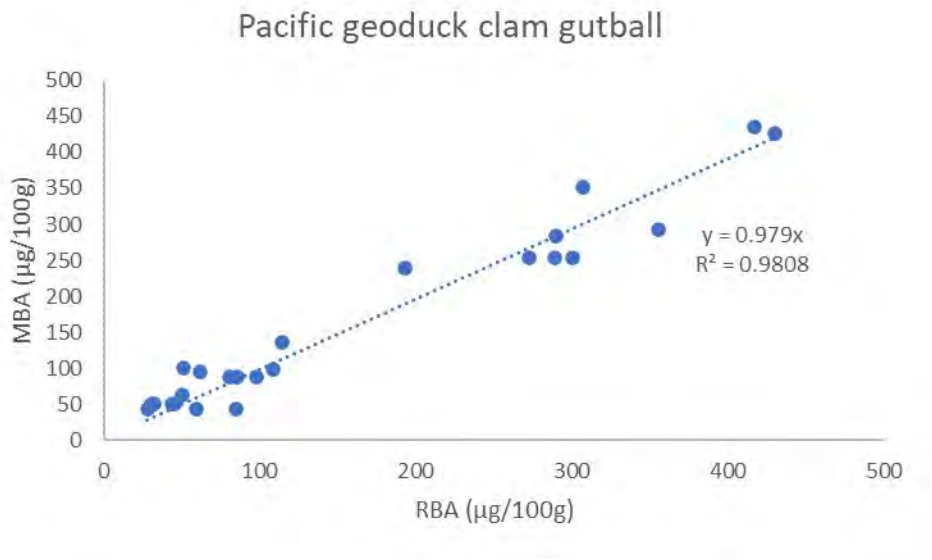
Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods

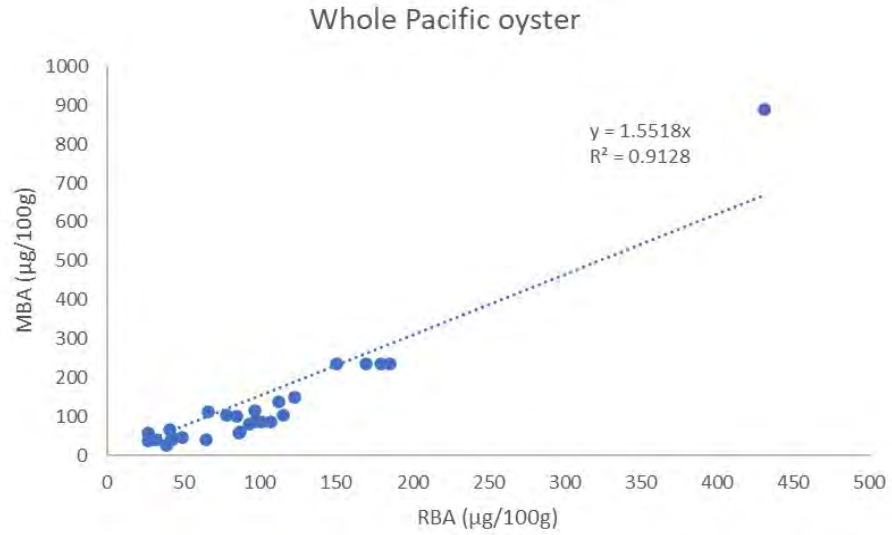
- Pacific geoduck clam gutball: 1.26
- Whole Pacific oyster: 1.29
- Whole hard clam: 1.29
- Razor clam meat: 1.02

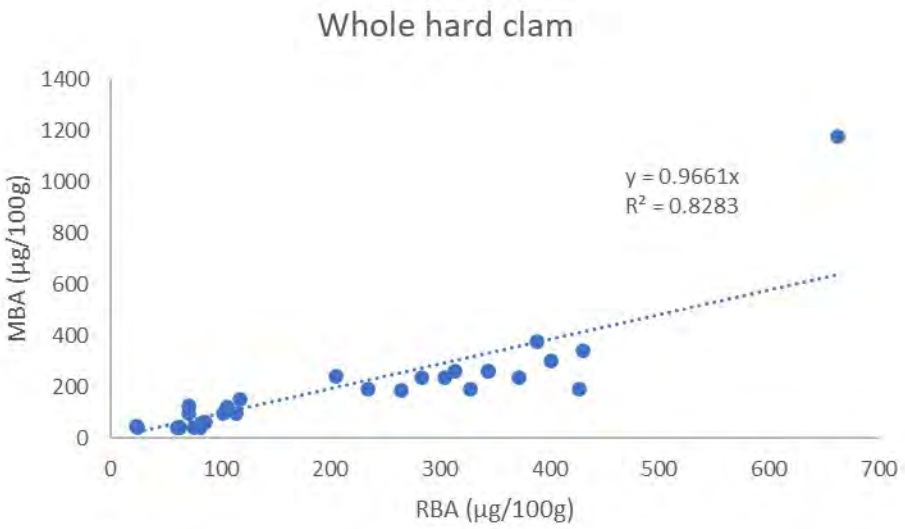
Is there a significant difference between the analytical methods Y/N

- Pacific geoduck clam gutball: No (p = 0.300)
- Whole Pacific oyster: No (p = 0.053)
- Whole hard clam: No (p = 0.0725)
- Razor clam meat: No (p = 0.086)

A total of 111 samples were tested using a previously established method with MBA for all shellfish matrix type except for razor clam meat, which used PCOX, and RBA was used for all shellfish matrix type: Pacific geoduck clam gutball (n=24), whole Pacific oyster (n=27), whole hard clam (n=29), and razor clam meat (n = 31).




Whole Pacific oyster
y = 1.5518x
R² = 0.9128
MBA (µg/100g)
RBA (µg/100g)
0
100
200
300
400
500
600
700
800
900
1000
0
50
100
150
200
250
300
350
400
450
500


Whole hard clam
y = 0.9661x
R² = 0.8283
MBA (µg/100g)
RBA (µg/100g)
0
200
400
600
800
1000
1200
1400
0
100
200
300
400
500
600
700

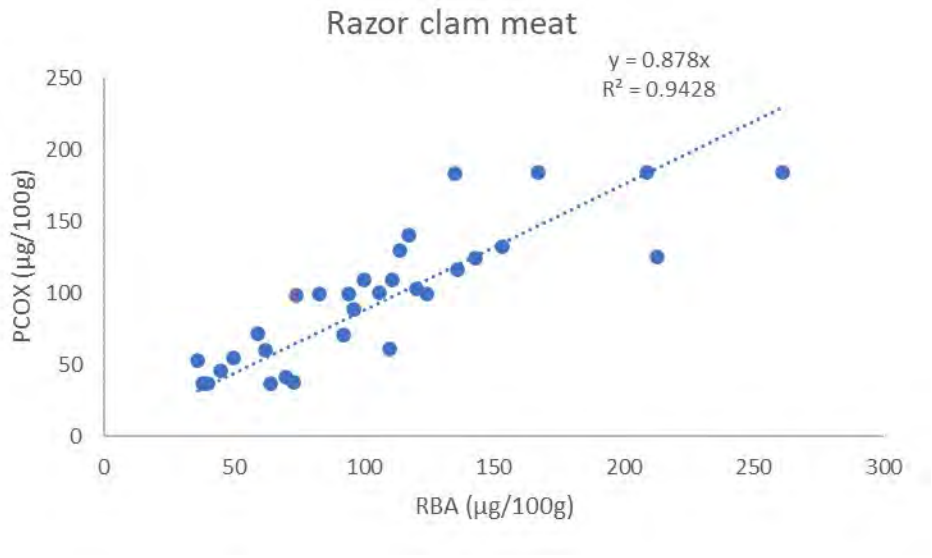


Figure 5.1. Linear regression of MBA versus RBA results in µg STX/100 g shellfish tissue for each shellfish matrix type except for razor clam meat, which was analyzed by PCOX and RBA.

Table 5.1. Average MBA and RBA concentrations for all shellfish matrix type except for razor clam meat (PCOX and RBA) in µg STX/100 g shellfish tissue, and average percent recovery (%) for each matrix type.

Matrix Type	*Avg. MBA Concentration*	*Avg. RBA Concentration*	*Average % Recovery*
Pacific Geoduck clam gutball	165.6	164.4	99.26
Whole Pacific oyster	127.4	98.38	77.22
Whole hard clam	191.3	239.0	124.9
Razor clam meat	97.26	106.3	109.3

Full Data:

Sample ID	Shellfish Matrix	Harvest Area	Harvest Date	Season	RBA (ug/100g)	MBA	% error	% recovery
CG31-1	geoduck	Agate Passage, Kitsap - WA	10/23/2023	Fall	289	254	13.78	113.8
CG31-2	geoduck	Agate Passage, Kitsap - WA	10/23/2023	Fall	301	254	18.50	118.5
CG31-3	geoduck	Agate Passage, Kitsap - WA	10/23/2023	Fall	273	254	7.480	107.5
CG32-1	geoduck	Agate Passage, Kitsap - WA	10/31/2023	Fall	290	283	2.473	102.5
CG33-1	geoduck	Beckett Beach, Kitsap - WA	10/29/2021	Fall	51	100	-49.00	51.00
CG34-1	geoduck	Dungeness West, Clallam - WA	9/25/2023	Fall	430	427	0.7030	100.7
CG35-1	geoduck	Beckett Beach, Kitsap - WA	11/9/2021	Fall	81	87	-6.897	93.10
CG35-2	geoduck	Beckett Beach, Kitsap - WA	11/9/2021	Fall	98	87	12.64	112.6
CG35-3	geoduck	Beckett Beach, Kitsap - WA	11/9/2021	Fall	86	87	-1.149	98.85
CG36-1	geoduck	Agate Pass, Kitsap - WA	10/8/2023	Fall	193	239	-19.25	80.75
CG37-1	geoduck	Manzanita Tract, Kitsap - WA	10/1/2023	Fall	417	435	-4.14	95.86
CG38-1	geoduck	President Point, Kitsap - WA	9/26/2021	Fall	32	50	-36.00	64.00
CG38-2	geoduck	President Point, Kitsap - WA	9/26/2021	Fall	44	50	-12.00	88.00
CG38-3	geoduck	President Point, Kitsap - WA	9/26/2021	Fall	45	50	-10.00	90.00
CG39-1	geoduck	Beckett Beach, Jefferson - WA	10/22/2021	Fall	114	136	-16.18	83.82
CG40-1	geoduck	Coon Bay, Kitsap - WA	9/26/2021	Fall	59	43	37.21	137.2
CG40-2	geoduck	Coon Bay, Kitsap - WA	9/26/2021	Fall	85	43	97.67	197.7
CG40-3	geoduck	Coon Bay, Kitsap - WA	9/26/2021	Fall	28	43	-34.88	65.12
CG41-1	geoduck	Beckett Beach, Jefferson - WA	9/25/2021	Fall	62	94	-34.04	65.96
CG42-1	geoduck	Beckett Beach, Jefferson - WA	11/19/2021	Fall	109	98	11.22	111.2
CG43-1	geoduck	Beckett Beach, Jefferson - WA	10/8/2021	Fall	307	352	-12.78	87.22
CG44-1	geoduck	Beckett Beach, Jefferson - WA	10/15/2021	Fall	356	293	21.50	121.5
CG45-1	geoduck	Beckett Beach, Jefferson - WA	11/12/2021	Fall	31	50	-38.00	62.00

CG46-1	geoduck	Beckett Beach, Jefferson - WA	6/22/2023	Summer	50	63	-20.64	79.37
OP19-3	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	101	87	16.09	116.1
OP19-4	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	97	87	11.49	111.5
OP19-5	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	107	87	22.99	123.0
OP20-1	oyster	Bay Center (Willapa Bay), Pacific - WA	5/29/2025	Spring	123	150	-18.00	82.00
OP21-1	oyster	Bruceport (Willapa Bay), Pacific - WA	5/30/2024	Spring	85	101	-15.84	84.16
OP22-1	oyster	Stony Point (Willapa Bay), Pacific - WA	5/30/2024	Spring	112	138	-18.84	81.16
OP23-1	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/30/2024	Spring	86	58	48.28	148.3
OP24-1	oyster	Nahcotta Area (Willapa Bay), Pacific - WA	5/31/2024	Spring	93	82	13.42	113.4
OP25-1	oyster	Tribal Center (Agate Pass), Kitsap - WA	9/19/2023	Summer	179	235	-23.83	76.17
OP25-2	oyster	Tribal Center (Agate Pass), Kitsap - WA	9/19/2023	Summer	170	235	-27.66	72.34
OP25-3	oyster	Tribal Center (Agate Pass), Kitsap - WA	9/19/2023	Summer	185	235	-21.28	78.72
OP26-1	oyster	Port Madison, Kitsap - WA	9/1/2023	Summer	150	237	-36.71	63.29
OP27-1	oyster	Kilisut Harbor, Jefferson - WA	9/15/2022	Summer	115	104	10.58	110.6
OP28-1	oyster	Drayton Harbor, Whatcom - WA	6/29/2022	Summer	42	47	-10.64	89.36
OP36-1	oyster	Agate Pass, Kitsap - WA	9/10/2023	Summer	431	888	-51.46	48.54
OP37-1	oyster	Agate Pass, Kitsap - WA	9/24/2023	Fall	78	104	-25.00	75.00
OP38-1	oyster	Samish Bay, Skagit - WA	9/28/2021	Fall	87	62	40.32	140.3
OP39-1	oyster	Samish Bay - West, Skagit - WA	9/28/2021	Fall	29	41	-29.27	70.73
OP40-1	oyster	Samish Bay - West, Skagit - WA	9/22/2021	Fall	41	68	-39.71	60.29

OP41-1	oyster	Samish Bay - West, Skagit - WA	9/27/2021	Fall	49	46	6.520	106.5
OP42-1	oyster	Samish Bay, Skagit - WA	9/12/2023	Summer	26.5	60	-55.83	44.17
OP43-1	oyster	Mill Point, Jefferson - WA	9/27/2021	Fall	32	40	-20.00	80.00
OP43-2	oyster	Mill Point, Jefferson - WA	9/27/2021	Fall	65	40	62.50	162.5
OP43-3	oyster	Mill Point, Jefferson - WA	9/27/2021	Fall	42	40	5.000	105.0
OP44-1	oyster	Bay Center Area, Pacific - WA	6/5/2024	Spring	97	115	-15.65	84.35
OP45-1	oyster	Stony Point, Pacific - WA	5/26/2024	Spring	66	114	-42.11	57.90
OP46-1	oyster	Chuckanut Bay, Whatcom - WA	5/28/2023	Spring	27	39	-30.77	69.23
CB16-1	butter clam	Samish Island, Skagit - WA	5/6/2022	Spring	63	40	57.50	157.5
CB16-2	butter clam	Samish Island, Skagit - WA	5/6/2022	Spring	61	40	52.50	152.5
CB16-3	butter clam	Samish Island, Skagit - WA	5/6/2022	Spring	76	40	90.00	190.0
CB18-1	butter clam	Camano State Park, Island - WA	4/18/2022	Spring	328	192	70.83	170.8
CB18-2	butter clam	Camano State Park, Island - WA	4/18/2022	Spring	427	192	122.40	222.4
CB18-3	butter clam	Camano State Park, Island - WA	4/18/2022	Spring	235	192	22.40	122.4
CB28-1	butter clam	Grays Marsh Beach, Grays Harbor - WA	5/16/2022	Spring	84	60	40.00	140.0
CB29-1	butter clam	Camano State Park, Island - WA	5/3/2022	Spring	401	300	33.67	133.7
CB30-1	butter clam	Mukilteo Tank Farm, Snohomish - WA	5/4/2022	Spring	431	344	25.29	125.3
CB31-1	butter clam	Camano State Park, Island - WA	5/16/2022	Spring	284	236	20.34	120.3
CB31-2	butter clam	Camano State Park, Island - WA	5/16/2022	Spring	305	236	29.24	129.2
CB31-3	butter clam	Camano State Park, Island - WA	5/16/2022	Spring	372	236	57.63	157.6
CB32-1	butter clam	Mukilteo Tank Farm, Snohomish - WA	5/17/2022	Spring	389	379	2.639	102.6
CB33-1	butter clam	Grays Marsh Beach, Clallam - WA	2/4/2024	Winter	314	260	20.77	120.8
CB34-1	butter clam	Grays Marsh Beach, Clallam -	2/13/2023	Winter	86	63	36.51	136.5

		WA						
CB35-1	butter clam	Camano State Park, Island - WA	2/13/2023	Winter	265	187	41.71	141.7
CB36-1	butter clam	Camano State Park, Island - WA	3/21/2022	Spring	344	261	31.80	131.8
CB37-1	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/17/2024	Winter	205	240	-14.58	85.42
CB38-1	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/8/2022	Winter	102	95	7.368	107.4
CB38-2	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/8/2022	Winter	71	95	-25.26	74.74
CB38-3	butter clam	Grays Marsh Beach, Grays Harbor - WA	3/8/2022	Winter	114	95	20.00	120.0
CB39-1	butter clam	Fort Flagler, Jefferson - WA	1/3/2022	Winter	81	43	88.37	188.4
CM40-1	manila clam	South End/Blyn, Clallam - WA	9/7/2023	Summer	663	1175	-43.57	56.43
CM41-1	manila clam	Penn Cove, Island - WA	8/19/2021	Summer	118	152	-22.37	77.63
CB42-1	butter clam	Mukilteo Tank Farm, Snohomish - WA	7/13/2022	Summer	344	264	30.30	130.3
CM43-1	manila clam	Samish Bay, Whatcom - WA	9/22/2021	Fall	23.5	47	-50.00	50.00
CM46-1	manila clam	Useless Bay, Island - WA	9/20/2021	Summer	24	40	-40.00	60.00
CM47-1	manila clam	Useless Bay, Island - WA	9/8/2021	Summer	106	123	-13.82	86.18
CM48-1	manila clam	Useless Bay, Island - WA	9/1/2021	Summer	71	126	-43.65	56.35

Sample ID	Shellfish Matrix	Harvest Area	Harvest Date	Season	RBA	PCOX	% error	% recovery
CR16-1	razor clam	Pacific, Pacific County Coast - WA	2/13/2024	Winter	167	184	-9.24	90.76
CR16-2	razor clam	Pacific County Coast, Pacific - WA	2/13/2024	Winter	261	184	41.85	141.8
CR16-3	razor clam	Pacific County Coast, Pacific - WA	2/13/2024	Winter	209	184	13.59	113.6
CR17-1	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	94	99	-5.051	94.95
CR17-2	razor clam	Grays Harbor, Grays Harbor -	9/5/2023	Summer	124	99	25.25	125.3

		WA						
CR17-3	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	83	99	-16.16	83.84
CR18-1	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	38	37	2.703	102.7
CR18-2	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	64	37	72.97	173.0
CR18-3	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	40	37	8.108	108.1
CR19-1	razor clam	Grays Harbor, Grays Harbor - WA	10/9/2023	Fall	213	125	70.40	170.4
CR20-1	razor clam	Pacific, Pacific County Coast - WA	10/23/2023	Fall	135	183	-26.23	73.77
CR21-1	razor clam	Willapa Spits, Pacific County Coast - WA	5/21/2025	Spring	153	132	15.91	115.9
CR22-1	razor clam	Grays Harbor, Grays Harbor - WA	11/6/2023	Fall	117	140	-16.43	83.57
CR23-1	razor clam	Pacific, Pacific County Coast - WA	9/25/2023	Fall	143	124	15.32	115.3
CR24-1	razor clam	Grays Harbor, Grays Harbor - WA	11/20/2023	Fall	114	130	-12.31	87.69
CR25-1	razor clam	Grays Harbor, Grays Harbor - WA	9/5/2023	Summer	136	116	17.24	117.2
CR26-1	razor clam	Pacific, Pacific County Coast - WA	4/30/2024	Spring	120	103	16.50	116.5
CR27-1	razor clam	Mocrocks, Grays Harbor - WA	6/3/2025	Spring	100	109	-8.257	91.74
CR28-1	razor clam	Grays Harbor, Grays Harbor - WA	2/27/2024	Winter	111	109	1.834	101.8
CR29-1	razor clam	Grays Harbor, Grays Harbor - WA	10/23/2023	Fall	106	100	6.000	106.0
CR30-1	razor clam	Pacific, Pacific County Coast - WA	2/27/2024	Winter	74	98	-24.49	75.51
CR31-1	razor clam	Grays Harbor, Grays Harbor -	2/7/2024	Winter	110	61	80.33	180.3

		WA						
CR32-1	razor clam	Grays Harbor, Grays Harbor - WA	1/17/2024	Winter	96	89	7.865	107.9
CR33-1	razor clam	Grays Harbor, Grays Harbor - WA	12/1/2023	Fall	92	71	29.57	129.6
CR34-1	razor clam	Mocrocks, Grays Harbor - WA	5/21/2025	Spring	59	72	-18.06	81.94
CR35-1	razor clam	Quinault Res, Grays Harbor - WA	5/20/2025	Spring	62	60	3.333	103.3
CR36-1	razor clam	Grays Harbor, Grays Harbor - WA	2/27/2024	Winter	73	38	92.10	192.1
CR37-1	razor clam	Grays Harbor, Grays Harbor - WA	11/6/2023	Fall	50	55	-9.091	90.91
CR38-1	razor clam	Pacific, Pacific County Coast - WA	4/30/2024	Spring	70	41	70.73	170.7
CR39-1	razor clam	Copalis, Grays Harbor - WA	6/3/2025	Spring	36	53	-32.08	67.92
CR40-1	razor clam	Grays Harbor, Grays Harbor - WA	1/2/2024	Winter	45	46	-2.174	97.83

Additional Information
References (Provide references that are pertinent and supplemental to the validation data submitted; these may include peer-reviewed publications in which the method was validated and/or applied, validation packages submitted to other entities, etc. Do not provide references in lieu of data in the "Validation Data" section.) See References listed on the next page.
Standard Operating Procedure (SOP) (Provide a detailed procedure adequate for replication in additional laboratories.) See Appendix I.
Laboratory Evaluation Checklist (Provide any additions and/or modifications to the current method checklist for laboratory evaluation based on inclusion of the new matrix/ces.) See Appendix V.
Overview of Quality Systems (Provide an overview of the quality assurance/quality control systems utilized in the developer(s)/submitter(s) laboratory.) See Appendix VII.

References

Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic shellfish toxins in shellfish. Journal of AOAC International 92(6): 1705-1713. See Appendix II.

Van Dolah et al. 2012. Determination of paralytic shellfish poisoning toxins in shellfish by receptor binding assay: Collaborative study. Journal of AOAC International 95(3): 795-812. See Appendix III.

OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org. See Appendix IV.

Appendix I: Standard Operating Procedure (SOP)

Matrix Extension: Step-by-Step Procedure

A. Sample Homogenate
    a. Each sample that was below the detection limit from the MBA (~40 µg STX/100g of shellfish) underwent mini saxitoxin extraction to be pre-screened by the RBA to detect true negative samples (<10µg/100g).
        i. Mini saxitoxin extraction was performed using the same protocol as Appendix A (Section A. Sample Extraction), by performing a 1:1 ratio of 0.5 g of tissue and 0.5 mL of 0.1 N HCl.
    b. Once the negative samples were identified, samples were combined to produce 30 sub-aliquots (~130 g of tissue) needed for spiking five homogenates for each shellfish matrix.
        1. We doubled the number of tissue samples for this study to maintain a backup supply, which safeguards against potential future issues such as lost, broken, or spilled samples.
        ii. To combine samples for one homogenate, it followed these criteria:
            1. The sample collection dates must be within a 30-day range and occur within the same season.
            2. The samples must be harvested from the same water system.
        iii. Homogenates were distinct, reflecting differences based on the season of collection, harvest area, or year.
    c. The positive samples that have MBA data (>40 µg STX/100g of shellfish) were distinct, reflecting differences based on the season of collection, harvest area, or year.
    d. All homogenates were blended by a blender and/or a homogenizer to liquify the homogenate for spikes.
    e. Once the homogenate is liquified, the designated amount of tissue is aliquoted into 50 mL falcon tubes for each spike level:
        i. 20 µg STX/100g of shellfish: 33 ± 0.3 grams of tissue
        ii. 40 µg STX/100g of shellfish: 33 ± 0.3 grams of tissue
        iii. 80 µg STX/100g of shellfish: 17 ± 0.3 grams of tissue
        iv. 160 µg STX/100g of shellfish: 17 ± 0.3 grams of tissue
        v. 320 µg STX/100g of shellfish: 17 ± 0.3 grams of tissue
B. Spike Procedure
    a. Every homogenate was spiked with RM 8642a FDA Saxitoxin Dihydrochloride Solution (103 µg/g ± 4 µg/g)
    b. Prior to spiking the pre-weighed tissue, the laboratory technician gravimetrically verified the dispensed volume of water to ensure the spiking accuracy was within 3% of the designated volume.
    c. The volume of the RM 8642a FDA saxitoxin dihydrochloride solution was calculated based on the desired concentration and tissue weight available.
        i. 20 µg STX/100g of shellfish: 64 µL
        ii. 40 µg STX/100g of shellfish: 128 µL
        iii. 80 µg STX/100g of shellfish: 132 µL

iv. 160 µg STX/100g of shellfish: 264 µL
v. 320 µg STX/100g of shellfish: 528 µL

d. Once spiked, each aliquoted homogenate was vortexed for approximately 2 minutes to ensure complete mixture of the spike and tissue.
e. Following the mixing of the sample, each sample was again aliquoted into 5 ± 0.3 grams, thereby achieving a minimum of three replicates for each spike level.
    i. Blank samples were not spiked and aliquoted into 5 ± 0.3 grams.
f. At least 16 samples for each homogenate (5) were frozen at -20℃ (total of 80 aliquots).
    i. In addition,10 more homogenates from different seasons, harvest areas, and years were spiked to a concentration of 80 µg STX/100g of shellfish and aliquoted into 5 ± 0.3 grams (20 aliquots).
    ii. 20 naturally incurred samples were aliquoted into 5 ± 0.3 grams.
g. All samples, except for backups, were shipped to Washington State Department of Health for RBA using the protocol listed in Appendix A.

Appendix II: Van Dolah et al. 2009

FOOD CHEMICAL CONTAMINANTS

Appendix II

Single-Laboratory Validation of the Microplate Receptor Binding Assay for Paralytic Shellfish Toxins in Shellfish

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A single-laboratory validation (SLV) study was conducted for the microplate receptor binding assay (RBA) for paralytic shellfish poisoning (PSP) toxins in shellfish. The basis of the assay is the competition between [^{3}H]saxitoxin (STX) and STX in a standard or sample for binding to the voltage dependent sodium channel. A calibration curve is generated by the addition of 0.01–1000 nM STX, which results in the concentration dependent decrease in [^{3}H]STX-receptor complexes formed and serves to quantify STX in unknown samples. This study established the LOQ, linearity, recovery, accuracy, and precision of the assay for determining PSP toxicity in shellfish extracts, as performed by a single analyst on multiple days. The standard curve obtained on 5 independent days resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%) and a dynamic range of 1.2–10.0 nM. The LOQ was 5.3 μg STX equivalents/100 g shellfish. Linearity, established by quantification of three levels of purified STX (1.5, 3, and 6 nM), yielded an r^2 of 0.97. Recovery from mussels spiked with three levels (40, 80, and 120 μg STX/100 g) averaged 121%. Repeatability (RSD_r), determined on six naturally contaminated shellfish samples on 5 independent days, was 17.7%. A method comparison with the AOAC mouse bioassay yielded r^2 = 0.98 (slope = 1.29) in the SLV study. The effects of the extraction method on RBA-based toxicity values were assessed on shellfish extracted for PSP toxins using the AOAC mouse bioassay method (0.1 M HCl) compared to that for the precolumn oxidation HPLC method (0.1% acetic acid). The two extraction methods showed linear correlation (r^2 = 0.99), with the HCl extraction method yielding slightly higher toxicity values (slope = 1.23). A similar relationship was observed between HPLC quantification of the HCl- and acetic acid-extracted samples (r^2 = 0.98, slope 1.19). The RBA also had excellent linear correlation with HPLC analyses (r^2 = 0.98 for HCl, r^2 = 0.99 for acetic acid), but gave somewhat higher values than HPLC using either extraction method (slope = 1.39 for HCl extracts, slope = 1.32 for acetic acid). Overall, the excellent linear correlations with the both mouse bioassay and HPLC method and sufficient interassay repeatability suggest that the RBA can be effective as a high throughput screen for estimating PSP toxicity in shellfish.

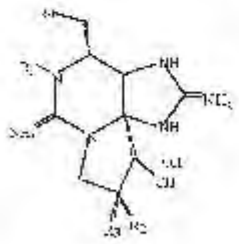
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Paralytic shellfish poisoning (PSP) is a seafood intoxication caused by the consumption of shellfish tainted with saxitoxins (STXs) produced by certain species of harmful algae. Saxitoxins are a suite of heterocyclic guanidinium toxins, of which currently more than 21 congeners are known (Figure 1). These congeners occur in varying proportions in the dinoflagellates that produce them and are further metabolized in shellfish that accumulate them, making analytical determination of PSP toxins in shellfish complex. The long-standing regulatory method for PSP toxins is the AOAC mouse bioassay (1), with a regulatory limit of 80 μg/100 g shellfish generally applied. Increasing resistance to whole animal testing has driven the need to develop alternative methods suitable for use in a high throughput monitoring or regulatory setting. In the past decade, several alternatives to the mouse bioassay have been developed and validated to various degrees. The precolumn oxidation HPLC method (2) has received First Action approval by AOAC as an Official Method for PSP (**2005.06**; 3) and has been accepted into the European Food Hygiene Regulations as an alternative to the mouse bioassay and further refined to optimize its use in the United Kingdom Official Control monitoring of PSP toxins in mussels (4). However, although the HPLC method performs well quantitatively, it is quite time consuming for high throughput screening needed by many monitoring programs. A qualitative lateral flow antibody test for PSP toxins with a detection limit of 40 μg/100 g, developed by

Jellett Rapid Testing Ltd (Chester Basin, NS, Canada), has been approved in the United States by the Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration (FDA) as a screening method. This method performed well in a comparison study with the mouse bioassay, with a false-positive rate of 6% and a false-negative rate of <0.1% (5), but it has not been put through a full AOAC collaborative trial, and does not provide quantitative analysis. To date, a suitable quantitative, high throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The current study establishes the single laboratory performance characteristics of the microplate receptor binding assay (RBA) for PSP toxins in shellfish and identifies it as a candidate for fulfilling the requirements of high throughput, quantitative analysis that measures a composite toxic potency in a manner analogous to the mouse bioassay.

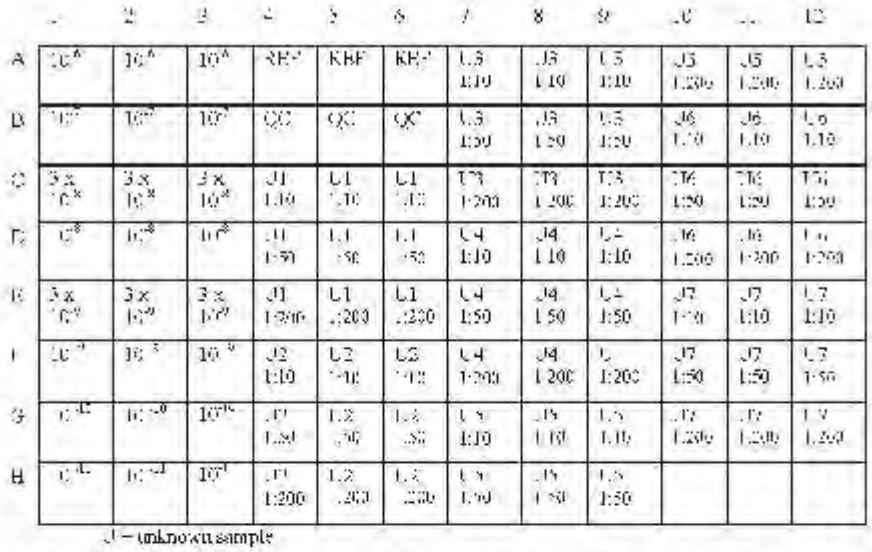
STX elicit their paralytic effects by binding to site 1 on the voltage dependent sodium channel, thereby blocking the transmission of neuronal and muscular action potentials. Because all STX congeners bind to site 1 with affinities proportional to their mouse intraperitoneal (IP) toxicity (6), a receptor binding competition assay can be used to measure the integrated toxic potency of STX congeners in a sample, independent of which toxin congeners are present. Moreover, any toxin metabolites originating in the shellfish matrix will also be detected by the assay according to their affinity for the sodium channel receptor. In this binding competition assay, [^{3}H]STX competes with unlabeled STX and/or its derivatives for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H]STX is removed by filtration and bound [^{3}H]STX is quantified by liquid scintillation counting. The percent reduction in [^{3}H]STX binding in the presence of unlabeled toxin is directly proportional to the amount of unlabeled toxin present. A standard curve is established using increasing concentrations of unlabeled STX, and the concentration of PSP toxins in an unknown sample is quantified using this standard curve.

The assay tested in this single laboratory trial is a modification of the method of Doucette et al. (7) to a 96-well microplate format described by Van Dolah et al. (8). Application of microplate scintillation counting to the PSP assay was first reported by Powell and Doucette (9), who applied it to phytoplankton analysis. The use of the microplate format, in conjunction with microplate scintillation counting, makes the assay suitable for use in a high throughput monitoring or regulatory setting. Several versions of the PSP receptor binding assay have undergone method comparisons in different laboratories with favorable correlations to the mouse bioassay and/or other assays for PSP toxins in shellfish. Suarez-Isla and Valez (10) showed excellent linear correlation ($r^2 = 0.97$) between the RBA and mouse bioassay of 41 shellfish extracts between 40 and 10 000 μg STX equivalents/100 g. Llewellyn et al. (11) found that the sodium channel receptor assay compared well to three other methods of analysis for PSP toxins in shellfish (HPLC, mouse bioassay, and N2A cytotoxicity assay). Ruberu et al. (12) optimized the microplate format assay for use in the Packard Top Count microplate scintillation counter (a single channel counter; GMI, Inc., Ramsey, MN), compared results with the same assay performed on the Wallac microplate counter (a two-channel coincidence counter; Perkin Elmer Wallace, Gaithersburg, MD), and provided further correlation data with



		R1	R2	R3	R4	MU/μmole
Carbamate	STX	H	H	H	$CONH_2$	2182
	Neo STX	OH	H	H	$CONH_2$	2285
	GTX1	OH	OSO_3^-	H	$CONH_2$	2758
	GTX2	H	OSO_3^-	H	$CONH_2$	732
	GTX3	H	H	OSO_3^-	$CONH_2$	1624
	GTX4	OH	H	OSO_3^-	$CONH_2$	1932
N-sulfocarbamoyl	GTX5 (B1)	H	H	H	$CONHSO_3^-$	180
	GTX6 (B2)	OH	H	H	$CONHSO_3^-$	-
	C1	H	OSO_3^-	H	$CONHSO_3^-$	16
	C2	H	H	OSO_3^-	$CONHSO_3^-$	277
	C3	OH	OSO_3^-	H	$CONHSO_3^-$	33
	C4	OH	H	OSO_3^-	$CONHSO_3^-$	43
Decarbamoyl	dcSTX	H	H	H	H	1274
	dcNeoSTX	OH	H	H	H	
	dcGTX1	OH	OSO_3^-	H	H	-
	dcGTX2	H	OSO_3^-	H	H	16.7
	dcGTX3	H	H	OSO_3^-	H	18.2
	dcGTX4	OH	H	OSO_3^-	H	
Decarbamoyl-N-sulfocarbamoyl	dcGTX5	H	H	H	H	-
	dcGTX6	H	H	OSO_3^-	H	
	dcGTX7	H	OSO_3^-	H	H	

Figure 1. Structures and toxic potency of 21 saxitoxin congeners. Toxic potency is listed as mouse units (MU)/μmole, where a mouse unit is defined as the minimum amount required to kill a 20 g mouse in 15 min when administered by IP injection. The table is modified from ref. 15.



U = unknown sample

Figure 2. Standardized plate layout recommended for the microplate RBA for PSP toxins in shellfish extracts. U = unknown sample.

the mouse bioassay. Usup et al. (13) utilized the microplate RBA method to compare predicted toxicity values in samples spiked with different STX congeners as assayed by the mouse bioassay and the RBA. Llewellyn (14) defined the competitive behavior of PSP toxin mixtures in receptor binding assays, using both the sodium channel and saxiphilin receptors, which explains their composite toxicity. However, none of these previous studies fully characterized assay performance according to AOAC single-laboratory validation (SLV) criteria that are the underpinning required for proceeding with an AOAC collaborative trial. Therefore, the current study was carried out to fulfill those requirements.

Experimental

Apparatus

(a) *Microplate scintillation counter.*—Wallac Microbeta, GMI Inc. (Ramsey, MN).

(b) *Microplate filtration manifold.*—Millipore (Bedford, MA).

(c) *Hot plate.*—Fisher Scientific (Suwannee, GA).

(d) *Countertop centrifuge.*—For 15 mL tubes, capable of 3000 × *g* (Fisher Scientific).

(e) *Microfilter filter plates (96 well) with 1.0 μm pore size type FB glass fiber filter/0.65 μm pore size Durapore support membrane.*—Cat. No. MSFB N6B 50 (Millipore Corp., Billerica, MA).

(f) *Microplate sealing tape.*—Cat. No. MATA HCL00 (Millipore Corp.).

(g) *Vortex mixer.*—Daigger Vortex Genie II (Daigger Scientific, Vernon Hills, IL).

(h) *Teflon/glass tissue homogenizer.*—Wheaton (Millville, NJ).

(i) *Polytron homogenizer.*—Brinkmann Instruments (Westbury, NY).

Reagents

(a) *Hydrochloric acid (HCl).*—0.1 M.

(b) *[^{3}H]STX.*—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (International Isotopes Clearinghouse, Leawood, KS).

(c) *STX diHCl.*—FDA reference standard (Office of Seafood, Laurel, MD) or National Research Council (NRC) of Canada Institute of Marine Biosciences (Halifax, NS, Canada).

(d) *Assay buffer.*—75 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cat. No. H9136]/140 mM NaCl, pH 7.5 (Sigma, St. Louis, MO).

(e) *Liquid scintillation cocktail.*—Optiphase (PerkinElmer Life Sciences, Downers Grove, IL).

Preparation of Samples (0.1 M HCl Extraction)

Shellfish samples were shucked and homogenized according to the AOAC mouse bioassay protocol (1). For the HCl extraction method, 5.0 (±0.1) g of tissue homogenate was transferred to a tared 15 mL conical polypropylene centrifuge tube. A 5.0 mL volume of 0.1 M HCl was added, and the sample was mixed on a Vortex mixer. The pH was checked to

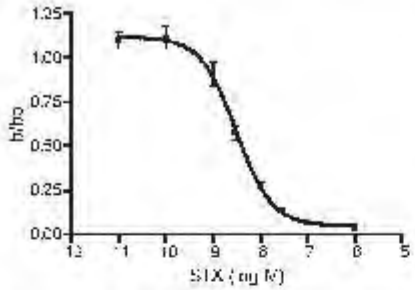


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

confirm it was between 3.0 and 4.0 in order to avoid alkalinization and destruction of the toxin, and adjusted with 1 M HCl or 0.1 M NaOH as needed. Tubes were placed in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Following removal from the boiling water bath, samples were allowed to cool to room temperature, and the pH was again confirmed to be between 3.0 and 4.0. The entire contents were then transferred to a graduated cylinder, diluted volumetrically to 10 mL, and centrifuged for 5 min at 1000 × *g*. The supernatant was transferred to a clean tube.

Preparation of Samples (Acetic Acid Extraction Method)

In a 50 mL plastic centrifuge tube, 5.0 ± 0.1 g homogenate was mixed with 3.0 mL 1% acetic acid on a vortex mixer. Tubes were capped loosely to avoid pressure buildup and placed in a boiling water bath for 5 min. Following removal from the water bath, samples were cooled in a beaker of cold water for 5 min, and then centrifuged for 10 min at 3000 × *g*. The supernatant was transferred to a 15 mL graduated conical test tube. A 3 mL amount of 1% acetic acid was added to the original tube with solid residue, mixed well on a vortex mixer, and centrifuged again for 10 min at 3000 × *g*. The second supernatant was combined with the first and diluted to 10 mL with water.

Preparation of Stock Solutions, Standards, and Reagents for Assay

(a) *Radioligand solution.*—[^{3}H]STX stock is provided in 50 µCi ampules, 24 Ci/mmol, 0.1 mCi/mL (4.17 µM). A 15 nM working stock of [^{3}H] STX was prepared fresh daily in 75 mM HEPES/140 mM NaCl (for 2.5 nM final in-well concentration).

(b) *STX standard curve.*—FDA STX dihydrochloride reference standard (100 µg/mL or 268.8 µM) used to prepare a bulk standard curve made up in advance and stored at 4°C for up to 1 month. The stock standard curve was made consisted of eight concentrations of STX in 0.003 M HCl [6×10^{-6}, 6×10^{-7}, 1.8×10^{-7}, 6×10^{-8}, 1.8×10^{-8}, 6×10^{-9}, 6×10^{-10}, 6×10^{-11}, and 0.003 M only HCl (reference)], which when diluted 1:6 in the assay, resulted in a standard curve of 0.01 nM–1000 nM STX. The reference provided a measure of total [^{3}H]STX binding in the absence of unlabeled STX.

(c) *Calibration standard (QC check).*—A reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) was prepared in 0.003 M hydrochloric acid, aliquotted in 1 mL volumes, and stored at 4°C for routine use (stable up to 1 month). On the day of the assay, 200 µL of each standard were pipetted into mini-dilution tubes for ease of pipetting into the microplate using an eight-channel pipettor.

(d) *Rat brain membrane homogenate.*—Cerebral cortices from 6-week-old male Holzman rats (Harlan Bioproducts, Indianapolis, IN) were homogenized on ice in a glass/Teflon tissue homogenizer in 75 mM HEPES/140 mM NaCl, pH 7.5, containing 0.1 mM PMSF (phenylmethanesulfonylfluoride;12.5 mL/brain) at 385 rpm for 10 strokes. Pooled homogenates were centrifuged at 20 000 × *g* for 15 min at 4°C and the pellet was resuspended in HEPES buffer (12.5 mL/brain) and rehomogenized on ice using a Polytron homogenizer set at 70% power for 20 s to ensure a fine suspension. The brain homogenate was aliquotted 2 mL/tube in cryovials and stored at –80°C. The protein concentration of the brain homogenate was determined using the Micro bicinchoninic acid (BCA) Assay (Pierce, Rockford, IL). For each assay, an aliquot of brain homogenate was thawed on ice and diluted with ice cold 75 nM HEPES/150 mM NaCl, pH 7.5, to yield a final protein concentration of 0.5 mg/mL in the assay.

Table 1. RBA measurements of calibration standards for assay linearity assessment (nM STX; *n* = 5)

Nominal	Mean	SD	RSD
1.5	1.7	0.16	10
3.0	3.0	0.52	17
6.0	6.0	0.34	6

Table 2. Recovery of analyte from spiked samples (µg STX equiv./100 g)

Nominal	Mean	SD	Measured RSD,	Recovery, %
0	<dl*			
40	47	6.6	18.7	115
80	103.7	21.8	21	129
120	145.5	15.2	10.5	121

* <dl = Less than LOQ (5 µg STX equiv./100 g).

Table 3. Comparison of receptor binding assay (RBA; $n = 5$) with AOAC mouse bioassay (MBA) of naturally contaminated shellfish (μg STX equiv./100 g)

Sample	MBA	RBA mean	SD	RSD
LP1	340	438	74	17
LP2	534	715	96	13
LP3	1158	1533	329	21
LP4	86	91	7	9
LP5	350	608	150	25
LP6	462	518	114	22

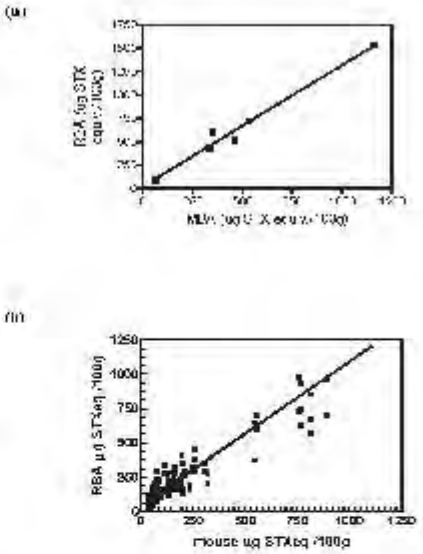


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days ($r^2 = 0.98$, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with a slope of 1.32.

Assay Procedure

(a) *Plate setup and incubation.*—A standardized plate layout was used for all assays (Figure 2). All standards, reference, QC check, and shellfish extracts were run in triplicate wells. For shellfish extracts, a standardized dilution series was run for each sample (1:10, 1:50, and 1:200), which ensured that at least one dilution would fall on the linear part of the competition curve for shellfish that contains between approximately 5 and 1500 μg STX equiv./100 g. Reagents were added in the following order: 35 μL STX standard or sample, then 35 μL [^{3}H]STX, followed by 140 μL brain homogenate. The addition of brain homogenate was carried out with sufficient force to ensure mixing of the well contents, but without risk of splashing. The plate was then covered and incubated at 4°C for 1 h.

(b) *Assay filtration and counting.*—The plate was filtered using a microplate vacuum filtration manifold, and each well rinsed twice with 200 μL ice-cold HEPES buffer at a filtration rate that ensured all wells were dry within 2–5 s. The microplate was then placed in a microplate scintillation counter cassette, and the bottom was sealed with plate sealing tape. Lastly, 50 μL scintillation cocktail was added to each well, and the top of the plate was sealed with sealing tape. The plate was allowed to sit for 30 min to ensure impregnation of the filters with scintillant prior to counting for 1 min/well in the microplate scintillation counter.

Data Analysis

Curve fitting was performed using a four-parameter logistic curve fitting model for a one-site receptor binding using Wallac Multicalc software. The software reports the in-well sample concentration in nM equiv. STX. Sample concentration was then calculated in μg STX equivalents/100 g shellfish using the following formulas:

$$(\text{nM equiv. STX}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} \rightarrow \text{nM equiv. STX in extract}$$

$$(\text{nM equiv. STX in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} \rightarrow \mu\text{g STX equiv./mL}$$

$$\mu\text{g STX equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish extracted}} \times 100 \rightarrow \mu\text{g STX equiv./100 g shellfish}$$

Critical Control Points

(*1*) For a ligand that interacts specifically at one receptor site, the slope of the resulting competition curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptable range of 0.8–1.2, linearity of the assay will be compromised, and quantification of the unknowns will be incorrect. Therefore, the assay should be re-run.

(*2*) The QC check standard should fall within ±30% of the stated value (3.0 nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.

Table 4. RBA-determined toxicities of nine naturally contaminated shellfish homogenates extracted using the 0.1 M HCl extraction method or the 1% acetic acid extraction method (μg STX equiv./100 g)

	HCl			Acetic acid		
Sample	Mean	SD	RSD	Mean	SD	RSD
1	11	4	36	19	7	39
2	600	143	24	488	104	21
3	690	142	21	584	167	29
4	136	8	6	131	41	31
5	152	27	18	167	21	13
6	302	87	29	270	72	27
7	340	88	26	264	63	24
8	262	79	30	252	48	19
9	63	26	41	54	19	34

(*3*) Sample quantification should be done only on dilutions that on the linear part of the curve [b/b_o = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and B_o is the maximum CPM)]. The RSD of the CPM must be <30%.

(*4*) For a given sample, if none of the sample dilutions falls within the linear range (i.e., the concentration is too high, b/b_o < 0.2), further dilutions must be made and the sample reanalyzed if a quantitative value is desired. If the sample concentration is too low to be quantified (i.e., b/b_o > 0.7) at sample dilution 1:10, the sample must be reported as below the LOQ.

Mouse Bioassay and HPLC Procedures

Shellfish samples extracted in parallel using the HCl and acetic acid extraction methods described above were analyzed using the standard protocols prescribed by the AOAC methods for mouse bioassay (1) or precolumn oxidation HPLC method (2).

Results and Discussion

Calibration Curve

To establish the dynamic range and repeatability of the calibration curve, five assays were performed by one analyst on separate days. The composite curve (Figure 3) resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%). Using the linear part of the curve (0.2–0.7 b/b_0) for quantification, a dynamic range of approximately one order of magnitude, 1.2–10.0 nM STX, was observed, as expected for a one-site binding assay. A QC check sample (3.0 nM STX) run in each assay averaged 3.0 ± 0.5 nM (RSD$_r$ = 17.3%), with a recovery of 99.3%.

LOQ

Shellfish extracts were diluted a minimum of 10-fold prior to analysis to minimize matrix effects that can result in false positives. The LOQ was empirically determined as the concentration, in a 10-fold diluted sample, that results in a b/b_o of 0.7. This is a more conservative cutoff than the 0.8 b/b_o frequently used in receptor assays and was used because quantification was unacceptably variable above this b/b_o cutoff. This results in an LOQ of approximately 5 μg equiv. STX/100 g shellfish, which provides a more than one order of magnitude margin relative to the regulatory limit of 80 μg/100 g.

Linearity

Linearity was assessed by five independent assays of three calibration standards that were expected to fall on the curve between 0.2 and 0.7 b/b_o: 1.5, 3.0, and 6.0 nM STX prepared from FDA STX diHCl standard. Expected and measured values are listed in Table 1. Linear regression yielded a slope of 0.98 and an r^2 of 0.97.

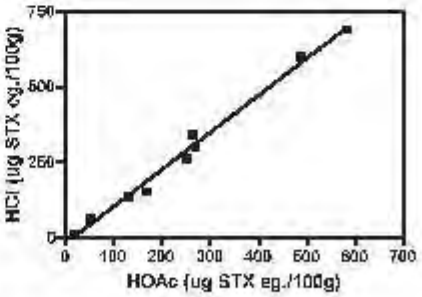


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

Table 5. HPLC analysis of nine naturally contaminated samples (1–9) extracted using 0.1 M HCl[a]

Sample	STX	NEO[b]	GTX1,4[c]	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HCl-1	3.5	0.0	0.0	7.3	0.0	0.0	10.8	6
HCl-2	231.6	23.9	42.8	324.5	28.3	249.7	900.4	412
HCl-3	220.8	53.7	74.9	436.3	43.4	338.1	1167.2	494
HCl-4	48.3	2.7	8.6	85.1	10.7	17.1	172.5	90
HCl-5	86.5	1.1	0.0	64.7	14.9	11.3	178.5	113
HCl-6	114.5	0.0	0.0	166.6	15.1	36.8	333.0	180
HCl-7	96.4	10.1	72.9	398.7	9.3	36.1	623.5	304
HCl-8	84.6	6.0	32.8	225.7	4.9	18.5	372.5	197
HCl-9	11.2	0.0	6.1	47.9	0.0	0.0	65.2	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.
[b] NEO = Neosaxitoxin.
[c] GTX = Gonyautoxin.

Recovery

Mussel tissue homogenates obtained from a local market were spiked with FDA STX diHCl standard at four levels bracketing the regulatory limit (0, 40, 80, and 120 μg/100 g) followed by thorough homogenization using a Polytron blender. Aliquots of spiked homogenate were stored at –80°C until extraction in 0.1 M HCl according to the protocol in the *Experimental* section. Extracts were analyzed in five assays performed on independent days. The mean recovery was 121% (Table 2).

Comparison of RBA-Reported Toxicity with the AOAC Mouse Bioassay

Six naturally contaminated shellfish samples were extracted in 0.1 M HCl according to the protocol in the *Experimental* section, and analyzed in five assays on independent days (Table 3). Three shellfish species were represented: clam *Mya arenaria* (whole) LP1, LP4; mussel *Mytilus edulis* (whole) LP2, LP3; and scallop *Placopecten magellanicus* (viscera) LP5, LP6. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 of 0.98 was obtained relative to the mouse bioassay, with a slope of 1.29 (Figure 4a).

A separate study of 110 naturally contaminated shellfish samples, extracted using the 0.1 M HCl method, and analyzed by RBA and mouse bioassay, yielded similar results with an r^2 of 0.88 and a slope of 1.32 (Figure 4b).

Effect of Extraction Method on RBA-Reported Toxicities

The recent approval of the precolumn oxidation HPLC method for PSP toxins as AOAC Official Method **2005.06** (3) and its potential recognition as a reference method for PSP

Table 6. HPLC analysis of the same nine naturally contaminated samples (1–9) extracted using 1% acetic acid[a]

Sample	STX	NEO	GTX1,4	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HOAc-1	3.4	0.0	0.0	7.3	0.0	0.0	10.7	6
HOAc-2	187.6	13.1	21.7	280.7	25.1	248.9	777.1	329
HOAc-3	175.2	35.6	79.2	335.9	37.2	237.7	900.9	393
HOAc-4	33.4	3.1	11.3	61.8	6.0	15.5	131.1	68
HOAc-5	59.3	3.1	0.0	67.6	10.8	19.3	160.0	89
HOAc-6	100.8	0.0	0.0	158.0	11.8	28.4	299.0	162
HOAc-7	67.4	11.2	42.7	228.4	5.2	15.6	370.5	192
HOAc-8	71.0	8.3	34.4	190.3	4.3	12.6	320.8	173
HOAc-9	11.2	0.0	11.7	38.1	0.0	61.0	122.1	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.

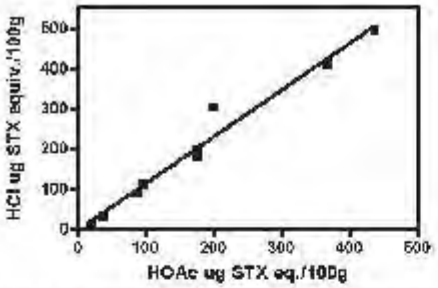


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

toxins prompted an investigation of the effects of extraction method on toxicity values reported by the RBA. Whereas the AOAC mouse bioassay prescribes shellfish extraction in 0.1 M HCl, the HPLC method uses extraction in 1% acetic acid. The 0.1 M HCl extraction procedure is known to result in the partial conversion of certain low-toxicity sulfocarbamoyl congeners to more highly toxic congeners in shellfish extracts, especially gonyautoxins, GTX5 and GTX6, to STX and neoSTX, and, thus, may result in somewhat higher toxicity values. To assess the effects of extraction procedure on RBA-reported toxicity, nine naturally contaminated shellfish samples (six blue mussel and three scallop) were homogenized and extracted independently using 0.1 M HCl and 1% acetic acid as described in the *Experimental* section. PSP toxicity in the extracts was then determined in four RBA assays run on independent days (Table 4). The between-assay RSD did not differ for samples prepared using the two extraction methods (25.8 and 26.3%, respectively). In general, the HCl extraction method resulted in slightly higher total toxicity values than reported for the acetic acid extracts (slope 1.23, r^2 = 0.99; Figure 5). The higher values reported for the HCl extracts are not explained by the conversion of sulfocarbamoyl toxins to more potent congeners in the HCl extracts, as can be seen in the toxin profiles determined by HPLC (Tables 5 and 6). Rather, the recovery of most congeners appears to be higher in the HCl extract. The higher concentrations reported in the HCl extract may reflect differences in the method by which volume is adjusted in the two extraction procedures. In the HCl method, final extract volume adjustment is made with the shellfish matrix present. In the acetic acid extraction, the matrix is first removed, the pellet re-extracted, the two extracts pooled, and then the final volume adjusted. HPLC analysis of the same samples showed a similar relationship between values reported for the HCl and acetic acid extracts (slope = 1.16, r^2 = 0.97; Figure 6) as seen in the RBA, with the HCl extracts containing greater STX equivalent/100 g.

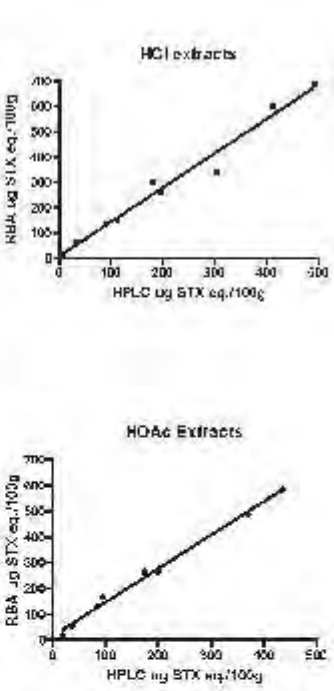


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

Comparison of RBA with HPLC

The RBA showed good linear correlation with HPLC analysis of both HCl (r^2 = 0.98, slope = 1.39) and acetic acid (r^2 = 0.99, slope = 1.32) extracts, in both cases giving somewhat higher toxicities than the HPLC method (Figure 7). A number of factors may contribute to the difference in results for total toxic potencies by these two methods. The higher toxicity values given by the RBA may result in part from the fact that the HPLC method uses the STX free base molecular weight (300 Da), whereas the receptor assay (and mouse bioassay) uses the STX dihydrochloride molecular weight (372 Da) to calculate concentration, which would result in approximately 20% higher values in the RBA. Additional differences may result from the use of FDA as compared to the NRC saxitoxin standards in the RBA and HPLC methods, respectively. Higher RBA results may also result from the dominance of the more potent PSP congeners over the weaker congeners in mixtures competing for binding to the receptor, as detailed in ref. 13, which reflects their binding affinities. In

contrast to this complex behavior, the HPLC method adds linearly the concentrations of each congener based on toxic potencies determined by mouse bioassay for isolated congeners. In some cases, e.g., 11-hydroxysulfate epimers, the concentrations of separate epimers pairs are not resolved by HPLC, although their potencies differ widely as do their ratios in shellfish samples. Lastly, higher toxicity values reported by the RBA may reflect the presence of congeners or metabolites not reported by the HPLC method.

Ruggedness

Although formal ruggedness testing was not carried out during this SLV study, several steps in the procedure might be noted that can affect the precision and accuracy of the results. First, it is important to clarify shellfish extracts by centrifugation prior to running the assay, particularly if extracts are stored refrigerated or frozen before analysis, as precipitates in the extract may cause nonspecific binding that may result in overestimates of PSP toxin concentrations. Second, since the rat brain homogenate is a suspension, it is important to ensure that it remains evenly suspended by frequent vortex mixing or pipetting prior to and during its addition to the plate. The rate of assay plate filtration should ensure that the wells clear in 2–5 s, and the rinse buffer should be ice cold in order to minimize the rate of toxin release from the receptor. Lastly, following addition of liquid scintillant to the microplate wells, it is essential to allow a minimum of 30 min for the scintillant to penetrate the filters before counting. Counting prematurely can result in increased variability between wells and lower counts/well, thus increasing RSD. A count time of 1 min/well was chosen for this study as a compromise between optimum RSD and assay throughput. Increasing the count time to 5 min/well has been shown to improve the between-well RSD in this assay when using the Packard Top Count scintillation counter, a single detector instrument with somewhat lower efficiency than the Wallac Microbeta used in the current study (11).

Summary

This SLV and method comparison study demonstrates excellent linear correlation ($r^2 > 0.98$) between the microplate receptor binding assay and both the mouse bioassay and the precolumn oxidation HPLC method for the determination of PSP toxins in shellfish. The microplate format of the assay, when coupled with microplate scintillation counting, provides a quantitative high throughput screening tool for PSP toxin testing in shellfish. The tendency of the RBA to overestimate PSP toxicity relative to the reference methods minimizes the chance of returning false negatives. Where RBA-measured toxicity results in STX equivalent values close to the regulatory limit, confirmation with a reference method is necessary if a regulatory decision is being made. Nonetheless, application of the assay as a high throughput screen can alleviate the unnecessarily large numbers of animals used for the mouse bioassay on negative samples and, similarly, alleviate the lengthy analysis of samples by HPLC at very high or very low concentrations. We propose that this method be collaboratively tested to establish if it is robust enough to be used in monitoring and regulatory laboratories.

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We thank Karen Mao and Steven Eaker for excellent technical support in the conduct of this study.

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Appendix III: Van Dolah et al., 2012

FOOD CHEMICAL CONTAMINANTS

Appendix III

Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study

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A collaborative study was conducted on a microplate format receptor binding assay (RBA) for paralytic shellfish toxins (PST). The assay quantifies the composite PST toxicity in shellfish samples based on the ability of sample extracts to compete with ^{3}H saxitoxin (STX) diHCl for binding to voltage-gated sodium channels in a rat brain membrane preparation. Quantification of binding can be carried out using either a microplate or traditional scintillation counter; both end points were included in this study. Nine laboratories from six countries completed the study. One laboratory analyzed the samples using the precolumn oxidation HPLC method (AOAC Method 2005.06) to determine the STX congener composition. Three laboratories performed the mouse bioassay (AOAC Method 959.08). The study focused on the ability of the assay to measure the PST toxicity of samples below, near, or slightly above the regulatory limit of 800 (µg STX diHCl equiv./kg). A total of 21 shellfish homogenates were extracted in 0.1 M HCl, and the extracts were analyzed by RBA in three assays on separate days. Samples included naturally contaminated shellfish samples of different species collected from several geographic regions, which contained varying STX congener profiles due to their exposure to different PST-producing dinoflagellate species or differences in toxin metabolism: blue mussel (*Mytilus edulis*) from the U.S. east and west coasts, California mussel (*Mytilus californianus*) from the U.S. west coast, chorito mussel (*Mytilus chilliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the west coast of the United States, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, from which only the hepatopancreas was homogenized. Among the naturally contaminated samples, five were blind duplicates used for calculation of RSD_r. The interlaboratory RSD_R of the assay for 21 samples tested in nine laboratories was 33.1%, yielding a HorRat value of 2.0. Removal of results for one laboratory that reported systematically low values resulted in an average RSD_R of 28.7% and average HorRat value of 1.8. Intralaboratory RSD_r, based on five blind duplicate samples tested in separate assays, was 25.1%. RSD_r obtained by individual laboratories ranged from 11.8 to 34.9%. Laboratories that are routine users of the assay performed better than nonroutine users, with an average RSD_r of 17.1%. Recovery of STX from spiked shellfish homogenates was 88.1–93.3%. Correlation with the mouse bioassay yielded a slope of 1.64 and correlation coefficient (r^2) of 0.84, while correlation with the precolumn oxidation HPLC method yielded a slope of 1.20 and an r^2 of 0.92. When samples were sorted according to increasing toxin concentration (µg STX diHCl equiv./kg) as assessed by the mouse bioassay, the RBA returned no false negatives relative to the 800 µg STX diHCl equiv./kg regulatory limit for shellfish. Currently, no validated methods other than the mouse bioassay directly measure a composite toxic potency for PST in shellfish. The results of this interlaboratory study demonstrate that the RBA is suitable for the routine determination of PST in shellfish in appropriately equipped laboratories.

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Paralytic shellfish poisoning (PSP) is caused by a suite of heterocyclic guanidinium toxins collectively called saxitoxins (STXs). Currently more than 21 congeners of STX are known; they occur in varying proportions in the dinoflagellates that produce them and may be further

Table 1. Shellfish homogenate samples analyzed for PSTs in the collaborative study[a]

Sample No.	Sample ID	Shellfish species and origin	Blind duplicate
1	MLV05	Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast	x
2	MLV06	California mussel (*Mytilus californianus*) from the U.S. west coast	x
3	MLV08	Green mussel (*Perna canaliculus*) from New Zealand	
4	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
5	MLV12	Blue mussel (*M. edulis*) east coast U.S., spiked with 200 µg/kg STX diHCl	
6	MLV14	Blue mussel (*M. edulis*) east coast U.S., spiked with 1200 µg/kg STX diHCl	
7	MLV16	Almeja clam (*Venus antique*) from Chile	
8	MLV01	Surf clam (*Spisula solidissima*) from the U.S. east coast	
9	MLV02	Chorito mussel (*M. chilensis*) from Chile	
10	MLV04	Scallop (*Plactopecten magellanicus*) from the U.S. east coast	
11	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
12	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
13	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
14	MLV13	Blue mussel (*M. edulis*) east coast U.S., spiked with 500 µg/kg STX diHCl	
15	MLV03	Chorito mussel (*M. chilensis*) from Chile	
16	MLV05	Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast	x
17	MLV06	California mussel (*M. californianus*) from the U.S. west coast	x
18	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
19	MLV10	Butterclam (*Saxidomus gigantea*) from the U.S. west coast	
20	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
21	MLV15	Blue mussel (*M. edulis*) negative control, east coast U.S.	

[a] Sample number identifies the individual samples analyzed in the assays, with 1–7 analyzed in assay 1, 8–14 in assay 2, and 15–21 in assay 3. Sample identification (MLV for multilaboratory validation) describes the 16 unique samples, among which five were assayed as blind duplicates, to make a total of 21 samples. Blind duplicates, run in different assays, are identified by an "x."

metabolized in shellfish that accumulate them, making analytical determination of paralytic shellfish toxins (PST) in shellfish complex. The long-standing regulatory method for PST is the AOAC mouse bioassay (1; AOAC Method **959.08**), with a regulatory limit of 800 µg STX di HCl equiv./kg shellfish generally applied, but established at 400 µg STX diHCl equiv./kg in certain countries (e.g., the Philippines). However, at concentrations near the regulatory limit, the mouse bioassay can significantly underestimate PST in shellfish (2). This, in addition to increasing resistance to live animal testing in both the United States and the European Union (EU), has increased the need to develop alternative methods suitable for use in a high-throughput monitoring or regulatory setting.

In the past decade, several alternatives to the mouse bioassay have been developed. In the EU, the mouse bioassay remains the reference method for PST in shellfish, but European Commission (EC) Regulation 1664/2006 specifies that other internationally recognized methods may be used. Two HPLC methods, a precolumn oxidation method (3, 4; AOAC Method **2005.06**) and a postcolumn oxidation method (5; AOAC Method **2011.02**), have been approved by AOAC as *Official Methods*℠ for PSP toxin analysis. The EC directive recognizes the precolumn oxidation HPLC method (AOAC Method **2005.06**) as an alternative to the mouse bioassay, but retains the mouse bioassay as the reference method in instances where results are challenged. HPLC methods separate and quantify individual STX congeners, which are then recombined according to their toxic equivalencies to yield a composite PST toxicity value. Although the HPLC methods perform well quantitatively, a high-throughput screening method capable of reporting toxic potency directly is still desirable for monitoring programs that often screen large numbers of negative samples. A qualitative lateral flow antibody test for PST with a reported detection limit of 400 µg STX equiv./kg was developed by Jellett Rapid Testing Ltd (Chester Basin, NS, Canada) and approved by the U.S. Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration as a screening method in specific circumstances. This method performed well in a comparison study with the mouse bioassay (6), but is not fully quantitative and has not been subjected to a full AOAC collaborative trial. To date, a suitable quantitative, high-throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The receptor binding assay (RBA) for PST is an excellent candidate for fulfilling the requirements of a high-throughput, quantitative assay that directly reports a composite toxic potency.

The basis of the RBA is the interaction between the toxins and their pharmacological target. All STX congeners bind to site 1 on the alpha subunit of the voltage-gated sodium channel with binding affinities proportional to their toxic potency (7). Therefore, an RBA can quantitatively measure the combined toxic potency of mixtures of STX congeners in a sample.

independent of the toxin congeners present (8). In the RBA for PST, tritiated STX ([^{3}H] STX) competes with unlabeled STX and/or its congeners for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H] STX is removed by filtration and receptor bound [^{3}H] STX quantified by liquid scintillation counting. The reduction in [^{3}H] STX binding is directly proportional to the amount of unlabeled toxin present. A standard curve is generated using increasing concentrations of nonradiolabeled STX standard from 10^{-10} to 10^{-6} M STX. The concentration of toxin in samples is determined in reference to the standard curve.

The assay being tested in this collaborative trial is a modification of the method of Doucette et al. (9) to incorporate a 96-well microtiter plate format, which increases sample throughput and minimizes error by reducing sample handling and pipetting steps. This microplate PST RBA was evaluated in a single-laboratory validation (SLV) study (10), which established an interassay repeatability (RSD_r) of 17.7% and good correlation with the mouse bioassay and precolumn oxidation HPLC methods. The toxin concentrations in shellfish tested in the SLV study ranged from near to well above the regulatory limit (approximately 900–15 000 μg STX diHCl equiv./kg). The current study focuses more specifically on the performance of the RBA in the critical range of shellfish toxicities below, near, and slightly above the regulatory limit (approximately 150–2400 μg STX diHCl equiv./kg).

The results of the collaborative study suggest that the RBA for PST is a suitable high-throughput screen for PST in shellfish. Although HPLC methods offer quantitative information on congener composition of samples, often the desired information is composite toxic potency, which requires the summation of individual congeners, corrected for their individual toxic equivalencies. The RBA provides a single integrated toxic potency value that reflects activity of all known and potential unknown congeners present in the sample. Use of the microtiter plate format, in conjunction with microplate scintillation counting, provides the ability to screen multiple samples simultaneously in a total assay time of less than 3 h. The assay format described in the current study provides for the quantitative determination of composite PST toxicity in seven shellfish extracts per 96-well microplate, each run in triplicate at three dilutions, covering toxicity ranges of approximately 35–6000 μg STX diHCl equiv./kg. In a high-throughput assay setting, multiple plates can be set up simultaneously, so that six assay plates can easily be accommodated each day by a single analyst, for a throughput of 42 samples/day. This compares favorably to an estimated throughput of 20–25 samples a day by the precolumn HPLC method (B. Niedzwiadek, Health Canada, personal communication) or 30–35 by mouse bioassay (B. Suarez, University of Chile, personal communication).

Collaborative Study

The focus of this study was to assess the performance of the RBA to determine PST toxicity in samples of commercially important shellfish at a range of concentrations below and above the regulatory limit. Twenty-one shellfish homogenates were included in the study, which represented 16 unique samples (Table 1). The homogenates included 12 naturally contaminated shellfish samples of different species collected from several geographic regions: blue mussel (*M. edulis*) from the U.S. east and west coasts, California mussel (*M. californianus*) from the U.S. west coast, chorito mussel (*M. chilliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus giganteus*) from U.S. west coast, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, which included hepatopancreas only. Among the naturally contaminated samples, five were blind duplicates tested on separate days that were used for calculation of RSD_r. Samples run as duplicates are indicated in Table 1. Three samples consisting of STX-spiked mussel homogenate (*M. edulis*) at levels that bracketed the regulatory limits of 800 μg/kg (500 and 1200 μg/kg spike) and 400 μg/kg (200 μg/kg spike) were included to calculate recovery. One sample was the negative control homogenate of *M. edulis* to which the STX spikes were added. All homogenates were extracted by the study participants and the extracts analyzed by RBA in three assays on separate days.

Study Participants

Ten laboratories from seven countries agreed to carry out RBAs for this study, including the United States, Italy, Australia, New Zealand, Thailand, the Philippines, and South Africa. Participants included laboratories from regulatory authorities, as well as government and academic laboratories with monitoring needs. Five of the participating laboratories (Laboratories 1–5) have this method well established and may be considered routine users. Two laboratories had previous experience running this format of the PST RBA, but have not implemented it routinely. One laboratory had previous experience with receptor assays, but had not used the microplate filtration format of the assay. One laboratory had no previous experience with RBAs. Three laboratories from different countries, United States, Chile, and Thailand, carried out the AOAC official mouse bioassay method (AOAC Method **959.08**) on the same set of samples. All mouse bioassay laboratories were experienced regulatory authorities with monitoring responsibilities. One laboratory (Health Canada) performed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**).

Preparation of Homogenates

All shellfish samples were thoroughly homogenized using a polytron blender. For spiked samples, saxitoxin standard reference material (STX diHCl) was added to the specified concentration, and the sample was thoroughly rehomogenized to ensure homogeneity. The toxin congener profiles and concentrations of all samples were determined by the precolumn oxidation HPLC method (performed by Health Canada). STX equivalents were determined by mouse bioassay (performed by Maine Department of Marine Resources). Subsamples of each homogenate (12 g) were packaged in polycarbonate tubes and stored at –80°C until shipment to collaborating laboratories by courier. All samples were coded prior to distributing to collaborating laboratories, with the codes to each laboratory being unique, and provided blind. Coding consisted of two letters followed by a number in the form X A1-7, X B1-7, and

X C1-7, where the X indicated the laboratory, the second letter indicated the three assays to be conducted, and the numerical code indicated sample number within that assay. Three practice homogenates were similarly produced.

Shipment of Study Material

The following reagents were provided to the collaborating laboratories in a single shipment containing enough dry ice to keep the contents frozen for 5 days: [^{3}H] STX; STX diHCl standard; rat brain membrane preparation; 21 coded shellfish homogenates; three practice homogenates; and a QC check sample consisting of 18 nM STX diHCl. Sufficient homogenate (12 g) was provided to ensure an accurate weight of material could be removed from the storage vial if an additional extraction were necessary due to unexpected circumstances. The identity of the samples was not released to collaborators. All reagents were received frozen and in good condition. Each participant received electronically a detailed assay protocol, comprehensive instructions for conducting the study and data reporting, and data reporting forms.

Analysis

Participants extracted all homogenates using a modification of the 0.1 M HCl extraction method used in the AOAC standard mouse bioassay protocol (modified only by scale). They were asked to perform three RBAs, each on separate days. Each assay consisted of one 96-well plate that included a standard curve, QC check sample, and seven shellfish extracts. All samples and standards were tested in triplicate wells. All shellfish extracts were run at three dilutions (1/10, 1/50, and 1/200), which ensured that at least one dilution would fall on the linear part of the standard curve. Participants were instructed to analyze samples coded A, B, or C in the first, second, or third assay, respectively, in numerical order. The five blind duplicate samples were coded so that they were tested in two independent assays, with the combination of assays differing between duplicates. Before performing the official study, participants were asked to run a practice assay that included three shellfish homogenates in the same format to ensure that any unexpected problems were encountered and addressed prior to the official study. The practice samples consisted of a negative control mussel homogenate (MLV15), and two naturally contaminated samples that were also included in the full study (MLV05 and MLV11). The identity of the practice samples was not made known to participants. Results of the practice run were submitted by e-mail to the coordinating laboratory for review before proceeding with the full study.

For the mouse bioassay, participants followed the AOAC official mouse bioassay method (AOAC Method **959.08**), with the exception of a modified 0.1 M HCl extraction protocol used in the RBA protocol, which was modified only by scale so that 5 mL 0.1 M HCl was added to 5 g of shellfish homogenate, with all other aspects of the extraction protocol being identical. The HPLC laboratory followed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**); however, final concentrations in μg/kg and μg STX equiv./kg were calculated using the formula weight of STX diHCl [372 daltons (da)], as opposed to the free base (299.3 da) in the standard HPLC protocol, to more directly compare with the RBA.

Data Analysis and Reporting

Participants were asked to report whether they used a standard or microplate scintillation counter for the study and, if a microplate counter was used, which model, because of differences in inherent counting efficiency between current commercially available counters. For data analysis, participants were instructed to use GraphPad Prism software (La Jolla, CA) or the on-board curve-fitting software provided with their microplate scintillation counter e.g., PerkinElmer Wallac MultiCalc (Gaithersburg, MD) or Packard Top Count software (Packard Instrument Co., Meriden, CT), and to report what software was used. For analysis, a four parameter logistic fit, also known as a sigmoidal dose response with variable slope, or Hill equation, was prescribed. Participants presented their analyzed data on the spreadsheet template provided, including assay quality parameters (slope, IC_{50}, and quantification of the QC check sample), between-well CVs for each sample dilution that fell within the linear part of the standard curve (0.2–0.7 B/B_0), and calculated values for these samples in the well (nM), in the extract (μg STX equiv./mL), and in the shellfish tissue (μg STX equiv./kg). Participants were also asked to report all raw count data so that all results could be analyzed by the coordinating laboratory using identical software (GraphPad Prism 4.0) to assess whether systematic differences in quantification arose from using different curve-fitting software. All data were reported via e-mail to the coordinating laboratory.

The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in dilutions and calculations and for use of the prescribed curve-fitting model. Obvious errors were corrected and the participant laboratory was consulted for concurrence. The reviewed results were then used for evaluation in the collaborative study.

Statistical Evaluation of the Collaborative Study

For each sample analyzed, outliers were first determined using the Grubbs test at a probability value of 1% (www.graphpad.com), with no more than one outlier removed, so that valid data remained from a minimum of eight laboratories. The mean, S_R, and RSD_R, and HorRat values were then calculated for each sample. For blind duplicates, the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0, was used to further evaluate for outliers and determine S_r and RSD_r. GraphPad Prism was used to determine correlation among the RBA, mouse bioassay, and HPLC results.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as μg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 μg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 μg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration.

All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(a) *Traditional or microplate scintillation counter.*

(b) *Micropipettors.*—1–1000 µL variable volumes and disposable tips.

(c) *Eight channel pipettor.*—5–200 µL variable volume and disposable tips.

(d) *96-Well microtiter filter plate.*—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50).

(e) *MultiScreen vacuum manifold.*—Millipore, Cat. No. NSVMHTS00.

(f) *Vacuum pump.*

(g) *Centrifuge tubes.*—15 and 50 mL, conical, plastic.

(h) *Mini dilution tubes in 96-tube array.*

(i) *Reagent reservoirs.*

(j) *Ice bucket and ice.*

(k) *Vortex mixer.*

(l) *Sealing tape.*—Millipore; Cat. No. MATA HCL00.

(m) *Volumetric flask.*—1 L.

(n) *–80°C freezer.*

(o) *Refrigerator.*

For traditional scintillation counter only:

(p) *MultiScreen punch device.*—Millipore; Cat No. MAMP 096 08.

(q) *MultiScreen disposable punch tips.*—Millipore; Cat. No. MADP 196 10.

(r) *MultiScreen punch kit B for 4 mL vials.*—Millipore; Cat. No. MAPK 896 0B.

(s) *Scintillation vials.*—4 mL.

For sample extraction:

(t) *Pipets.*

(u) *Centrifuge tubes.*—15 mL, conical, plastic.

(v) *Vacuum pump or house vacuum.*

(w) *pH meter or pH paper.*

(x) *Hot plate.*

(y) *Graduated centrifuge tubes.*—15 mL.

(z) *Centrifuge and rotor for 15 mL tubes.*

C. Reagents

(a) *[^{3}H] STX.*—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, or International Isotopes Clearinghouse, Leawood, KS).

(b) *STX diHCl.*—NIST RM 8642 (www.nist.gov).

(c) *3-Morpholinopropanesulfonic acid (MOPS).*—Sigma (St. Louis, MO; Cat. No. M3183-500G), or equivalent.

(d) *Choline chloride.*—Sigma (Cat. No. C7527-500G), or equivalent.

(e) *Rat brain membrane preparation.*—See *Appendix.*

For traditional counter:

(f) *Scintiverse BD liquid scintillation cocktail.*—Fisher Scientific (Waltham, MA; Cat. No. SX-18), or equivalent.

For microplate counter:

(g) *Optiphase liquid scintillation cocktail.*—PerkinElmer Life Sciences (Downers Grove, IL; Cat. No. 1200-139), or equivalent.

For sample extraction:

(h) *Hydrochloric acid (HCl).*—1.0 and 0.1 M.

(i) *Sodium hydroxide.*—0.1 M.

(j) *Water.*—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × g for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in receptor assay.

E. Preparation of Stock Solutions and Standards

(a) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(b) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

Assay	Sample No.	Sample ID	Lab 1	2	3	4	5	6	7	8	9	All labs Mean	S_R	RSD_R, %	HorRat	Labs 1–8 Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	820	410	690	1070	630	680	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV08	1100	1340	1320	1440	1260	1720	2080	2130	890	1478	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	180	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	880	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[a]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	346	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	490	340	840	490	240	800	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[a]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[a]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1490	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	689	23.3	1.7
	20	MLV11	430	350	490	280	550	620	1148[a]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%, not used in calculations.
[b] Outlier, not used in calculations.
[c] ND = Not detected.

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	480	620	
7	530	880	3090	1240	1750	1150	1460	1830	230*	1149*	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	670	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

* Outlier; not used in calculation.

buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(c) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(d) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(e) *Rat brain membrane preparation.*—Prepare rat brain membrane preparation in bulk (*see Appendix: Rat Brain Membrane Preparation*) and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(a) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_o on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(b) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(c) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8" Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Lab	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230*	1150*			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

* Outlier; not used in calculations.

ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(d) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure 2011.27), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC_{50}) \times \text{Hill slope}}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_0; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure 2011.27); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is total ligand binding in CPM (here represented as B/B_0, or bound/max bound). A curve fitting package such as Prism (GraphPad Software, Inc.) is recommended. For the microplate counter users, receptor

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	–0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD).

(a) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_0 of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_0 represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_0 of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl equiv./kg shellfish, from the in-well nM concentration obtained from the curve fitting software using the following formulas:

$$(\text{nM STX equiv}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCL equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	950	940	1060	1130	1040	750	1460	1320	810	1051	618	485
7	660	930	1080	870	840	1320	1490	2420	490	960	685	528
2	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	931	595
17	1290	1520	1460	970	1800	2520	1470	870	1250	1460	931	595
4	860	680	950	870	980	1120	1460	820	590	926	1070	653
12	810	1190	1130	810	1630	1390	1880	1120	870	1203	1070	653
11	1260	1540	1220	1380	1760	1530	1660	1210	840	1444	965	714
18	1010	1600	1390	1000	1720	1860	1520	2150	890	1452	965	714
8	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	834	752
9	830	1180	1130	1150	1130	1780	1340	980	690	1134	802	792
19	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	2000	1027
10	2440	2840	2910	1740	2150	1800	2690	2490	1210	2252	1890	1060

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

(a) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(b) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(c) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(d) QC check should be 3 nM STX ± 30% (in-well concentration). Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(a) Sample quantification should be done only on dilutions that fall within B/B_0 of 0.2–0.7. In the event that all sample dilutions fall below B/B_0 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_0 > 0.7$), the sample is reported as below LOD. If more

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)*

Microplate row	Microplate column											
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-6}	3×10^{-6}	3×10^{-6}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-6}	10^{-6}	10^{-6}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-6}	3×10^{-6}	3×10^{-6}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-6}	10^{-6}	10^{-6}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

* REF = Reference; QC = quality control check; U = unknown sample. [*Note:* The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(b) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int.* **95**, 795(2012)

Results and Discussion

Sample Characterization

All shellfish homogenates (MLV1–16) were analyzed by HPLC using the precolumn oxidation method (AOAC Method **2005.06**) to determine toxin congener profiles and quantify total PST as μg STX diHCl equiv./kg prior to initiation of the study (Table 2). It is noteworthy that the clear majority of samples, irrespective of shellfish species and location, were dominated largely by STX and GTX2,3 whereas the N1-hydroxylated congeners NEO and GTX1,4 were virtually absent, except in blue mussel from the U.S. west coast. The most unusual profile was observed in green mussel, which was dominated by the weakly toxic N-sulfo-carbamoyl congeners C1,2. The samples were analyzed by the AOAC mouse bioassay (AOAC Method **959.08**) by three laboratories that routinely perform the mouse bioassay for regulatory purposes (Table 3). The mouse bioassay detection limit is approximately 400 μg STX diHCl equiv./kg (one laboratory reported values as low as 290 μg STX equiv./kg). Because the study design included samples that bracketed the lower regulatory limit of 400 μg STX diHCl equiv./kg, several samples were reported as being below the mouse bioassay detection limit. For samples in which all values were above the detection threshold, the between-laboratory RSD_R of the mouse bioassay was 18.9%.

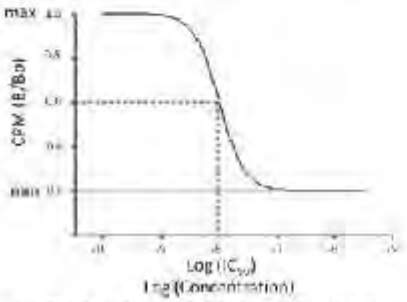


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

Data Reporting and Initial RBA Data Review

Nine of the 10 laboratories that received the study materials completed the study and reported results. All nine carried out the practice assay and reported results to the coordinating laboratory, which evaluated the results and provided feedback to the participating laboratories before initiating the full study. Following completion of the full study, the participating laboratories provided all raw and calculated data for each of

the three assays performed via e-mail to the coordinating laboratory. The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in sample dilutions and calculations, and for the use of the prescribed curve-fitting model. One laboratory used a sigmoidal curve-fitting model with the slope set to 1 (one-site binding curve in Prism), rather than the prescribed four-parameter logistic fit. In this case, the raw data were reanalyzed by the coordinating laboratory using the prescribed method. Obvious errors in calculation were corrected, such as accounting for the two-fold sample dilution resulting from the extraction process. In some cases, the participating laboratory carried out a fourth assay due to variability or inconsistency among dilutions for selected samples. In these cases, the value reported from the repeat (fourth) assay was used. One laboratory had consistent disagreement between the 1/50 and 1/200 dilutions when both fell within B/B_0 0.2–0.7. In all cases the 1/200 dilution overestimated almost two-fold relative to the 1/50 dilution, suggesting a systematic dilution error. In standard practice, these samples should be rerun. However, the instructions did not direct the participants to do so. Therefore, where there was corroborative evidence for the value reported by the 1/50 dilution, based on the 1/10 dilution, the 1/200 dilution was omitted. Where there was no basis on which to exclude the 1/200 value, an average value was calculated. This tended to result in an overestimate, and in two cases resulted in statistical outliers.

Overall Performance of the Method: Reproducibility

Table **2011.27A** summarizes the results obtained for 21 individual shellfish samples analyzed in three RBAs, determined by nine participating laboratories. Samples 1–7 were analyzed in the first assay, samples 8–14 in the second assay, and samples 15–21 in the third assay. Among these samples were five blind duplicates, treated here as individual unknown samples. One sample (marked by an footnote a in Table **2011.27A**) had a high variability in CPM between wells that was not attributable to any known cause, and was, therefore, omitted from analysis. Outliers identified by Grubbs test ($P < 0.01$) were excluded from the analysis (marked by footnote b in Table **2011.27A**). The overall RSD_R among all 21 independent samples was 33.2%, resulting in an average HorRat value of 2.0 (Table **2011.27A**). The HorRat values on individual samples ranged from 1.4 to 3.3, with a median value of 1.8. There was no apparent trend in reproducibility according to sample concentration or among shellfish species. If only the laboratories that are routine users of the RBA for PST (Laboratories 1–5) are included in the analysis, the average RSD_R is 23.1%, resulting in an average HorRat value of 1.4. Laboratory 9 tended to report the lowest values among the participating laboratories (14 of 21 samples), and although its individual sample values were not found to be statistical outliers, removing the results of this laboratory reduces all but one HorRat value (which remains unchanged), yielding an average HorRat value of 1.8 (range 1.0–2.8; Table **2011.27A**). Removal of any other single laboratory's results does not appreciably change the overall study performance. The reason for the systematically low values reported by Laboratory 9 is not clear, since the assay parameters fall well within those reported by the other laboratories. Given that assay parameters are within normal range, one possible source of systematic error could be incomplete extraction or pH adjustment of extracts, either of which would result in lower toxicity values.

A comparison of the RBA reproducibility with that of existing AOAC *Official Methods* is instructive. The AOAC collaborative study of the mouse bioassay (11), which entailed the analysis of seven samples representing three levels of STX-spiked shellfish by 11 participating laboratories, yielded a similar average RSD_R of 22%. More recent proficiency tests of the mouse bioassay performed in European regulatory laboratories report RSD_R of 2.3–38.3% on three samples run by eight laboratories (2) and RSD_R of 18.1–44.8% on two samples run by 20 laboratories (12). The mouse bioassay RSD_R values obtained in the current study ranged from 1.1 to 46.3% (average 19%) for three laboratories. The collaborative studies of the HPLC methods report reproducibility values for individual PST congeners, but do not report reproducibility of the composite toxic potency values. Collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an average RSD_R of 27.0% and HorRat value of 1.3 (range 0.8–2.1) for STX following C18 cleanup, but the reproducibility of other congeners varied considerably, with the maximum HorRat value (4.7), exceeding the highest HorRat value obtained by RBA (3.3).

Because composite toxic potency values were not reported in the studies of the HPLC methods, it is uncertain how this variability influences the composite toxic potency calculated from these methods. The average and ranges of HorRat values obtained for different congeners were: neoSTX–1.7 (range 1.2–2.5); dcSTX–1.1 (range 0.6–2.1); GTX1,4–1.9 (range 1.1–4.2), GTX2,3–1.4 (range 0.8–1.9); B1–1.1 (range 0.7–1.9); and C1,2–1.6 (range 0.9–4.5). Because of the variability obtained in neoSTX, GTX1,4, C3,4, and B2, AOAC Method **2005.06** calls for a second SPE-COOH cleanup of samples suspected of containing these congeners, after which reproducibility improved somewhat: neoSTX–1.8 (range 1.3–2.1); GTX1,4–1.3 (range 1.0–2.1); and C3,4–1.3 (range 0.8–1.8). The postcolumn oxidation HPLC method (AOAC Method **2011.02**) reported an average HorRat value of 0.6 for STX. In this method, neoSTX with an average HorRat of 1.9 (range 0.6–4.0) and GTX4 with an average HorRat of 1.6 (range 1.0–2.9) had reproducibility values that may affect the overall composite potency values. The maximum HorRat value (4.0) reported in this study also exceeded the maximum value reported in the RBA.

In summary, with the removal of Laboratory 9, the overall reproducibility of the RBA falls within the performance measures achieved by the established AOAC *Official Methods* for PST. The difference in reproducibility achieved by the laboratories that are routine users of the assay and participants who are not routine users of the method highlights the importance of training if this method were to be implemented in a regulatory setting.

Within-Laboratory Repeatability

Within-laboratory variability (RSD_r) was determined on five samples that were provided as blind duplicates. Participants were unaware that blind duplicates were included among the coded samples received. The duplicate samples were coded so that they were analyzed in separate assays, with different duplicate pairs falling into different assays (Table 1). One outlier was found among the results of the blind duplicates by Cochran's

test, $P < 0.025$ (Laboratory 7, sample MLV11) using the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0. An overall RSD_r of 25.1% was observed, with an RSD_R of 32.9%, yielding a HorRat value of 2.0, similar to that of the overall study (Table **2011.27B**). When the performance of individual laboratories was evaluated separately, the average RSD_r was 22.2%, with individual laboratories varying from 11.8 to 34.4% (Table **2011.27C**). Routine users of the microplate format of the PST RBA (Laboratories 1–5) obtained an average RSD_r of 17.1%, which is similar to that obtained in the SLV study (10), and lower than that obtained by nonroutine users (Laboratories 6–9), which averaged 26.1% and ranged as high as 34.4%. The AOAC collaborative study of the mouse bioassay (11) did not report RSD_r; however, analysis of the data from that study using AOAC INTERNATIONAL's Interlaboratory Study Workbook for Blind Duplicates results in an average RSD_r of 16.5% for three STX-spiked samples. Proficiency testing of the mouse bioassay performed in eight French laboratories reported an average RSD_r of 8.3% on three samples (2). The analysis of blind duplicates in the collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an RSD_r of 15.2% for STX following SPE C18 cleanup and an average RSD_r of 16.4% across all congeners, which ranged from 6.0 to 31.7%. Following SPE–COOH cleanup, repeatability was similar, with RSD_r of 17.2% across all congeners. The intralaboratory repeatability values obtained in the postcolumn oxidation HPLC method (AOAC Method **2011.02**) averaged 6.4% for STX; most other congeners were similar, with neoSTX being the only congener that showed a somewhat higher RSD_r of 23.3%.

In summary, the within-laboratory repeatability of the RBA was found to be acceptable, with all but two laboratories achieving an RSD_r of 23.3% or less, and the routine users of the assay achieving an average RSD_r of 17.1%.

Spike Recovery

Three samples included in the study were homogenates of blue mussel spiked with STX diHCl at concentrations intended to bracket the regulatory limits of 800 μg STX equiv./kg used by most countries and 400 μg STX equiv./kg imposed in the Philippines. Nominal concentrations in the spiked samples were 200, 500, and 1200 μg STX equiv./kg. Also included in the study was the blue mussel homogenate to which the STX spikes had been added, which was determined to be negative for STX by the precolumn oxidation HPLC method. The negative control homogenate was reported as nondetectable by eight of nine laboratories. Recovery of spiked STX by the RBA was 84.4, 93.3, and 88.1%, respectively, for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, and yielded a slope of 0.87 and r^2 of 0.86 (Figure 2). In the current study, the mouse bioassay reported < detection limit, and 68.6 and 40.5% recovery for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels. The AOAC collaborative study of the mouse bioassay (11) reported recoveries of 62.3% at spike levels similar to those in the current study (equivalent to 1000 μg STX diHCl equiv./kg) but higher recoveries of 81.5 and 96.0% were achieved at higher spike levels equivalent to 4000 and 8000 μg STX diHCl equiv./kg.

The observed poor recovery in the mouse bioassay at concentrations near and below the regulatory limit has been observed in other studies (2), and has been attributed to a salt or protective effect of the shellfish matrix, which, for concentrations at or below the regulatory limit of 800 μg/kg, is injected undiluted into the mouse. The spike recovery observed in the precolumn HPLC method in this study is also somewhat low, with 54.0, 62, and 51.5% recovery at the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, respectively. The AOAC collaborative study of the precolumn HPLC method reported 74.4–76.8% at similar spike levels following SPE C18 cleanup and 63.7–68.2% following SPE-COOH cleanup (3, 4). In comparison, the postcolumn HPLC method reported 88–104% recovery of STX spiked at levels somewhat lower than the current study. The higher recovery of the RBA than the HPLC method in the current study may reflect the use of the 0.1 M HCl extraction method in the RBA as compared to the acetic acid extraction used in the HPLC methods.

We previously established in the SLV study that the RBA performs well with shellfish extracted using either method (10). In that study, the RBA reported slightly higher toxicity values for shellfish extracts made using the 0.1 M HCl method than the acetic acid extraction, yielding a correlation of 0.99 with a slope of 1.23 (10). The higher toxicity reported by the RBA in 0.1 M HCl extracts may reflect the hydrolysis of less toxic congeners to more toxic congeners.

Assay Parameters and Quality Metrics

Table **2011.27D** summarizes the assay parameters and quality metrics for all laboratories. Eight of nine laboratories used microplate scintillation counters. Laboratory 4 used the manual counting method in which the microplate well filters are punched out, using an eight-place punch system, into traditional 4 mL scintillation vials and counted. Its performance using the manual counting method (RSD, 17.4%) was similar to or better than that of the laboratories using the microplate method, indicating that using the manual counting method does not affect the performance of the assay. Similarly, there was no apparent difference in assay parameters when the Packard Top Count (single detector) was used, compared to the Wallac Microbeta (coincidence detector), although the reference CPM values obtained on the Top Count generally were somewhat lower due to differences in counting efficiency inherent in the differences in detector geometry. Eight of nine laboratories used GraphPad Prism for curve-fitting, while only Laboratory 5 used Wallac MultiCalc software. Values reported by Laboratory 5 fell well within the range of values reported by laboratories using Prism.

All assays resulted in slopes between –0.8 and –1.2, as specified in the protocol. This specification reflects the fact that in a competitive binding assay for a ligand that interacts specifically at a single receptor site, the slope of the resulting standard curve should theoretically be 1.0. Although curve-fitting software packages often include a one-site binding curve that fixes the slope at 1.0, we specified in the protocol the use of the four-parameter logistic fit (also known as sigmoidal dose-response with variable slope), because it more readily identifies problems with the standard curve that may skew results. Laboratory 9 reported results using a one-site binding curve fit; in this case, the coordinating laboratory recalculated their raw data using the four-parameter logistic fit. The protocol also calls for RSD% < 30 on all standards. Most analysts did not experience variability problems in the standard wells. Infrequent high RSDs were most often associated with the well

Table 2. Congener profiles in shellfish homogenates included in the collaborative study[a]

Sample name	Species	STX	NEO	dcSTX	GTX1,4	GTX2,3	dcGTX2,3	B1	C1,2	C3,4	Total PSP	µg STX diHCl equiv./kg
MLV01	Surf clam	639.8		74.0		226.2	207.0				1146.9	864.3
MLV02	Almeja clam	298.3				1290.1		266.6			1855.0	802.1
MLV03	Chorito mussel	77.6				310.4					388.0	195.5
MLV04	Atlantic sea scallop	831.6				2785.6					3617.3	1880.2
MLV05	Atlantic sea scallop	193.8				576.2					770.0	412.8
MLV06	California mussel	912.8		10.9		0.0		233.8			1157.5	931.3
MLV07	Blue mussel, U.S. east coast	548.2				1097.3					1645.5	965.2
MLV08	Green mussel	164.2		63.5			272.3	454.8	3629.0		4419.6	340.8
MLV09	Blue mussel, U.S. west coast	432.3	124.9	8.7	353.7	727.8		506.4			2153.8	1070.9
MLV10	Butter clam	1783.5		40.6		533.2		203.5			2540.8	2000.9
MLV11	Almeja clam	159.1		12.2		185.5					356.8	236.9
MLV12	Blue mussel spike	108.4									108.4	108.4
MLV13	Blue mussel spike	310.2									310.2	310.2
MLV14	Blue mussel spike	618.5									618.5	618.5
MLV15	Blue mussel blank										0.0	0.0
MLV16	Chorito mussel	389.8		14.3		754.1					1158.1	884.9

[a] Values for individual congeners are in µg/kg. Values for composite toxicity are in µg STX diHO equiv./kg. Abbreviations for congeners are as follows: STX – saxitoxin; NEO – neosaxitoxin; dcSTX – decarbamoyl saxitoxin; GTX1,4 – gonyautoxin 1 and gonyautoxin 4; GTX2,3 – gonyautoxin 2 and gonyautoxin 3; B1 – gonyautoxin 5 (also known as sulfocarbamoyl STX B1); C1,2 – sulfocarbamoyl STX C1 and sulfocarbamoyl STX C2; C3,4 – sulfocarbamoyl STX C3 and sulfocarbamoyl STX C4.

Table 3. Mouse bioassay results on collaborative study samples from three laboratories[a]

Sample No.	Sample ID	MBA Lab A	MBA Lab B	MBA Lab C	MBA Avg.	MBA s_R	MBA RSD_R, %
1	MLV05	400	415	340	385	39.7	10.3
2	MLV06	550	597	540	562	30.4	5.4
3	MLV08	440	<dl[b]	370	405	49.5	12.2
4	MLV09	670	612	760	681	74.6	11.0
5	MLV12	<dl	<dl	<dl	—	—	—
6	MLV14	489	489	480	486	5.2	1.1
7	MLV16	585	585	470	547	66.4	12.1
8	MLV01	750	716	600	689	78.6	11.4
9	MLV02	670	1115	590	792	282.9	35.7
10	MLV04	2040	<dl	1080	1560	678.8	43.5
11	MLV07	1480	748	670	966	446.8	46.3
12	MLV09	—	594	670	602	11.3	1.9
13	MLV11	380	379	<dl	380	—	—
14	MLV13	<dl	343	<dl	343	—	—
15	MLV03	400	364	<dl	382	—	—
16	MLV05	—	396	370	383	18.4	4.8
17	MLV06	—	702	630	666	50.9	7.6
18	MLV07	—	<dl	690	690	—	—
19	MLV10	1320	690	870	1027	254.2	24.8
20	MLV11	—	364	290	327	52.3	16.0
21	MLV15	<dl	<dl	<dl	—	—	—

[a] Values are in µg STX diHCl equiv./kg.

[b] dl = Detection limit.

in column 1 of the 96-well plate. Most analysts removed the suspect well from the curve-fitting process. When the RSD for a given standard was near the stated cutoff (e.g., 31–33%), and left in the curve-fitting process, there was no apparent effect on the curve parameters listed as criteria for assay acceptance.

The average IC_{50} among all 27 assays was 1.9 + 0.45 nM (RSD_R 23.5%). The other assay quality metric called for by the protocol is the analysis of the QC check sample, which should be 3 ± 0.9 nM STX (30% RSD, in-well concentration). Four of the 27 assays had QC values outside the stated limits, with no obvious error responsible for the variability. Among these, Laboratory 7 reported 6.5 nM for the QC check in assay 3 and an IC_{50} of 3.4 nM, which was outside the norm. Similarly, Laboratory 8 reported a QC of 1.5 nM in assay 2 and a low IC_{50} of 1.4 nM, which is at the lower edge of acceptability. In general practice, these values would trigger repeating the assay. However, because of the minimal number of laboratories participating in the study, both of these assays were retained in the study. In neither case were the reported sample values systematically higher or lower than those reported in the other assays.

LOD and LOQ

The LOD was calculated based on the measurement of the negative control shellfish matrix (MLV15) using the blank + 3×SD approach according to Eurachem guidelines (13), as recently applied to AOAC Method 2006.02, an ELISA for domoic acid in shellfish using a similar four-parameter logistic curve (14). All laboratories reported <dl for this sample using the prescribed cutoff of B/B_0 <0.7 for quantification, with the exception of Laboratory 8, which was removed as an outlier as determined by Grubbs test ($P < 0.01$). If these samples are instead quantified using the B/B_0 values obtained, a mean of 5.5 ng/mL is obtained with an SD of 5.7 ng/mL, resulting in an LOD of 45 µg STX diHCl equiv./kg. Using the blank + 10×SD definition, an LOQ of 126 µg STX di HCl equiv./kg is thus obtained. We previously established empirically that a 1/10 dilution of shellfish extracts is sufficient to remove matrix effects in the RBA (10), when a quantification cutoff of B/B_0<0.7 is used. This is the basis for the ten-fold minimum sample dilution used in the current study. The IC_{70} values (B/B_0 0.7) for all standard curves run in the study are presented in Table 2011.27D. An average of 0.80 ± 0.188 nM STX diHCl was obtained across all assays, following the removal of one outlier based on the Grubbs test ($P < 0.01$). Applying the blank + 3×SD to this value, an LOD of 64 µg STX diHCl equiv./kg is obtained; applying the blank + 10×SD to this value results in an LOQ of 131 µg STX diHCl equiv./kg for a sample diluted 1/10 and extracted as indicated in the study, in fair agreement with the value calculated above.

Correlation with HPLC and Mouse Bioassay

Comparison of the RBA results with the mouse bioassay

Nominal	Avg	S_d	RSD_R,%	Recovery,%
200	169	58	34.6	84.4
500	466	133	28.5	93.3
1200	1057	228	21.7	88.1

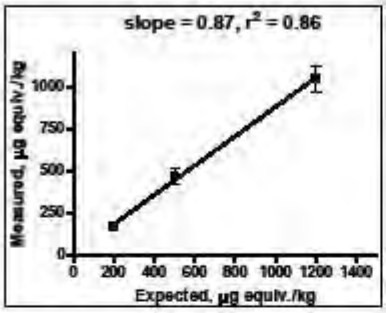


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

results yielded an r^2 of 0.84 and a slope of 1.64, indicating that the RBA reports somewhat higher STX equivalents in shellfish, relative to the mouse bioassay (Figure 3). This overestimate has been previously reported for both RBA and HPLC methods (2, 9) at the STX levels near or below the regulatory limit, which are the focus of the current study. Consistent with these findings, the HPLC method also reported higher values than the mouse bioassay in this study, with a slope of 1.33 and an r^2 of 0.84. RBA results correlated better with the precolumn oxidation HPLC method, with a slope of 1.20 and an r^2 of 0.92.

RBA Yielded No False Negatives Relative to the Regulatory Limit

When the data from the three methods were sorted by increasing µg STX diHCl equiv./kg as reported by the mouse bioassay, the RBA did not report any false negatives when compared to the regulatory limit of 800 µg STX equiv./kg (Table 2011.27E). When compared with the precolumn oxidation HPLC method, only Laboratory 9 reported values lower than the HPLC method. The fact that the RBA reports somewhat higher toxicity than the mouse bioassay or HPLC at levels near or below the regulatory limit is beneficial from a food safety standpoint. The higher values reported presumably arise from better recoveries, as demonstrated above. From a shellfish producer's perspective, the improved detection limits relative to the mouse bioassay and better recovery of low toxin levels compared to the HPLC can help to provide advance warning of developing toxicity, allowing producers to harvest early, delay harvest, or move cultures, as appropriate.

Participants' Comments

Laboratory 6 participated in the study without previous experience running receptor assays, and in doing so, identified several points needing clarification that have since been added to the proposed *Official Method* as enumerated in this report: (*1*) The vacuum required for filtration was not specified at 4–8" Hg, which is critical because insufficient vacuum pressure results in too slow a clearance of the wells, whereas too much pressure results in an airlock and no filtration at all. (*2*) Scintillation counting time for the microplates is 1 min/well. (*3*) Instructions have been added regarding how to calculate sample concentration if more than one dilution falls within B/B_0 0.2–0.7; specifically, an average value should be calculated from all sample dilutions falling within B/B_0 0.2–0.7. When corrected for dilution, serial sample dilutions should yield similar quantification. The absence of linearity between sample dilutions indicates either error in dilution or sample matrix interference; however, at the minimum sample dilutions recommended in the proposed *Official Method*, matrix effects from shellfish homogenates have not been encountered (10). In the current study, the nonlinearity of dilutions experienced in several samples by Laboratory 8 was not observed by the other laboratories, suggesting a systematic sample dilution issue rather than a sample matrix problem. Although experienced in RBAs in general, Laboratory 8 had not previously run the microplate filtration format of the assay for PST.

Laboratory 9, which reported generally lower values than the other laboratories, although familiar with the assay, had not performed it in more than a year. The lower values reported do not appear to be associated with conduct of the assay, or scintillation conduct of the assay, or scintillation counting, since the assay metrics are well within the averages reported by the other laboratories. Insufficient boiling or pH adjustment of sample extracts are a possible explanation. These points identified by the study participants should be added to the critical steps identified in the SLV study (10) that can affect precision and accuracy of the assay results, including: (*1*) ensure that the water is strongly boiling during extraction; (*2*) carefully adjust pH of extracts; (*3*) ensure even distribution of the membrane preparation across the microplate by frequent vortex-mixing or pipetting before and during its addition to the plate; (*4*) the wells must clear within 2–5 s during filtration; (*5*) the wash buffer should be ice-cold to minimize the rate of toxin release from the receptor; and (*6*) following addition of scintillant to the wells, incubate a minimum of 30 min to ensure that the scintillant fully penetrates the filters before counting.

Recommendations

The collaborative study of the RBA for PST was completed by nine laboratories representing six countries. Collaborators quantified PST as a composite toxicity value reported in µg STX di HCl equiv./kg in a variety of shellfish species from different regions of the world, containing varied toxin congener profiles. The study included laboratories with extensive experience as well as others with little or no previous experience. The study also included both microplate and scintillation counters as end points, because either instrument type could potentially be used by test laboratories. The study demonstrates that the RBA yields adequate repeatability, reproducibility, and recovery for routine determination and monitoring of PST in shellfish. The greater precision attained by laboratories that received prior training on the RBA and routinely implement this assay suggests that

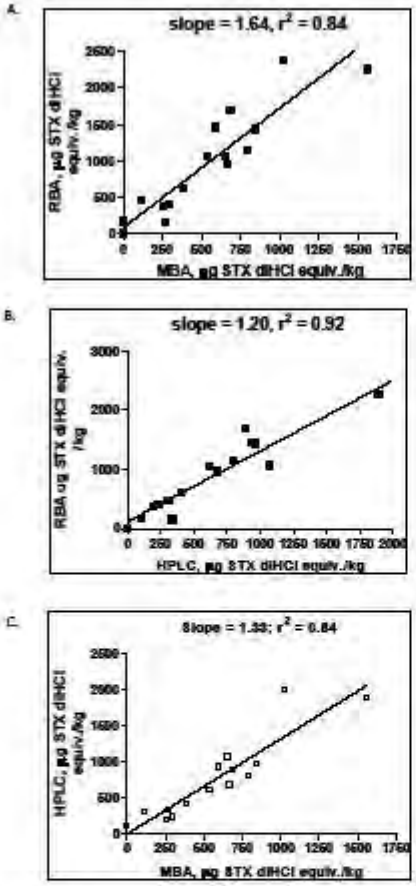


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

the overall interlaboratory reproducibility can be further improved. It is recommended that this method be accepted by AOAC INTERNATIONAL as Official First Action for the determination of PST in shellfish.

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Appendix: Rat Brain Membrane Preparation

The rat brain membrane preparation used in this assay can be produced in bulk, aliquotted, and stored at –80°C until use. Under this storage condition, the preparation is stable for a minimum of 6 months. The following protocol provides sufficient membrane preparation for a minimum of 125 plates and can be scaled up or down as needed.

A. Apparatus

(a) *Teflon/glass homogenizer.*—Motorized tapered Teflon pestle and glass tube, 15 mL.

(b) *Motorized tissue homogenizer.*—Polytron or small hand-held blender.

(c) *High-speed centrifuge and fixed angle rotor.*—Capable of 20000 × *g* (rcf).

(d) *Centrifuge tubes.*—12–15 mL rated for >20000 × *g* (rcf).

(e) *Plastic cryovials.*—2 mL.

(f) *Graduated beaker.*—300 or 500 mL.

(g) *Pipets.*—Disposable 5 and 10 mL.

(h) *Forceps.*

B. Reagents

(a) *20 Rat brains.*—Male, 6-week-old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA; http://hilltoplabs.com) or equivalent.

(b) *MOPS.*—pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G).

(c) *Choline chloride.*—100 mM (Sigma; Cat. No. C7527-500G).

(d) *Phenyl methylsulfonyl fluoride (PMSF).*—Sigma; Cat. No. P7626.

(e) *Isopropanol.*

C. Procedure

(*1*) Prepare 1 L 100 mM MOPS buffer, pH 7.4, containing 100 mM choline chloride (detailed protocol in E, above) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol; dissolve 0.174 g PMSF in 10 mL isopropanol to make 100 mM stock. Aliquot and store at –20°C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh on the day of use.

(*2*) Remove medulla and cerebellum from each brain using forceps and discard. Place the cerebral cortex (*see* Figure 1) in a small amount of ice-cold buffer and place on ice.

(*3*) Place one cerebral cortex in 12.5 mL MOPS/choline Cl/PMSF, pH 7.4, in glass/teflon homogenizer (two brains in 25 mL buffer will fit into 30 mL homogenizer tube). Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes (more if necessary to homogenize brain; there should be no visible chunks remaining in the homogenate). Keep tube in ice at all times. Pour homogenized tissue into 250 mL beaker on ice and repeat procedure with remaining cortices.

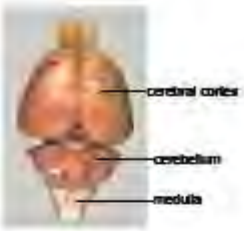


Figure 1. Rat brain.

(*4*) Transfer pooled homogenized tissue to centrifuge tubes, balance the tubes (pairwise; use ice-cold buffer to balance), and centrifuge at 20000 × g for 15 min at 4°C.

(*5*) Aspirate the supernatant and resuspend the pellets in ice-cold MOPS/choline Cl/PMSF buffer, using an adequate amount (~5 mL) to fully resuspend the pellet (can use clean glass stir rod to break up pellet), not exceeding 10 mL per brain.

(*6*) Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume to 200 mL total (keep on ice).

(*7*) Keeping the beaker on ice, Polytron (or use a small hand-held blender at low speed) at 70% full speed for 20 s to obtain a consistent homogenate.

(*8*) Aliquot 2 mL/tube into cryovials. It is critical to keep the preparation well mixed while dispensing, e.g., prior to each aliquot to ensure equal allocation of protein/receptors to each vial. Keep cryotubes on ice.

(*9*) Freeze and store at –80°C. This preparation is stable for at least 6 months. Use a permanent marker to label the preparation date on the storage container.

D. Protein Assay

(a) Determine protein concentration of membrane preparation using Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) protein assay kit or equivalent protein assay (Thermo Fisher, Rockford, IL). The above protocol should yield 6–8 mg protein/mL of rat membrane preparation.

(b) Determine membrane dilution needed for the assay: The protein concentration in the daily working stock for the assay should be 1 mg/mL (this is diluted in the assay to yield 0.5 mg/mL in-assay concentration). Based on the protein concentration determined in the protein assay, determine the dilution needed to achieve 1 mg/mL. This is the dilution used in section E(e) above for all assays using this lot of membrane preparation. The protocol above typically yields a protein concentration that requires a dilution of 1/6–1/8. (Do not use less than 1/4 dilution or filtration wells may become clogged.) Protein concentration will need to be determined for each new batch of membrane preparation.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration. All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(a) *Traditional or microplate scintillation counter.*

(b) *Micropipettors.*—1–1000 µL variable volumes and disposable tips.

(c) *Eight channel pipettor.*—5–200 µL variable volume and disposable tips.

(d) *96-Well microtiter filter plate.*—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA, USA; Cat. No. MSFB N6B 50).

(e) *MultiScreen vacuum manifold.*—Millipore; Cat. No. NSVMHTS00.

(f) *Vacuum pump.*

(g) *Centrifuge tubes.*—15 and 50 mL, conical, plastic.

(h) *Mini dilution tubes in 96-tube array.*

(i) *Reagent reservoirs.*

(j) *Ice bucket and ice.*

(k) *Vortex mixer.*

(l) *Sealing tape.*—Millipore; Cat. No. MATA HCL00.

(m) *Volumetric flask.*—1 L.

(n) *–80°C freezer.*

(o) *Refrigerator.*

For traditional scintillation counter only:

(p) *MultiScreen punch device.*—Millipore; Cat. No. MAMP 096 08.

(q) *MultiScreen disposable punch tips.*—Millipore; Cat. No. MADP 196 10.

(r) *MultiScreen punch kit B for 4 mL vials.*—Millipore; Cat. No. MAPK 896 0B.

(s) *Scintillation vials.*—4 mL.

For sample extraction:

(t) *Pipets.*

(u) *Centrifuge tubes.*—15 mL, conical, plastic.

(v) *Vacuum pump or house vacuum.*

(w) *pH meter or pH paper.*

(x) *Hot plate.*

(y) *Graduated centrifuge tubes.*—15 mL.

(z) *Centrifuge and rotor for 15 mL tubes.*

C. Reagents

(a) *[^{3}H] STX.*—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, USA, or International Isotopes Clearinghouse, Leawood, KS, USA).

(b) *STX diHCl.*—NIST RM 8642 (www.nist.gov).

(c) *3-Morpholinopropanesulfonic acid (MOPS).*—Sigma (St. Louis, MO, USA; Cat. No. M3183-500G), or equivalent.

(d) *Choline chloride.*—Sigma (Cat. No. C7527-500G), or equivalent.

(e) *Rat brain membrane preparation.—Appendix 1 [J. AOAC Int.* (future issue)].

For traditional counter:

(f) *Scintiverse BD liquid scintillation cocktail.*—Fisher Scientific (Waltham, MA, USA; Cat. No. SX-18), or equivalent.

For microplate counter:

(g) *Optiphase liquid scintillation cocktail.*—PerkinElmer Life Sciences (Downers Grove, IL, USA; Cat. No. 1200-139), or equivalent.

For sample extraction:

(h) *Hydrochloric acid (HCl).*—1.0 and 0.1 M.

(i) *Sodium hydroxide.*—0.1 M.

(j) *Water.*—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in

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Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv/kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

Assay	Sample No.	Sample ID	Lab 1	2	3	4	5	6	7	8	9	All labs Mean	S_R	RSD_R, %	HorRat	Labs 1–8 Mean	S_r	RSD_r, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	860	1478	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	90	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	256	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	280	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1460	2420[a]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1820	1580	1110	1700	3180	1400	2780	820	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2680	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1830	1390	1880	1120	670	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[a]	580	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[a]	90	385	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1481	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2680	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	669	23.3	1.7
	20	MLV11	430	350	480	280	550	620	1149[a]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[a]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

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Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	560	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	860	2090	1240	1750	1150	1460	1830	230*	1149*	
8	660	940	2130	870	1210	2150	820	1120	500	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		336		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R, %		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

* Outlier; not used in calculation.

receptor assay.

E. Preparation of Stock Solutions and Standards

(a) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(b) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(c) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table 2011.27F) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table 2011.27F).

(d) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(e) *Rat brain membrane preparation.*—Prepare rat brain membrane preparation in bulk [*Appendix 1; J. AOAC Int.* (future issue)] and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(a) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_0 on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table 2011.27G).

(b) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to

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Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Laboratory	ID	Day 1	Day 2	Mean	s,	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230*	1150*			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

* Outlier; not used in calculations.

dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(c) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8" Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(d) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC50)\times \text{Hill slope}}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (Graph Pad Software, Inc.) is recommended. For the microplate counter users, receptor assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD, USA).

(a) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl

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Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{75}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	−0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	−1.0	2.0	2.6	733	0.96	None			
	3	−1.1	2.1	3.2	1038	0.92	None			
2	1	−1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	−1.2	2.2	3.9	1260	0.85	None			
	3	−1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	−1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	−0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2068	0.80	None			
4	1	−0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	−1.2	1.7	3.2[a]	1611	0.77	None			
	3	−0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	−0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	−1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	−1.2	1.8	2.9	1052	0.67	None			
6	1	−1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	−1.0	2.0	4.0[b]	1124	1.08	None			
	3	−1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	−0.8	1.0	2.8[b]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	−1.0	1.6	2.7	619	0.70	None			
	3	−0.9	2.1	3.2[a]	693	0.82	None			
8	1	−1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	−1.1	1.4	1.5[b]	1095	0.78	None			
	3	−1.1	2.4	2.3	886	1.04	None			
9	1	−1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	−1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	−1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

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Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	540	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	**490**	**960**	585	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	**590**	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1580**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	**690**	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

equiv./kg shellfish, using the following formulas:

$$(\text{nM STX equiv.}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv. in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCl equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

(a) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(b) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(c) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(d) QC check should be 3 nM STX ± 30% (in-well concentration).

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

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Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

Microplate row	Microplate column											
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [Note: The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

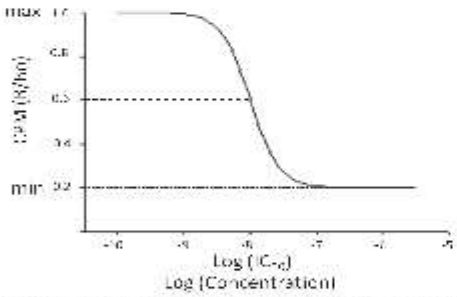


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}

Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(a) Sample quantification should be done only on dilutions that fall within B/B_0 of 0.2–0.7. In the event that all sample dilutions fall below B/B_0 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_0 > 0.7$), the sample is reported as below LOD. If more than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(b) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int.* **95**, 795(2012)

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Appendix V: Shellfish Laboratory Evaluation Checklist

<table>
<tr><td colspan="3">PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV</td></tr>
<tr><td colspan="3">SHELLFISH LABORATORY EVALUATION CHECKLIST
Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP)</td></tr>
<tr><td colspan="3">LABORATORY:</td></tr>
<tr><td colspan="3">ADDRESS:</td></tr>
<tr><td colspan="2">TELEPHONE:</td><td>FAX:</td></tr>
<tr><td colspan="3">EMAIL:</td></tr>
<tr><td>DATE OF EVALUATION:</td><td>DATE OF REPORT:</td><td>LAST EVALUATION:</td></tr>
<tr><td colspan="2">LABORATORY REPRESENTED BY:</td><td>TITLE:</td></tr>
<tr><td colspan="2"></td><td></td></tr>
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<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2">LABORATORY EVALUATION OFFICER:</td><td>SHELLFISH SPECIALIST:</td></tr>
<tr><td colspan="2">OTHER OFFICIALS PRESENT:</td><td>TITLE:</td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="2"></td><td></td></tr>
<tr><td colspan="3">Items which do not conform are noted by: Conformity is noted by a “√”
C- Critical K - Key O - Other N/A- Not Applicable</td></tr>
</table>

PART I – QUALITY ASSURANCE			
CODE	**REF**	**ITEM**	
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3		1.1.1 Written Plan: (check √ those items which apply).
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			b. Internal quality control measures for equipment, calibration, maintenance, repair, performance and rejection criteria established.
			c. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			d. Internal performance assessment.
			e. External performance assessment.
C	**2**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**2.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		2.2.2 In state/county laboratories, the analyst(s) meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**2.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		2.2.4 In commercial/private laboratories, the analyst(s) must have at least a high school diploma and at least three months of experience in laboratory sciences.

C	**6**		**2.2.5 Training regarding radiation laboratory safety, handling and disposal practices is documented and records are maintained.**
C	**15**		**2.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitoxin in this assay. Alternatively, the laboratory uses less than 50 µCi per year and adheres to the American Radiolabeled Chemical (ARC) exemption status.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**
		1.4 Laboratory Equipment	
C	**4**		**1.4.1 Any lab equipment that may come into contact with [^{3}H]-STX at any point in the preparation or assay procedures must be specially labelled and must remain in the work area designated for low-level radiation work.**
O	5		1.4.2 The pH meter has a standard accuracy of 0.1 pH units.
K	7		1.4.3 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8		1.4.4 The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1		1.4.5 The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.

K	1		1.4.6 The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	9		1.4.7 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.
K	6		1.4.8 pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6		1.4.9 The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3		1.4.10 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
K	2		1.4.12 Refrigerator temperatures are maintained between 0 and 4 °C. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.
K	1		1.4.13 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**4, 6, 10**		**1.4.14 Freezer temperature used to store [^{3}H] STX standard, rat brain membrane tissue preparation, interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -80 °C or below. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.**
K	6, 10		1.4.15 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.16 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.

O	8		1.4.17 All glassware is clean.
C	**3**		**1.4.18 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.19 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
C	**6**		**1.4.20 Micropipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.**
C	**11**		**1.4.21 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.22 Minimum radiation safety equipment and protocols include the following: A wipe-test is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
			1.5 Reference Solution Reagent Storage, Preparation and Security
C	**12**		**1.5.1 [^{3}H] STX standard is stored in a freezer at -80 °C or below.**
C	**10**		**1.5.2 Concentration of ^{3}[H]STX standard is calculated from the lot information provided by the supplier with each batch.**
K	6		1.5.3 Unopened diHCl STX standard may be stored at room temperature or refrigerated.
C	**10**		**1.5.4 Preparation of MOPS assay buffer includes the following:** a. **100 mM MOPS/L.** b. **100 mM choline chloride/L.** c. **pH adjustment to 7.4 with NaOH.** d. **Refrigerated storage at 4 °C.** e. **Maintained ice cold while in use.**
C	**10**		**1.5.6 Bulk standard curve dilutions are stored at 4 °C for up to one (1) month.**

K	1		1.5.7 Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice).* b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____ c. Water contains <100 CFU/mL using the heterotrophic plate count method.
	1.6 Rat Brain Membrane Tissue Preparation and Storage		
C	**10**		**1.6.1 MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to MOPS/choline chloride fresh on the day of use**.
C	**10**		**1.6.2 The cerebral cortex of 6-week old Sprague-Dawley rats is used in membrane tissue preparations, placed in iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL) and homogenized with no visible chunks remaining in the homogenate. This procedure is repeated until twenty (20) rat brains have been processed.**
C	**10**		**1.6.3 The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**
K	10		1.6.4 The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).
K	10		1.6.5 The resuspended rat brain tissue preparations are pooled and the centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue.
K	10		1.6.6 The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.
K	10		1.6.7 The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed for 20 seconds to obtain a homogeneous membrane tissue preparation.

C	10		1.6.8 Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months
	1.7 Rat Brain Membrane Tissue Protein Receptor Determination		
C	**10**		**1.7.1 The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube method) or equivalent.**
C	**10**		**1.7.2 The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined.**
K	10		1.7.3 Dilutions of the protein/receptor concentration of the rat brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous.
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS - RBA			
	2.1 Collection and Transportation of Samples		
C	**5**		**2.1.1 A representative sample of shellfish is collected.**
K	5		2.1.2 Shellfish samples are collected in clean, waterproof, puncture resistant containers, loosely sealed.
K	5		2.1.3 Shellfish samples are labeled with the collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**5**		**2.1.4 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**

K	6, 13		2.1.5 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
	2.2 Preparation of Samples for Analysis – Homogenization		
C	**5, 6**		**2.2.1 At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8 Pieces of shell and drainage are discarded.

C	**5, 6**		**2.2.9 Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender with all freeze thaw liquid for homogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender with all freeze-thaw liquid for homogenization.**
K	6, 13		2.2.10 Homogenates should be extracted immediately. If homogenates must be stored, they should be frozen.
	2.3 Preparation of Samples for Analysis – Extraction		
K	5, 10		2.3.1 0.1 M HCl is used for extractions.
K	5, 10		2.3.2 At least five (5) grams of tissue +/- 0.1g is extracted using a 1:1 mass to volume ratio of 0.1 M HCl.
C	**10**		**2.3.3 The pH of the sample is checked and adjusted as necessary to between 3.0– 4.0.**
C	**10**		**2.3.4 Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**
C	**6**		**2.3.5 The sample is promptly brought to a boil at 99.0 +/- 1.0 °C and gently boiled for 5 minutes.**
O	6		2.3.6 The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.7 The sample is allowed to cool to room temperature.
C	**10**		**2.3.8 The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted, if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	5, 10		2.3.9 The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water).
K	10		2.3.10 The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; then b. an aliquot of the sample is centrifuged at 3,000 x g for 10

			minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.11 The sample extract is analyzed immediately, refrigerated at 4°C in a sealed container for up to 24 hours, or frozen at -20°C.
	2.4 Sample Assay		
K	6		2.4.1 One analyst performs the entire plate set-up for the assay.
K	6		2.4.2 Microtubes containing dilutions and samples are vortexed immediately before dispensing.
K	10		2.4.3 The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).
K	10		**2.4.4 The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension**
K	10		2.4.5 Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.
C	**10**		**2.4.6 The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.7 Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane.
K	10		2.4.8 All wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, in order to ensure even pressure and filtration across the plate.
C	**10**		**2.4.9 Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate).**
K	10		2.4.10 [^{3}H] STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.
C	10		**2.4.11 An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).**

K	10		2.4.12 Standard curve fitting is calculated using appropriate software program.
C	10		**2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.15 The IC50 is in acceptable range (2.0 nM +/- 30%). If the IC50 is outside this range, the assay results are rejected and the assay must be repeated**
C	**10**		**2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.**
K	4		2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.
	2.5 Calculation of Sample Toxicity		
C	**10**		**2.5.1 When more than one dilution falls within B/Bo of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**
C	**10**		**2.5.2 Sample toxicity is calculated as follows:** **(nM STX eq) x (sample dilution) x [(210 µL total volume/35 µL sample] = mM STX eq in extract** **(nM STX diHCl eq in extract) x (1L/1000 mL) x (372 ng/nmol) x (1 µg/1000 ng) = µg STX diHCl eq/mL** **µg STX diHCl equiv./mL x (mL extract/g shellfish) x (1000 g/kg) = µg STX diHCl equiv./kg**
C	**14**		**2.5.3 Any value equal to or greater than 80 µg STX diHCl eq/100 g) of sample is actionable.**

C	**14**		**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:**

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LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
RECEPTOR BINDING ASSAY FOR PSP COMPONENT: (Part I-II)	
A. Results	____
Total # of Critical (C) Nonconformities	_____
Total # of Key (K) Nonconformities	_____
Total # of Critical (C), Key (K), and Other (O) Nonconformities	_____

B. Criteria for Determining Laboratory Status of the RBA for PSP:

1. **Conforms Status:** The RBA for PSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a.
   b.
   c.

2. **Provisionally Conforms Status:** The RBA for PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a.
   b.
   c.

3. **Does Not Conform Status**: The RBA for PSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a.
   b
   c.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

Appendix A: RBA Step-by-Step Procedure

A. Sample Extraction
   a. The extraction detailed below represents a small scale MBA procedure. The actual MBA extraction could be used instead of the small scale version described here.
   b. Accurately weigh 5.0 g of tissue homogenate into a tared, labeled 15 ml conical tube.
   c. Add 5.0 ml of 0.1 M HCl, vortex, and check pH.
      i. If necessary, adjust pH to 3.0-4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing.
   d. Place the tube in a beaker of boiling water on hot plate (or in a water bath) for 5 min with the caps loosened.
   e. Remove and cool to room temperature.
   f. Check pH and, if necessary, adjust cooled mixture to 3.0-4.0 as described above.
   g. Transfer entire contents to a labeled, graduated centrifuge tube and dilute volumetrically to 10 ml.
   h. Gently stir contents to homogeneity and then allow to settle until a portion of supernatant is translucent and can be decanted free of solids.
   i. Pour 5-7 ml of the translucent supernatant into a labeled centrifuge tube.
   j. Centrifuge at 3000 x g for 10 min.
   k. Retain clarified supernatant and transfer to a clean, labeled centrifuge tube.
   l. Store extracts at -20 °C until tested in RBA.

B. Preparation of Stock Solutions and Standards
   a. Assay buffer: 100 mM MOPS/100 mM choline chloride, pH 7.4
      i. Weigh 20.9 g MOPS and 13.96 g choline chloride and add to 900 ml distilled or milli-Q water.
      ii. Adjust pH to 7.4 with NaOH while stirring.
      iii. Bring to a final volume of 1 L with distilled or milli-Q water.
      iv. Store at 4 °C.
   b. Radioligand solution: 3H-STX
      i. Calculate the concentration of 3H-STX stock provided by the supplier. Suppliers generally provide specific activity in Ci/mmol (~10-30 Ci/mmol) and activity in mCi/ml (~0.05-0.1 mCi/ml), from which the molar concentration can be calculated.
      ii. Prepare 4 ml of a 15 nM working stock of 3H-STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate.
      iii. Measure total counts of each working stock prior to running an assay. Add 36 µl of working stock 3H-STX in buffer to a liquid scintillation counter vial with 4 ml scintillant and count on a traditional liquid scintillation counter to confirm correct dilution. The CPM should be consistent and within 15% of expected value.
   c. Unlabeled STX standard working solution: The STX diHCl standard (NIST RM 8642 STX diHCl) is provided at a concentration of 268.8 µM (100 µg/ml).

i. A bulk standard curve can be made up in advance and stored at 4 °C for up to one month. The use of a bulk standard curve minimizes time needed for routine analyses and improves repeatability.
ii. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µl in 50 ml) and use for the serial dilutions.
iii. Serial dilutions should result in the following stock concentrations (M):
    1. 6 x 10-6 [100 µl 268.8 µM STX + 4.38 ml 0.003 M HCl]
    2. 6 x 10-7 [500 µl 6 x 10-6 M STX + 4.5 ml 0.003 M HCl]
    3. 1.8 x 10-7 [1.5 ml 6 x 10-7 M STX + 3.5 ml 0.003 M HCl]
    4. 6 x 10-8 [500 µl 6 x 10-7 M STX + 4.5 ml 0.003 M HCl]
    5. 1.8 x 10-8 [500 µl 1.8 x 10-7 M STX + 4.5 ml 0.003 M HCl]
    6. 6 x 10-9 [500 µl 6 x 10-8 M STX + 4.5 ml 0.003 M HCl]
    7. 6 x 10-10 [500 µl 6 x 10-9 M STX + 4.5 ml 0.003 M HCl]
    8. 5 ml 0.003 M HCl.

d. Interassay calibration standard (QC check): Reference standard STX (1.8 x 10-8 M STX) in 3 mM HCl. For long-term storage keep at -80 °C; for routine use (up to one month), store at 4 °C.

e. Rat brain membrane preparation: Prepare bulk rat brain membrane preparations (Appendix B) and store at -80 °C.
i. Thaw an aliquot of rat brain preparation on ice.
ii. Dilute membrane preparation with cold (4 °C) 100 mM MOPS/100 mM choline chloride, pH 7.4 to yield a working stock with a protein concentration of 1.0 mg/ml.
iii. Vortex vigorously to achieve a visibly homogeneous suspension.
iv. Keep the diluted membrane preparation on ice.

C. Performing the Assay

a. Plate setup: When possible use a multichannel pipet to minimize effort and increase consistency.
i. Run standards, samples, and QC check in triplicate.
ii. For quantitation, multiple dilutions per extract should be analyzed in order to obtain a value that falls within the dynamic range of the assay. A minimum sample extract dilution of 1:10 is recommended to minimize potential matrix effects.
iii. Use of a standard plate layout (Figure 1) is recommended. This will improve ease of analysis and can help maximize the number of samples/standards that can be analyzed per plate.

b. Addition of samples/standards: Add in the following order to each well
i. 35 µl assay buffer
ii. 35 µl STX standard/QC check/sample extract
iii. 35 µl 3H-STX
iv. 105 µl membrane preparation (ensure solution is homogeneous)
v. Cover the plate and incubate at 4 °C for 1 h.

c. Assay filtration: Use the vacuum manifold attached to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process.
i. Set the vacuum pressure gauge on the pump or manifold to ~4-8” Hg (~135-270 millibar).

ii. Place the 96-well plate on the vacuum manifold.
iii. Fill any empty wells with 200 µl MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate.
iv. Turn on vacuum. Optimum vacuum will pull the wells dry in 2-5 s.
v. With vacuum pump running, quickly rinse each well twice with 200 µl ice cold MOPS/choline chloride buffer using a multichannel pipet. Maintain vacuum until liquid is removed.

d. Preparation of the assay for counting: Remove the plastic bottom from the plate and blot the plate bottom once on absorbent towel.
   i. For counting in microplate scintillation counter:
      1. Seal the bottom of a counting cassette with sealing tape.
      2. Place the microplate in the counting cassette.
      3. Add 50 µl scintillation cocktail per well using multichannel pipet.
      4. Seal the top of the plate with sealing tape.
      5. Incubate for 30 min at room temperature.
      6. Place the plate in the scintillation counter and count for 1 min per well.
   ii. For counting in traditional scintillation counter:
      1. Place the microplate in the MultiScreen punch system apparatus and place the disposable punch tips on top of the microplate.
      2. Punch the filters from the wells into scintillation vials and fill with 4 ml scintillation cocktail.
      3. Place caps on the vials and vortex.
      4. Allow vials to sit overnight in the dark.
      5. Count using a tritium window in a traditional scintillation counter.

D. Analysis of Data
   a. Curve fitting: Perform curve fitting using a four-parameter logistic fit (sigmoidal dose response curve with variable slope).
      i. $y = \text{min} + (\text{max-min})/1+10^{(\text{x-log } IC_{50})\text{Hill slope}}$
      ii. where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX (also known as B_0); min is the bottom plateau, equal to nonspecific binding in CPM in the presence of saturating nonradiolabeled STX; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is the total igand binding in CPM (B/B_0).
   b. Sample quantification: Sample quantification is only carried out on dilutions having a B/B_0 in the range of 0.2-0.7.
      i. Where B represents the bound 3H-STX in CPM in the sample and B_0 represents the max bound 3H-STX in the sample.
      ii. Sample concentration is calculated in µg STX diHCl equivalents (eq)/kg shellfish as described below:

(nM STX eq) x (sample dilution) x [(210 µl total volume)/35 µl sample]
= nM STX eq in extract

(nM STX diHCl eq in extract) x (1 L/1000 ml) x (372 ng/nmol) X (1 µg/1000 ng)

= g STX diHCl eq/ml

µg STX diCHl eq/ml x (ml extract/g shellfish) x (1000g/kg)
= µg STX diHCl eq/kg

Figure 1. Example plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U4 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

Concentrations indicate those of the STX standard curve; REF = reference; QC = quality control; U = unknown sample (with dilutions indicated). The same standard curve made be used for additional plates run on the same day using the same reagents (i.e., 11 samples can be run on subsequent plates).

Appendix B: Rat Brain Membrane Preparations

B. Equipment/Supplies
   a. Teflon/glass homogenizer: Tapered Teflon pestle and glass tube, 15 ml
   b. Motorized tissue homogenizer: Polytron or small hand-held blender
   c. High-speed centrifuge and fixed angle rotor: capable of 20,000 x g
   d. Centrifuge tubes: 12-15 ml, rated for >20,000 x g
   e. Plastic cryovials: 2 ml
   f. Glass beaker: 300-500 ml
   g. Pipets: disposable 5 and 10 ml
   h. Forceps.

C. Reagents
   a. 20 rat brains: male, 6-week old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA) or equivalent
   b. MOPS: pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G)
   c. Choline chloride: 100 mM (Sigma; Cat. No. C7527-500G)
   d. Phenyl methylsulfonyl fluoride (PMSF): (Sigma; Cat. No. P7626)
   e. Isopropanol.

D. Procedure
   a. Prepare 1 L of 100 mM MOPS, pH 7.4, containing 100 mM choline chloride (as described in Appendix A) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol: dissolve 0.174 g PMSF in 10 ml isopropanol to make 100 mM stock. Aliquot stock and store at -20 °C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh in the day of use.
   b. Remove the medulla and cerebellum from each brain using forceps and discard. Place cerebral cortex in a small amount of ice-cold buffer and place on ice.
   c. Place one cerebral cortex in 12.5 ml MOPS/choline Cl/PMSF, pH 7.4, in glass/Teflon homogenizer. Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes and ensure there are no visible chinks remaining in the homogenate. Keep tube in ice at all times. Pour homogenized tissue into 250 ml beaker on ice and repeat procedure with remaining cortices.
   d. Transfer pooled homogenate tissue to centrifuge tubes, balance the tubes (pairwise: using ice-cold buffer to balance), and centrifuge at 20,000 x g for 15 min at 4 °C.
   e. Aspirate the supernatant and resuspend pellets in ice-cold MOPS/choline Cl/PMSF, using an adequate amount to fully resuspend the pellet (5-10 ml per brain).
   f. Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume up to 200 ml (keep on ice).
   g. Keeping the beaker on ice, polytron (or homogenize with small handheld blender) at 70% full speed for 20 s to obtain a homogeneous solution.
   h. Aliquot 2 ml per tube into cryovials. It is critical to keep the preparation well mixed while dispensing. Keep cryotubes on ice.
   i. Freeze and store at -80 □C. This preparation is stable for at least 6 months.

E. Protein Assay

a. Determine the protein concentration of the membrane preparation using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) or equivalent. The above protocol should yield ~6-8 mg protein/ml of rat membrane preparation.
b. Determine the membrane dilution needed for the assay. The protein concentration in the daily working stock should be 1 mg/ml (which yields a diluted concentration of 0.5 mg/ml in-assay concentration). Based on the protein concentration determined using the protein assay, dilute rat membrane preparation with buffer to 1 mg/ml. It is this diluted membrane preparation that is used in the assay.
c. Protein concentrations must be determined and new dilutions calculated accordingly for each new batch of membranes prepared.

Appendix C: Radiation Safety Requirements

A. All users must follow all local, state, and federal laws and procedures regarding receipt, use and disposal of isotopes.
B. All users must be trained in and follow all in-house safety procedures for working with radiolabeled materials.
C. All isotopes and work stations where isotopes are used should be controlled access areas. Any one with access to the area must also receive radiation safety training.
D. Freezers where the isotopes are stored must be locked.
E. Personal protective equipment must include lab coats (designated specifically for use with radioactive materials), safety glasses, and gloves.
F. Radioactive materials will only be handled and manipulated in designated areas, which have been clearly identified and labeled accordingly.
G. Work with source radiation material must be conducted in a fume hood.
H. Radioactive materials will be stored and/or carried in secondary containment.
I. When possible, disposable supplies such as pipet tips, absorbent paper, and kim wipes will be used so that contaminated supplies can be readily disposed of as radioactive waste.
J. Wipe surveys will be conducted at the end of each experiment as well as monthly to ensure that there is no contamination in the laboratory.
K. The filter plates used in the assay will be designated as solid radioactive waste, while the washes from the filter plates (containing buffer and unbound 3H-STX) will be handled as liquid radioactive waste. There will be a dry active waste container to hold contaminated items such as the plates, gloves, absorbent paper and kim wipes. There will be a liquid waste jug to hold the contaminated liquid radioactive waste.
L. All wastes must be disposed of according to state and local laws.

Appendix VI. Abbreviations and Acronyms

3H-STX	Tritiated saxitoxin
AOAC	Association of Analytical Communities
ARC	American Radiolabeled Chemicals
B	Bound CPM
B_0	Maximum bound CPM
CFSAN	Center for Food Safety & Applied Nutrition
CPM	Counts per minute
diHCl	Dihydrochloride
Eq	Equivalents
HCl	Hydrochloric acid
IC_{50}	Inhibitory concentration at which CPMs are at 50% max
LC-FD	Liquid chromatography with fluorescence detection
LOD	Limit of detection
LOQ	Limit of quantitation
MBA	Mouse bioassay
MOPS	3-Morpholinopropanesulfonic acid
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NSSP	National Shellfish Sanitation Program
OMA	Official method of analysis
PMSF	Phenyl methylsulfonyl fluoride
PCOX	Post-column oxidation liquid chromatography with fluorescence detection
Pre-COX	Pre-column oxidation liquid chromatography with fluorescence detection
PSP	Paralytic shellfish poisoning
PSTs	Paralytic shellfish toxins
QA	Quality assurance
QC	Quality control
QS	Quality System
RBA	Receptor binding assay
RSD	Relative standard deviation
SLV	Single laboratory validation
STX	Saxitoxin

Appendix VII: Overview of Quality Systems

Public Health Laboratories

Quality Management Plan



Washington State Department of Health
Public Health Laboratories

For more information or additional copies contact:

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1610 NE 150th Street
Shoreline, WA 98155-9701

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69790.12 *Quality Management Plan*

Washington State Department of Health

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Although every effort has been made to provide valid information in this manual the Public Health Laboratories makes no assurance as to the accuracy of data and statements in this document. The user assumes all responsibility for verification and validation of the information provided.

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1 INTRODUCTION

The Washington State Department of Health, Public Health Laboratories (PHL) is committed to the continuous improvement of quality management and assurance. A quality management program must systematically evaluate the quality and appropriateness of laboratory services. The program must be designed to identify and resolve important problems in patient care and identify opportunities to improve patient care. Quality Assurance (QA) is defined as planned or systematic actions necessary to provide adequate confidence that a product or service will satisfy defined needs.

A detailed monitoring and evaluation process enables the Laboratories to effectively use their resources to manage the quality of the service they provide. This process involves the ongoing examination of services provided, identification of deficiencies in the services, and improvement, as necessary, of the quality of the services. Appropriate monitoring and evaluation activities are ongoing and integrated with other managerial and planning activities throughout the organization.

The monitoring and evaluation process assists in identifying patterns of service that may not be evident when only a case-by-case review is performed. The process may also identify situations in which a case review is likely to be most useful in identifying correctable deficiencies in service and opportunities to improve service. Although this process will not identify every case of substandard service, monitoring, and evaluation help the organization distinguish situations on which its attention could be most productively focused.

2 MISSION OF THE PUBLIC HEALTH LABORATORIES

PHL's vision is to be a world-class laboratory in providing diagnostic and analytical measurements to support the Washington State Department of Health's programs and Local Health Jurisdictions to improve and protect the health of citizens of Washington State.

PHL's mission is to put the best diagnostic and analytical tests to accurately and rapidly provide timely results to our partners by:

- Continually interacting and supporting the public health community.
- Soliciting and encouraging all interested parties to communicate with us.
- Building, maintaining, and sharing technical expertise in the latest laboratory technology available to the public health community.
- Providing a wide range of diagnostic and analytical functions for the assessment and surveillance of infectious/communicable, heritable/genetic, and chronic diseases as well as environmental contamination.
- Improving the QA and analytical performance of clinical, surveillance, and environmental laboratories through training and consultation as well as providing scientific and managerial leadership in developing public health policy.

Our Quality Management Plan (QMP) is designed to support our vision, mission, and values as well as help us provide the highest level of analytical support to our many partners throughout the state of Washington as well as the country.

3 STATED GOALS AND OBJECTIVES OF QUALITY ASSURANCE PROGRAM

PHL Quality Assurance program has an obligation to the customers that the PHL serves. QA includes all actions planned and implemented to provide confident test results and exemplary customer service. It is those planned or systematic actions necessary to provide adequate confidence that a product or service

will satisfy defined needs. This includes control of all processes and procedures that occur within the Laboratories, from the time the sample/specimen is received, through the analytical testing, to the reporting and archiving of data. QA also extends beyond the physical confines of the Laboratories, from the time a test is requested, through the collection process, to the medical or scientific use and interpretation of the test results.

The QA Program was established to facilitate achieving the highest standards of quality customer service. The program has been developed to provide a:

- Continuous program monitoring and evaluation of QA activities
- Working structure for identification and resolution of QA concerns
- Means for integrating and monitoring information between support and scientific areas
- Mechanism for integrating the QA within PHL management and operation
- Framework such that the standards established by the Laboratories will meet or exceed the standards and requirements for certification of all accrediting agencies

4 QUALITY MANAGEMENT RESPONSIBILITIES

The PHL Deputy Director has the overall responsibility for the PHL QA Program. The QA team is the organizational structure that supports the Deputy Director in carrying out this responsibility. The Deputy Director has designated the QA Officer and the QA team to be responsible for the review of the PHL QA program. The Office Directors and Supervisors are responsible for the implementation and feedback for the QA program. The QA Officer reports directly to the Deputy Director. Currently, the Deputy Director also serves as CLIA Director.

4.1 Clinical and Technical Consultant

The primary role of Clinical Consultant for communicable disease-related issues at the PHL is filled by Dr. Scott Lindquist MD, MPH Washington State Epidemiologist for Communicable Diseases. Dr. Lindquist and the staff of the Office of Communicable Disease Epidemiology are physically located within the PHL building and are available for consultation during Laboratory operating hours and on-call during weekends and holidays.

In addition to Dr. Lindquist, Epidemiologists within the Office of Communicable Disease Epidemiology are available for client and laboratory consultation relating to laboratory testing for communicable diseases, foodborne illness, STI, etc.

The Clinical Consultants are:

- Marcia Goldoft, MD, DOH Epidemiologist
- Scott Lindquist, MD State of Washington Epidemiologist
- Michael Bender, MD, PhD - Pediatric Hematologist - Fred Hutchinson Cancer Research Center
- Patricia Fechner, MD, Pediatric Endocrinologist – Seattle Children's Hospital
- The Cystic Fibrosis clinical consultant is Margaret Rosenfeld, MD, MPH, Pediatrics Pulmonologist at Seattle Children's Hospital and Regional Medical Center, Associate Professor, Pediatrics/Pulmonary, University of Washington, School of Medicine
- Sihoun Hahn, MD, PhD, Director, Biochemical Genetics Program, Professor, Department of Pediatrics, Division of Genetic Medicine, University of Washington School of Medicine, Seattle Children's Hospital
- Maria Teresa de la Morena MD, Immunologist at Seattle Children's Hospital
- Seth Perlman MD, Neurologist Seattle Children's Hospital

4.2 Delegation of Duties

The Office Directors in Microbiology, Newborn Screening, and Environmental Laboratory Sciences serve as **Technical Consultants** and **General Supervisors** along with the PHL Deputy Director for the laboratory's CAP accreditation. They are delegated appropriate duties by using the form:
69790.21 *Delegation Form Office Director*

The Office Directors delegate appropriate duties to the supervisors using **69790.22** *Delegation Form Supervisor*. The supervisor delegates appropriate duties to the Leads using **69790.125** *Delegation Form Lead*. Additional delegation forms are:
69790.18 *Delegation Form Deputy Director*
69790.19 *Delegation Form IT Applications*
69790.20 *Delegation Form IT Hardware*

These delegation form templates are kept in document control and approved by the Deputy Director prior to the signature workflow for approving the delegation of duties to an individual.

4.3 Quality Assurance Monthly Meeting

The PHL Deputy Director, upper management, and QA team meet monthly, all staff are invited to attend via Teams. This has enabled staff who were previously unable to attend because of time constraints, meeting room capacity, etc. to attend. The agenda is presented each month and the meeting minutes are available to all staff on the PHL QA SharePoint page. The meeting minutes are approved at the following month's QA meeting.

4.4 QA Officer

The QA Officer is the primary contact for all QA-related activities at PHL. This position coordinates both internal and external inspections as well as PHL's response to any deficiencies and recommendations noted. The duties of the QA Officer include but are not limited to chair of the QA team, presentation of the monthly QA meeting, and revision of the QMP as necessary with approval from the Deputy Director. The QA Officer participates in QA-related training, guides and reviews the performance of the biennial client survey, assists with new method validation/verification, and directs follow-up of nonconformance reports. The QA Officer is responsible for administrating MediaLab software, which is PHL's cloud-based document control platform.

4.5 Office Directors and Supervisors

Office Directors and Supervisors have the responsibility of reviewing the accuracy and authenticity of the data generated for laboratory reporting and test method validation/verification. They are responsible for ensuring the daily quality control activities have been performed accordingly, to ensure that the test results are accurate and reliable. Supervisors and lead workers have the primary responsibility of managing daily testing operations for their respective units.

4.6 Public Health Laboratories Staff

All staff are responsible for:

- Reporting to their leads or supervisors QA problems encountered while performing their duties and responsibilities.
- Assisting in the resolution of problems.
- Providing suggestions for ongoing monitoring and developing improvements in service.
- Providing pertinent information to their supervisors as it relates to QA.
- Signing the Proficiency testing (PT) attestation for samples analyzed.

- Completing a **69790.14** *PT Quality Improvement Assessment Sheet* when results are not graded as Passing or Acceptable.
- Completing a **69790.793** *PHL Customer Feedback Form* for client complaints and compliments (in MediaLab IQE).
- Reading and following the rules and guidance in the QMP and requesting variances from the QA Officer, if necessary.
- Completing the **69790.67** *New Test & Instrument Checklist* before for new procedures are placed online.
- Reporting laboratory quality issues or concerns. See **69790.945** *How to Record a New Nonconforming Event in MediaLab* for details on how to use the IQE module in MediaLab to report quality issues and concerns.
- Submitting feedback intended to improve QA systems at the PHL using the *Internal (Laboratory Staff) Customer Feedback Form* found on the PHL QA Welcome page at PHL QA Welcome! (sharepoint.com). Staff feedback may be submitted anonymously using this form.

4.7 Laboratory Safety

The Laboratory Safety Program is administered by the Safety Office with assistance from the Laboratory Safety Committee. The Safety Office helps to ensure a safe work environment for staff and clients. The Safety Officer provides training, information, technical assistance, policy development, and continual oversight that enables management to maintain a safe and effective workplace environment at the PHL. The Safety Officer helps to ensure that the activities of the laboratories are compliant with statutory requirements. The PHL promotes safety and quality as an institutional core value and ensures that analytical activities are conducted in compliance with applicable policies and regulations.

Please refer to the PHL manuals listed below for further information regarding safety practices.

• Safety & Health Manual	69790.38
• Biosafety Manual	69790.17
• Chemical Hygiene Plan	69790.32
• Respirator Protection Program	69790.37
• Emergency Response Plan	69790.34
• Radiation Safety Manual	69790.178
• Confined Space Program	69790.33
• Hearing Conservation Program	69790.36
• VHP Management Plan	69790.3876

5 DOCUMENT CONTROL

5.1 Document Control Process

The PHL uses an electronic cloud-based software system called MediaLab (www.medialab.com). All employees stationed at the PHL have been given access to MediaLab with varying permission levels of access. The document control process is described in **69790.1057** *Document Control*.

5.2 Quality Management Program Review

The Deputy Director is responsible for the annual Quality Management Program (QMP) review and approval, along with input from the QA Officer, QA team, Office Directors, and Supervisors. Upon a

change to the Deputy Director position, the new appointee will re-review and approve the QMP, over a reasonable period of time (typically 90 days after appointment to the Deputy Director position).

A paper copy of the QMP is located at the front office. The QA Officer is responsible for replacing any old copies when a new version of the QMP is issued.

5.3 Procedures, Forms & Records

Supervisors are responsible for adhering to the authorized document control process, and for ensuring that testing personnel have read the policies and procedures relevant to their job activities, as well as conducting biennial procedure reviews in the assigned section, along with the Office Director. All procedures and forms should adhere to regulatory guidelines governing that unit. Next generation sequencing (NGS) procedures should include the classification process and interpretation of sequence variants.

5.4 Management and correction of laboratory records

The detailed process is described in **69790.1057** *Document Control.*

5.5 Document Retention

The detailed process is described in **69790.1308** *Record and Specimen Retention Policy.*

5.6 Electronic Signature

Per Department of Health Policy number 17.008, electronic signatures may be used where feasible and especially in cases where the document workflow is already conducted electronically. Many methods of implementing an E-Signature process are accepted under this policy. At the PHL, the most common method is the Clickwrap method and the symbol indicating intent to sign is /s/.

6 DESCRIPTION OF SERVICES

The PHL consists of four technical offices:

- Office of Operations
- Office of Public Health Microbiology
- Office of Environmental Laboratory Sciences
- Office of Newborn Screening

6.1 Laboratory Director

The Laboratory Director provides operational and strategic direction and leadership. The office consists of the Deputy Director, administrative support staff (payroll, human resources, travel, etc.), and the QA Officer.

6.2 Office of Operations

The Office of Operations (OPS) provides auxiliary services such as glassware sterilization and preparation, mailroom activities, facilities management, and media preparation. OPS also includes the Safety Office and security. The Safety Office reports to the OPS Office Director.

6.3 Office of Public Health Microbiology

The Office of Public Health Microbiology (MICRO) supports and assists programs of the Department of Health, other state agencies, federal agencies, private and public laboratories, and local community health departments by providing analytical and diagnostic services on infectious diseases and communicable outbreak response.

6.4 Office of Environmental Laboratory Sciences

The Office of Environmental Laboratory Sciences (ELS) supports the Department of Health Programs, local health jurisdictions, and the general public in supplying qualitative and quantitative testing on the microbial, radiological, and chemical conditions in the environment.

6.5 Office of Newborn Screening

State law and regulation (Chapter 246-650 WAC) requires hospitals to submit a blood specimen from every newborn to the WA Newborn Screening Laboratory (NBS). Parents may opt out, based on religious tenets or practices by signing a refusal form. The NBS is required to perform appropriate screening tests to detect:

Amino Acids disorders: Phenylketonuria (PKU), Maple syrup urine disease (MSUD), Homocystinuria (HCY), Argininosuccinic academia (ASA), Citrullinemia (CIT), Tyrosinemia type 1 (TYR-1)

Fatty acid oxidation disorders: Medium chain acylCoA dehydrogenase (MCADD), Carnitine uptake defect (CUD), Long chain L-3-hydroxy acyl-CoA dehydrogenase (LCHAD) deficiency, Trifunctional protein (TFP) deficiency, very long chain acyl-CoA dehydrogenase (VLCAD) deficiency.

Organic acid disorders: 3-Hydroxy-3methylglutaric aciduria (HMG), Beta –ketothiolase deficiency (BKT), Glutaric acidemia type 1 (GA-1), Isovaleric acidemia (IVA), Methylmalonic acidemias (CblA, CblB & Mutase), Multiple carboxylase deficiency (MCD), Propionic acidemia (PROP) AND

Other disorders: Congenital hypothyroidism (CH), Congenital adrenal hyperplasia (CAH), Hemoglobinopathies, Galactosemia, Biotinidase deficiency, Cystic fibrosis (CF), Severe combined immunodeficiency (SCID), X-linked adrenoleukodystrophy (X-ALD), Pompe disease & Mucopolysaccharidosis type I (MPS-I), spinal muscular atrophy (SMA) and other heritable disorders as determined by the State Board of Health.

7 IMPORTANT ASPECTS OF SERVICE

The services provided by the PHL support the initiatives to develop healthy children and protect Washingtonians from environmental hazards and contamination, infectious, communicable, genetic, and chronic diseases.

8 LICENSURE, ACCREDITATION, AND CERTIFICATION

Accreditation or certification is the recognition for meeting certain predetermined standards conferred upon a facility by a nationally recognized agency. Standards of the accrediting agency assure quality for performance in both administration and analytical testing. The basic principle of accreditation is that the laboratory complies with the accrediting agency's standards continuously.

The laboratories are licensed, accredited, certified, or inspected by the following agencies:

- WA Medical Test Site Licensure Program, CLIA
- College of American Pathologists, CAP
- State of California Clinical and Environmental Testing
- Washington State Department of Ecology, WA DOE
- United States Food and Drug Administration, FDA
- Federal Emergency Management Administration, FEMA
- State of Washington Office of Radiation Protection
- Center for Disease Control Select Agent Program, SA
- Department of Transportation, DOT

- ANSI National Accreditation Board, ANAB ISO/IEC 17025

These agencies periodically inspect the laboratories they certify. Inspections determine the degree to which a laboratory is following the agency's standards.

Any deficiency revealed during an inspection is addressed and corrected by the appropriate laboratory section within an agreed-upon timeframe with the inspecting agency Follow-up report(s) for corrected deficiencies are sent to the certifying agency with a copy maintained on the P: drive or in the IQE module of MediaLab.

Successful accreditation is dependent on satisfactory performance on Proficiency Tests. Unsuccessful performance on Proficiency Tests may result in the loss of accreditation status and cessation of the testing.

8.1 Compliance Policies

The PHL shall comply with applicable laws and regulations such as the Washington State Labor & Industries, OSHA worker safety regulations, and Clinical Laboratory Improvement Amendments (CLIA). WA PHL develops, implements, and maintains policies, processes, and procedures throughout the organization that are designed to meet relevant requirements.

8.2 Medicare/Medical Test Site Licensure Program

Clinical laboratories in Washington State are required to be licensed under the WA Medical Test Site Licensure Program. Laboratory inspections are performed by inspectors from the Department of Health Office of Laboratory Quality Assurance or by CLIA-deemed accrediting agencies.

The requirements and standards are described in the federal register, dated Friday, February 28, 1992, in Part II Department of Health and Human Services, titled Clinical Laboratory Improvements Amendment, 1988 (CLIA88). A copy is maintained in the QA Officer's office.

The PHL sections performing clinical testing are licensed by the WA Medical Testing Site Licensure Program and biennially inspected by the College of American Pathologists (CAP). Internal audits are performed during the year when no external inspection is scheduled. A detailed audit procedure can be found in **69790.957** *CAP Internal Inspection*.

The summary reports from the internal inspection are prepared by the QA Officer and submitted to the external inspectors upon request.

8.3 College of American Pathologists

CAP is a well-recognized clinical laboratory-certifying agency and CLIA-deemed entity. The PHL has maintained accreditation through CAP since the 1970's. Further details on CAP accreditation can be found in **69790.958** *CAP Accreditation*.

8.4 Department of Ecology, Washington State

The WA DOE Laboratory Accreditation Section accredits laboratories performing testing of drinking water throughout the state. The PHL is accredited by the DOE for performing lead testing in drinking water, i.e., school drinking water testing. The DOE also regulates hazardous waste management.

8.5 Food and Drug Administration

Under provisions of the National Shellfish Sanitation Program, State Laboratory evaluations are the responsibility of the FDA to ensure the uniform application of standard procedures and methods in split samples, the examination of shellfish growing waters, quality control of market shellfish, and the monitoring of depuration systems and paralytic seafood poisoning. An additional purpose of laboratory

evaluations is to determine the adequacy of facilities, equipment, and personnel to perform the level of analytical testing necessary to meet the State's Shellfish Program requirements.

A satisfactory evaluation indicates that the agency recognizes that the laboratory 1) complies with recommended procedures and 2) has the capabilities to produce quality analytical results in support of the State's Shellfish Control Program.

The FDA recommends on-site evaluations at least every three years or more frequently if necessary. Inspections follow guidelines set by the Food and Drug Administration and typically include a review of laboratory apparatus, materials, media preparation, bacteriological procedures, and laboratory techniques. The analysts must also demonstrate competence in performing appropriate tests as described in the fourth edition of the *Recommended Procedures for the Examination of Sea Water and Shellfish, APHA Official Methods of Analysis of the Association of Official Analytical Chemists*, Section 46.117-46.119, Thirteenth Edition, 1980, AOAC, and *Interim Guides for the Depuration of the Northern Quahaug, Mercenaria*, Marine Health Sciences Laboratory, 1986.

The FDA may also inspect laboratories that do reference testing on blood supply products.

8.6 Federal Emergency Management Administration

The FEMA evaluates the PHL Environmental Radiation laboratory, as a part of the State of Washington Radiation Emergency Response Team. Every twelve years through observation of the laboratory performance during a simulated drill, FEMA evaluates the laboratory preparedness for the radiation emergency response by their ability to follow the Laboratory Standard Operating Procedures (*Radiation Emergency Response Manual,* a hard copy).

8.7 Select Agent Inspections

As part of the registration process for the CDC Select Agent Program, the PHL is subject to unannounced inspections, at any time, by inspectors from the Select Agent Program. There are no routinely scheduled inspections, annual or otherwise.

8.8 Department of Transportation

As part of the requirement to ship diagnostic and infectious substances, the PHL can be inspected at any time to ensure that they are compliant with shipping regulations.

8.9 Washington Radiation Inspections

The State of Washington Office of Radiation Program issues a license every two years to the PHL to handle radioactive materials. Prior to the renewal of the licensures the PHL will be inspected by the Office of Radiation Protection.

8.10 ANSI National Accreditation Board

As of Nov 2019, the PHL Radiation Laboratory is accredited by ANAB ISO/IEC 17025.

9 PERSONNEL

9.1 Educational and Experience Requirements

Personnel education and experience qualifications are a major aspect of QA. It is the policy of the PHL to comply with all applicable state and local laws and regulations regarding personnel policies. Sufficient education and/or job experience is required for each position at the PHL, including those of clinical consultants whether employed by DOH or under contract. For detailed job requirements see the appropriate Washington State Department of Personnel website:

http://www.dop.wa.gov/CompClass/JobClassesSalaries/Pages/ClassifiedJobListing.aspx (accessed June 2024).

Certain positions have specific educational requirements the lab must follow to maintain accreditation and license to perform testing. The Center for Medicare and Medicaid Services (CMS) requires specific credentials for clinical testing personnel. Those requirements can be found in **69790.5052** *CAP Personnel Requirements*.

Required qualifications for a position are documented in the position posting and job description. At the time of recruitment and hiring, Human Resources collects and records proof of position qualifications which may include but are not limited to diplomas, transcripts, board certifications, licenses, and work history.

Some units within the PHL have responsibilities that occur outside of regular business hours (after hours, weekends, and holidays) when Supervisor and Lead staffing is minimal. To ensure testing is completed in a satisfactory manner only qualified employees who have completed training and competency requirements for their job duties are permitted to work outside of regular business hours.

9.2 Office Organization

The PHL is an office within the division of Disease Control and Health Statistics in the State of Washington Department of Health. The Laboratories are under the directorship of Laboratory Director, Dr. Vince Aoki, and consist of four offices: Public Health Microbiology, Environmental Laboratory Sciences, Newborn Screening, and Operations. The Office Directors currently serve roles as General Supervisor and Technical Supervisor as defined by the CLIA/CAP. Level 4 Microbiologists and Chemists act as subject matter experts (SME) in their specialty of testing, providing technical advice to Office Directors. Diagnostic testing is performed by Microbiologists, Chemists, and Laboratory Technicians.

Public Health Microbiology

The Office of Public Health Microbiology is divided into the following laboratory sections: Reference Bacteriology / Antibiotic Resistance laboratory, Sexually Transmitted Diseases/Tuberculosis laboratory, Molecular/AMD (Advanced Molecular Detection)/Virology laboratory, Enteric/Food/Parasitology laboratory, Bioterrorism/Biowatch laboratory and High Throughput (Covid)/ Central Accessioning laboratory. Each laboratory has a Microbiologist 4 as a Supervisor and a Microbiologist 3 as a Lead.

Environmental Laboratory Sciences

The Office of Environmental Laboratory Sciences is divided into the following laboratory sections: Shellfish Biotoxins, Marine Water Bacteriology, Chemical Incident Response, and Environmental and Radiation Chemistry. Each section has a Supervisor (Chemist 4), who reports directly to the Office Director of Environmental Laboratory Sciences.

Newborn Screening

The Office of Newborn Screening is divided into three sections: laboratory, operations, and follow-up. The follow-up section is supervised by the Follow-Up Supervisor (Epidemiologist 2), the operations section is supervised by the NBS Program Manager (MA5), the laboratory is supervised by two laboratory supervisors (Chemist 4) and the entire program is supported by a NBS epidemiologist (Epi2). A laboratory quality assurance coordinator (WMS1) provides quality oversight to the NBS laboratory operations. The laboratory is further divided into sections based on testing methodology. These are MS/MS (amino acid disorders, fatty-acid oxidation disorders, organic acid disorders & X-linked adrenoleukodystrophy), Autodelfia (CH, CAH & CF), isoelectric focusing for hemoglobinopathies (reflex

to HPLC and DNA testing), enzyme activity assays (biotinidase deficiency and galactosemia), DNA-based tests (SCID & SMA) and digital microfluidics (Pompe disease & MPS-I).

Operations

The Office of Operations is divided into three units: Facilities, Support Services, and Safety Office.

An organizational chart is available through the Microsoft Teams or Outlook contact cards and in Service Central under "My Org Chart".

9.3 Training and Competency

From a laboratory perspective, training is the documented process where an employee becomes proficient in performing an assay or protocol safely according to the parameters outlined. Successful completion of training is required prior to performing duties. Training documents are kept by Supervisors in each section in the Compass module of MediaLab

Ensuring that all analysts are competent to perform their analyses is basic to Quality Assurance. To be effective, the competency assessment must be performed throughout the year (twice for the first year of employment, then annually). Duties related to the training and competency evaluation are delegated to the Office Directors.

Detailed procedure can be found in **69790.965** *Personnel Training and Competency*.

9.4 Performance Evaluation

The State Department of Personnel requires that all state employees complete a performance evaluation annually. PHL employees are evaluated by their respective supervisor. Evaluations are maintained by the Department of Personnel in Olympia.

9.5 Color Deficiency

All technical personnel performing testing or other tasks that require color discrimination are to be evaluated for difficulty with visual color discrimination. Evaluation is not required for personnel that do not perform such functions. The evaluation may be limited to discrimination of those colored items pertinent to the job. The Ishihara Test for Color Deficiency is utilized, and a record of the testing is recorded on the Color Deficiency Form. Currently, the Administrative Assistant to the PHL Director (AA4) is responsible for color deficiency testing as a part of onboarding orientation. If the AA4 is unavailable, color deficiency testing will be administered by Office Administrative Assistance 3 (AA3). In circumstances where social distancing is necessary, an online version of the Ishihara test is used. The employee will copy their test results from the online module to a modified **69790.2077** *Ishihara's Online Test for Color-Deficiency* form. The original result form will be kept in the employee's medical file. The AA4 will provide a notification to the employee as well as their supervisor indicating a pass or failure. If the employee fails, they are not disqualified from performing the test, especially if this is a part of their essential duties. If an employee fails the test, The Reasonable Accommodations Manager should be contacted to engage in the reasonable accommodations process and further steps.

The online Ishihara Test for Color Deficiency can be found at the below link:

https://www.colorlitelens.com/ishihara-test.

10 SAMPLE TEST MANAGEMENT

It is the WA PHL policy that all testing is compliant with applicable state and local laws and regulations. Test requests shall be made from authorized personnel such as hospitals, health care clinics, and various government agencies. A signed Patient Consent Form for testing may be required to process specimens.

More specific testing information can be found in the individual procedures in the laboratory and on the laboratory test menu website.

Most samples and specimens are delivered to the PHL by U.S. mail, UPS, or by a courier service. Further details can be found in **69790.468** *Specimen Handling in Central Accessioning*.

The PHL will make an effort to provide necessary training to our customers in specimen and sample collection. Information for the collection and handling of laboratory specimens is available in the Public Health Laboratories Directory of Services as well as the PHL Lab Test Menu website. The QA Officer and unit Supervisors are responsible for maintaining updated information posted on the website.

https://doh.wa.gov/sites/default/files/legacy/Documents/Pubs//301-016-PHLDirectoryServices.pdf (accessed June 2022)

https://doh.wa.gov/public-health-healthcare-providers/public-health-laboratories/lab-test-menu (accessed June 2022)

10.1 Unique Identification of Specimens and Samples

All specimens and samples must be labeled at the time of collection to provide unique identification. For clinical specimens, two unique identifiers are to be used to label the primary specimen container.

10.2 Limited Exceptions in Specimen and Sample Labeling

If laboratory personnel identify a potential error on a specimen or sample label, the best practice is to reject this specimen/sample and recollect the specimen/sample. However, there may be circumstances when recollection is not possible or practical. Circumstances under which clerical correction of the information on a specimen/sample label is permitted are determined by a supervisor in a given specialty of testing. A record of all such corrections should be maintained on the original specimen/sample paperwork and laboratory log. The PHL promptly notifies the submitter and asks them to investigate the reason for the errors in specimen/sample labeling. If needed, recommendations can be made for the submitter to develop corrective/preventive action as appropriate, including an education of personnel who collect specimens/samples.

The PHL has created a **69790.46** *Specimen Label Deficiency Authorization Form* to address the issue when the PHL receives a specimen with missing or discrepant labeling. This form clearly states that the submitters shall take full responsibility for the identity of the patient from whom this specimen was collected. Laboratory results will not be reported out until this form has been completed and received by the PHL.

10.3 Retaining Samples

Laboratory samples are to be retained for a defined period as listed in **69790.1308** *Record and Specimen Retention Policy*.

10.4 Specimens Sent to Reference Laboratories

In several instances, the PHL must send specimens, nucleic acid extractions, aliquots, cultures, NGS testing processes (analytical wet bench, analytical bioinformatics, interpretation), samples, etc. to a reference laboratory for additional testing.

Shipment of specimens, samples, shipping documentation, and shipment receipt is predominately handled by Central Accessioning (CA) and in rare circumstances by the laboratory unit directly. The Office of Newborn Screening accessions their own samples in the N-wing. The CA Supervisor ensures the appropriate communication with recipient laboratories regarding technical details for the particular requirement on shipment, if applicable. The Deputy Director is responsible for monitoring the quality of test results received from reference laboratories. The detailed shipping procedure is described in

69790.467 *Central Accessioning Shipping* and the procedure for receipt and distribution of reports from reference laboratories is described in **69790.466** *Central Accessioning Reporting Send Out*.

NGS bioinformatics data files may be transferred to reference laboratories. The conditions and details when a data file can be transferred to a reference laboratory are detailed in the specific NGS method standard operating procedure (SOP). Procedures for file transfer are found in **69790.3152** *Bioinformatics – IT Infrastructure*. Data files are only transferred using secure and approved methods (eg, FASTQ file format and secure file transfer systems such as PulseNet FTP or BaseSpace Sequence Hub). Records of file transfer and receipt to reference laboratories must be kept.

Lab reports from all the referred testing laboratories are retained according to the DOH retention policy, **69790.1308** *Record and Specimen Retention Policy*.

The essential elements of referred test results are reported by the WA PHL as received from the referral laboratory, without alterations that could affect clinical interpretation. Transcribed results from the referral laboratory report, the test result(s), interpretation, and information directly related to the interpretation are copied as reported by the referral laboratory.

10.5 Selection of Reference Laboratories

The selection of reference laboratories is under the authority of the CLIA director and is designated on a case-by-case basis. Any diagnostics testing sent out shall be pre-authorized by the CLIA director and the CLIA director shall evaluate the authenticity of the recipient reference laboratory.

The PHL is fully responsible for the selection and transport of the specimen to reference laboratories. PHL will properly follow all requisition, collection and handling specifications of the reference laboratories, and state and federal transportation regulations.

The selection and evaluation of reference laboratories must include the following criteria:

- The reference laboratory must meet appropriate accreditation requirements for the area of testing they are performing. For instance, clinical laboratories must meet CLIA-88 testing requirements and the laboratory must be a CLIA-certified laboratory or a laboratory meeting equivalent requirements as determined by the Center for Medicaid Services. Environmental laboratories must meet the Environmental Protection Agency, State of Washington Department of Ecology, Food and Drug Administration, Office of Radiation Protection, or ISO 17025 certification requirements.
- The PHL is responsible for verifying that the reference laboratory has the appropriate certification.
- Laboratory quality of performance must be accounted for. This can be done by previous experience with the laboratory, other users of the laboratory, contacting the accreditation agency for the laboratory, requesting documentation of proficiency performance from the reference laboratory, or past accreditation inspections.
- Turnaround times must meet the testing needs of the PHL and their clients.

PHL is responsible for closely controlling pre-analytic variables to maintain specimen integrity. Also, it may be necessary to collect specific patient information required by the testing laboratory.

PHL ensures access to reference laboratory test reports either via direct access to the reference laboratory portal or via keeping a hard copy of the original reports. Results are typically released directly to ordering providers from the reference laboratory, or PHL releases the original report from the reference laboratory without alterations. This is captured in document **69790.466** *Central Accessioning Reporting Send Out Results*.

10.6 Identification of specimens, specimen types, samples, and aliquots

A system must be in place to positively identify all patient specimens, specimen types, samples, and aliquots at all times. Each specimen and sample container must identify the patient uniquely. This may be text-based, numeric, bar-coded, etc. The form of this system is entirely at the discretion of each laboratory, so long as all primary collection containers and their aliquots have a unique label that can audit back to full the particulars of patient or sample identification, collection date, specimen type, etc. Practical considerations of container size may limit the extent of such details. Aliquots must not be returned to the original container for specimens to be used for molecular-based testing. For specimens used for non-molecular types of testing, the laboratory must consider contamination and the potential for specimen mix-up when defining this in its procedure. If previously aliquoted specimens are used for additional testing, the procedure must define when and how they can be used.

When a specimen aliquot is made, care must be taken to prevent cross-contamination of the aliquot containers. No aliquot is ever returned to the original container.

10.7 Accessioning Specimens and Samples

The date (and time, if appropriate) that the specimen or sample was received by the laboratory is to be recorded. The specimens and samples for the Offices of Microbiology and Environmental Laboratory Sciences are received and accessioned in the central accessioning facility, B103. Newborn Screening accessions their specimens in N-wing (N22) after having been received in the central receiving facility (B103). Detailed accessioning process is found in **69790.465** *Central Accessioning Data Entry* and NBS **69790.368** *Specimen Processing*.

Clinical laboratory specimens are to be analyzed only at the written or electronic request of an authorized person. An authorized person would be a physician licensed under chapter 18.71 RCW, Physicians; chapter 18.57 RCW, Osteopathy—Osteopathic medicine and surgery; or chapter 18.22 RCW, Podiatric medicine and surgery; advanced registered nurse practitioner, licensed under chapter 18.79 RCW, Nursing care; Midwife licensed under chapter 18.50 RCW, Midwifery; Physician assistant licensed under chapter 18.71A RCW, Physician assistants; Naturopath licensed under chapter 18.36A RCW, Naturopathy; or Dentist licensed under chapter 18.32 RCW, Dentistry. WAC 246-338-020, *Licensure — Types of medical test site licenses*, was used as a guide in determining who is an authorized person. If this authorization is lacking, the Public Health Laboratories will follow up and get the appropriate authorization.

Extracted nucleic acid specimens for clinical testing are only accepted from a CLIA-certified (or equivalent) laboratory and are clearly indicated on the test requisition form.

Starting January 1, 2024, to be in compliance with the Washington State Notifiable Conditions Rule Chapter 246-101 WAC, the PHL Microbiology Office will no longer accept paper-based test requisitions for clinical test submissions. Specimen submissions are to be completed through the electronic test ordering portal, Lab Web Portal (LWP), which will collect all required demographic data included in the Notifiable Conditions Rule. First-time users of the LWP can sign up on the PHL Lab Test Menu home page at Lab Test Menu | Washington State Department of Health. Paper-based requisitions will be accepted with preauthorization from the PHL on a limited basis if the submitter cannot access the LWP or due to some other barrier preventing the use of the LWP.

This change does not apply to Newborn Screening submissions. Environmental Laboratory Sciences submissions are currently being transitioned to LWP, once this transition is complete, paper-based requisitions will be phased out.

10.8 Review of Specimen and Sample Acceptance

An integral part of providing timely results is the receipt of samples/specimens by the laboratory that are properly collected, labeled, packaged, and shipped. Each laboratory unit monitors the unsatisfactory package/specimen to collect and do appropriate follow-up, such as re-training submitters regarding specimen shipment.

The condition of the specimen is recorded in the laboratory information management system (LIMS) system and a report is generated to the submitting physician or the follow-up team (NBS) that the specimen was unsuitable for testing. Starting January 1, 2024, Microbiology clinical specimens submitted with a paper requisition will be rejected unless preauthorized to do so (see section 10.7).

To improve this process, some laboratory units track the frequencies of the unsatisfactory package/ specimen to collect and analyze the data when unacceptable samples or specimens are received by the PHL. An example of such a log is **69790.501** *Specimen Rejection Log* in the Reference laboratory in the Microbiology office. The root cause analysis and/or statistical analysis to identify the potential trends will assist PHL in determining where to focus our quality process improvement efforts. In addition, such data analysis will provide each office with guidelines on which quality indicators to track yearly. The QA Officer is responsible for summarizing findings in the annual *Quality Management Plan Effectiveness Report*.

10.9 Verbal Test Orders

It is the PHL policy that personnel receiving verbal or phone orders for testing must read back the entire order to verify the accuracy of the transcription. All verbal order is required to submit a written test order, prior to release test results. Exceptions may be case-by-case approved by the CLIA director. If modifications of existing orders are verbally made by submitters, such as adding a test panel, such changes are noted in the comment section of a final report.

10.10 Specimen and Sample containers and analytical interference

Specimen and sample containers are to be assessed to ensure that they do not interfere with the assays performed. Exhaustive testing is not mandated for each laboratory, rather, this may be done through some combination of:

- direct testing by the laboratory
- review of the clinical literature
- evaluation of information from manufacturers.

Documentation of a summary of their analytical interference evaluation is to be kept either in the method validation report and/or in the testing procedure(s) for which the specimen containers are utilized.

10.11 Analyst Identification

The analyst, testing system and date of test at all stages of a test must be able to be identified. At PHL, this can be done by either initialing/dating paper worksheets, reports, etc. or tracked by the computer, depending on the assay.

10.12 Reporting Results

The laboratory communicates diseases of particular significance promptly and retains records of the communication. The method of reporting results may vary with each respective Office. Detailed procedures for reporting results are found in Office or Section specific SOPs and/or in individual test SOPs. All patient results are handled and reported in compliance with Health Insurance and Portability Accountability Act (HIPAA) requirements and the respective regulatory or certifying body requirements (eg, CLIA, ISO).

Result reports will include the method(s) used, analyte(s) tested for, test result(s), and when appropriate reference intervals, and the clinical interpretation. All report template content and format are reviewed by the Deputy Director prior to test implementation and any edits post-implementation. Preliminary results must be documented with the same level of detail as final reports.

Reports interpreting NGS sequence variants must comply with a standardized written algorithm. This algorithm is used to determine the clinical significance of any detected organisms and genes linked to resistance and pathogenicity. When sequencing identifies normal microbiota in a patient sample it is clearly differentiated from pathogenic organisms in the report. Each NGS SOP must address this policy in the reporting structure. Before releasing reports, any microbial phenotypes identified through traditional methods are to be checked against the phenotype identified through NGS.

Disclaimers for Reports and Amended Reports are kept in MediaLab for document control. They will follow the full approval process and be linked to the SOP to which they pertain to. Once approved, the supervisor will forward the updated disclaimer to the LIS manager to update the LIS report.

10.13 Amended Reports

All revised preliminary or final reports for previously reported incorrect or incomplete results are to be identified as amended, regardless of the format of the reports (paper, verbal, e-FAX etc.). In the case of multiple sequential corrections of a single test result, all corrections are to be referenced in sequential order on subsequent reports. Reports that display amended results must clearly indicate the new result is a change from a previously reported result. Submitters are immediately notified of the amendment of the previous results.

The amended and original data must be clearly identified as such, and the original data is to be readily accessible to the user for comparison, such as the original data must be present in the amended report (for paper reports) or linked electronically or logically to the amended information (in electronic reports). The precise format of corrected reports is at the discretion of the individual laboratory.

Events with amended reports are documented using MediaLab/IQE platform. QA Officer reviews and determines the necessary follow-ups. Detailed procedure for non-conformance reporting is found in **69790.945** *Non-Conformance Report* and **69790.946** *Managing Non-Conforming Work with Continuous Quality Improvement.*

10.14 Retrieval of Information

Copies or files of reported results are to be retained by the laboratory in a manner that permits prompt retrieval of the information. The length of time that reported data is retained in the laboratory may vary. Detailed procedure for the record retention is found in **69790.1308** *Record and Specimen Retention Policy.*

10.15 Availability of Test Methods to Clients

If a client request is made to obtain a laboratory's current test methods and/or performance specifications, the section Supervisor and/or Office Director shall address their needs.

10.16 Change in Analytical Methodology

If the laboratory changes its analytical methodology so that test results or their interpretations may be significantly different, the change is to be explained to clients. This can be accomplished in any of several different ways, depending on local circumstances. Some methods may include a change in the *Directory of Services*, directed mailings, laboratory newsletters, or part of the test report itself.

10.17 Reagent Labeling, Storage & Expiration

Reagents and solutions are to be labeled, stored according to manufacturer specifications, and used by the expiration date. Detailed procedure is found in **69790.1038** *Reagent Handling*.

10.18 Unknown Submitter

When specimens are received without the submitter's identification (name, address), specimen will be rejected for testing. The exception to this rule is newborn screening specimens are tested even if the submitter's identification is blank because many of the newborn screening conditions can be life-threatening during the first weeks of life. Newborn screening staff can usually identify a specimen's submitter by a barcode search.

10.19 Loss of Critical Documents

Upon determining laboratory documents critical to reproducing data are lost, immediately notify the incident to the section Supervisor, Office Director, and QA Officer. Type of laboratory document, root cause of the incident and any other information of importance to the investigation is documented. Non-conformance is reported via MediaLab IQE platform for QA review. The Office Director is responsible for notifying appropriate stakeholders including submitters.

10.20 Testing Control Specimens

Control specimens are tested in the same manner and by the same personnel who routinely perform testing. Each operator does not necessarily need to perform QC daily, so long as each instrument and/or test system has QC performed at required frequencies, and all operators participate in performing QC on a regular basis.

10.21 Specimen Handing for Molecular Procedures

Due to the high sensitivity and potential for contamination in molecular testing involving nucleic acid special laboratory hygiene and sample handling procedures shall be followed as detailed in **69790.1229** *Laboratory Contamination Prevention and Monitoring: Nucleic Acid Testing*.

11 ANALYTICAL PROCEDURES

Each office is required by their certifying agencies to have a procedure for all analytical work they perform. The procedures may cover a grouping of testing or be very specific in its emphasis. All PHL procedures must follow the PHL Procedure format as described in ***69790.107*** *Results Generating PHL Procedure Template* and **69790.70** *Non-results Generating PHL Procedure Template* .

11.1 Intermittent Testing

When a test is put back into production, the following requirements must be met:

- PT or alternative assessment performed within 30 days prior to restarting patient testing.
- Method performance specifications verified, as applicable, within 30 days prior to restarting patient testing.
- Competency assessed for analysts within 12 months prior to restarting patient testing.

This applies to tests that are taken out of production for a certain period of time (for example, seasonal testing for influenza). A test is considered to be taken out of production when (1) patient testing is not offered AND (2) PT or alternative assessment, as applicable, is suspended.

For tests for which PT is required by CAP, if a PT challenge is not offered during the 30-day period prior to restarting patient testing, the laboratory may perform an alternative assessment of the test. In such a case, the laboratory must participate in the next scheduled PT event.

12 QUALITY CONTROL

Quality control more narrowly describes the techniques and activities by which a department validates and documents proof of the quality of a specific test or service, most notably testing results. The quality control system is monitored using both statistical and non-statistical techniques. Quality control should be viewed as a system or Quality Control Program that includes many different procedures, such as checking reagent lot numbers, monitoring temperatures of refrigerators, freezers, and heating baths and measuring quality control samples of known values. Control of analytical testing may be broadly divided into internal and external types.

Results are validated and monitored in real-time using internal control systems that include information from stable control samples as well as from other samples/specimens. The main objective of internal quality control is to ensure the day-to-day consistency of measurements.

External quality control refers to a process of retrospectively comparing results from different laboratories through the use of an external source. The major purpose of external quality control is to assess accuracy by comparing results with other laboratories with similar and different methodologies. External Controls are useful to determine lot to lot variability of testing kits.

Documents related to the Total Quality Control Program (TQCP) are:

69790.1365 *TQCP Implementation Guidance*
69790.1356 *TQCP IQCP Flow Plan*
69790.1357 *TQCP Summary Sheet*
69790.1358 *TQCP Summary Example & Notes*
69790.1359 *Westgard Sigma Rules*
69790.1360 *Quality Control MTS Checklist*
69790.1373 *Sigma Quality Determination of Test Systems*

Individual Quality Control Programs allow the laboratory to customize the quality control practices of a test system. An IQCP consists of three steps critical to ensuring QC practices are compliant and reduce potential errors or problems. These three steps include a risk assessment, definition of the quality control plan (QCP) and ongoing monitoring and assessment of the QCP. For more information on developing and IQCP for a test system, review the documents listed below.

Documents related to the Individual Quality Control Program (IQCP) are:
69790.1060 *IQCP Implementation Guidance*
69790.1041 *IQCP Assay Summary Form*

12.1 Proficiency Testing

The objectives of proficiency testing are:

- Provide a measure of the quality of performance for individual laboratories
- Promote improvement of performance
- Allow potential customers and accreditation agencies to evaluate/compare laboratory performance
- Provide a training tool for employees that are being trained and evaluated in the procedure

PT or an alternative performance assessment (for analytes with no commercially available PT provider) will be performed on all regulated analytes performed at the PHL by those who routinely perform the testing. The alternative PT's will be handled in the same manner as with an external vendor, twice per

year (approximately every 6 months). Alternate PTs should always contain at least two to five test specimens per event.

Detailed procedure for PT process is in **69790.1330** *Proficiency Testing*.

Centers for Disease Control
CDC distributes samples to be tested for Blood Lead, Fluoride Analyses, Newborn Screening, Bioterrorism and Chemical Terrorism (Center of Toxicology, Quebec, Canada). The CDC is not a proficiency-testing agency *per se*. Although there is no accreditation or certification involved with the testing of analytes from CDC, the PHL participates in the survey. Summaries of the testing performed are sent to the CDC for review. The QA Officer will be informed when these PT samples have arrived as well as when the results are sent to the PHL.

College of American Pathologists
To perform successfully on a College of American Pathologists' testing survey, the laboratory must achieve at least an 80% success rate. Usually, five different analyses are required per testing event. Currently, most surveys are distributed 3 times per year.

Environmental Resource Associates (ERA)
ERA provides material for the quality control and PT needs of environmental chemical, radiochemical and pharmaceutical laboratories. ERA provides the products and support to help with accreditation or the verification of the quality of critical pharmaceutical analyses. ERA is the oldest and largest company in the industry with the most accreditations, staff, instrumentation, and facilities.
http://www.eraqc.com/ (accessed June 2022).

Food and Drug Administration
PT is performed annually for both the Food and Shellfish microbiology laboratories. The FDA Laboratory Quality Assurance Branch, 6502 south Archer Rd, Summit-Argo, IL 60501-1957, provides Food Split Sample Proficiency Test and the Shellfish Split Sample Proficiency Tests.

Inter-laboratory Comparison (Alternative PT)
Biannual alternative PTs are set up when there is no commercial proficiency test available. Usually, participants take turns in preparing and sending out the proficiency specimen to all participating laboratories. Acceptance criteria must be established based upon mutual agreement among participants and a summary of comparison results must be generated and sent to all participants.

Laboratory Proficiency (REML)
REML provides the PT for the water microbiology laboratory. Each method in the water microbiology laboratory will receive one set of samples with ten samples per set. The water microbiology laboratory can receive these samples all at one time or at staggered intervals. Acceptable test results are scored against the assigned values.

Laboratory Response Network (LRN)
LRN was established by the Department of Health and Human Services, CDC in accordance with Presidential Decision Directive 39, which outlined national anti-terrorism policies and assigned specific missions to federal departments and agencies. Through a collaborative effort involving LRN founding partners, the Federal Bureau of Investigation, and the Association of Public Health Laboratories, the LRN became operational in August 1999. Its objective was to ensure an effective laboratory response to bioterrorism by helping to improve the nation's public health laboratory infrastructure, which had limited ability to respond to bioterrorism. Today, the LRN is charged with the task of maintaining an integrated network of state and local public health, federal, military, and international laboratories that can respond to bioterrorism, chemical terrorism, and other public health emergencies.

https://emergency.cdc.gov/lrn/index.asp (accessed June 2022)

www.inspq.qc.ca/en (accessed June 2022)

- **LRN Select Agents**: the LRN sends proficiency events three to four times per year with five samples per event.

LRN Chemical Terrorism: PT specimen for cyanide in blood, organophosphate nerve agent metabolites (OPNA) in urine, and volatile organic compounds (VOCs) in blood, OPNA in serum, Abrine/Ricinine in Urine, Tetramine in Urine, Mercury in Urine, Toxic Element in Urine (Urine Metals), and Blood Metals are received three times a year on a pre-announced schedule from LRN contractors. Once a year the LRN-C will also send an unannounced PT from one of the methods to selected laboratories. Each proficiency set is comprised of ten samples per set. The Wadsworth Center in New York (CDC contractor) provides matrix-matched Priority Metals Quality Assessment Screen (PMQAS) PT specimen for multiple trace elements in urine and blood matrices, three times per year. Each proficiency set varies, at times all samples have analytes (typically metals-urine or blood matrix) spiked into the samples, but it is not uncommon to receive one or more (sometimes two) non spiked samples. It is typical to receive one or more non spiked samples for the rest of the LRN-C PTs, but it is usually two (not always) for those methods. Blood metal's PT samples are provided by WSLH (WI State Lab of Hygiene). Mixed Analyte Proficiency Evaluation Program (MAPEP, US Department of Energy).

The PHL Environmental Radiation Laboratory participates in MAPEP, conducted by the US Department of Energy Radiological and Environmental Measures Laboratory (RESL). The analytical performance is evaluated on the relative bias of the laboratory results vs. reference value for radiological and inorganic analytes.

Model Performance Evaluation Program (MPEP)
The CDC provides the MPEP program for PT testing for retroviral, AID related and *Mycobacterium tuberculosis* testing. Well characterized samples resembling patient specimens encountered in clinical testing are sent out as two surveys per year.

- **Retroviral and AIDS Related Testing**: HIV-1 antibody; HTLV-I/II antibody; HIV-1 RNA; HIV-1 p24 antigen; and T-lymphocyte immunotyping evaluations
- **Mycobacterium Testing**: five culture panels consisting of strains of *Mycobacterium tuberculosis* and non-tuberculosis mycobacteria.

www.cdc.gov/tb/topic/laboratory/mpep/default.htm (accessed June 2022)

National Institutes of Standards (NIST)
Founded in 1901, NIST is a non-regulatory federal agency within the U.S. Commerce Department's Technology Administration. NIST's mission is to promote U.S. innovation and industrial competitiveness by advancing measurement science, standards, and technology in ways that enhance economic security and improve our quality of life. The PHL Radiation Laboratory participates in the NIST Radiation Inter-comparison Program. This program prepares and distributes PT samples of different matrices, such as air, water, soil, urine, and for radiochemical analytes. This program was founded for developing laboratory capability to respond to radiation emergencies.

www.nist.gov/ (accessed June 2022)

WISCONSIN STATE LABORATORY OF HYGIENE (WSLH)
WSLH provides clinical, environmental, and industrial analytical services, specialized public health procedures, reference testing, training, technical assistance and consultation for private and public health agencies. To perform successfully on a WSLH proficiency survey, the laboratory must achieve at least an 80% success rate. Each event consists of six analytes, there are three events per year with the exception of blood metals PT which only receives three analytes per event.

www.slh.wisc.edu/ (accessed June 2022)

TEXAS DEPARTMENT OF STATE HEATLH SERVICES – MUTATIONAL ANALYSIS

NBS conducts PT testing in conjunction with the Texas Newborn Screening Program. Two times a year (May and November) the NBS sends the Texas State Laboratory previously tested blood spots to verify our results as followings: one specimen with the following GALT genotypes - Q188R, K285N, N314D, S135L or not detected; three blood spots each with Hb S, Hb C, or Hb E mutations. The paper results are reviewed and signed off by the CLIA Director. Documentation is in MediaLab IQE module (previously found in P:\EHSPHL\PHL\NEWBORN\COMMON\DNA\QC\PTs\Exchange Program).

12.2 Monitoring of Laboratory and Equipment Environmental Conditions

If the environmental conditions in a laboratory are not stable, the substances being used may physically change or equipment may malfunction resulting in unreliable test results. Adequate environmental monitoring is critical to ensuring quality results are produced by the laboratories.

Monitoring of environmental conditions required by the test method, test kit, or equipment manufacturer must occur, be documented, and a monthly review completed by the supervisor or their designee. Environmental conditions include but are not limited to temperature, humidity, and CO_2. Monitoring must be done with calibrated equipment suitable for the range required by the test method, reagents, specimens, or equipment used in the laboratory.

The PHL uses the SensoScientific monitoring system for temperature, humidity, and CO_2 monitoring. Because temperature is one of the most universally impactful conditions in the laboratory, local backup min/max thermometers are to be installed in equipment identified as critical by the laboratory sections. To learn more about the SensoScientific environmental monitoring system refer to the following documents.

- **69790.2235** *SensoScientific Cloud Monitoring System User Manual*
- **69790.2513** *PHL SensoScientific Equipment Monitoring Job Aid*
- **69790.3874** *Sensoscientific Probe Removal and Installation Job Aid*
- **69790.81** *Temperature Monitoring System*

Guidelines For Monitoring

Follow the guidelines below for laboratory spaces or equipment with specific environmental requirements for temperature, humidity, or CO_2.

- Establish a specific operating range appropriate to the test method, reagents, or specimens and the length of time those conditions may be out of range before a data alarm is triggered.
- Use SensoScientific for continuous monitoring and notifications when measurements go out of range.
- If the condition being monitored is outside the acceptable range, investigate the cause and take appropriate action to ensure test results remain viable. Use the SensoScientific Cloud application to document the root cause and corrective actions taken.
- Follow the manufacturer's instructions for operating thermometers, probes, and monitoring equipment.
- All non-certified thermometers used for clinical specimen storage must be checked against a NIST thermometer before use. All NIST thermometers used in the PHL must have a certificate of analysis, which requires them to be renewed yearly or biennially.
- Mercury thermometers should not be used unless there is a specific reason they need to be used. Mercury spill kits must be present wherever mercury thermometers are used.
- SensoScientific CO_2 probe measurements must be verified at a minimum annually with a calibrated instrument or method.

12.3 Calibration Check of Thermometers

Calibration checks of thermometers are done annually by calibrating the thermometer to a NIST traceable thermometer. This is to be done by sending the thermometer for calibration to a vendor who can provide a calibration certificate. If requested, manufacturers can provide a certificate of calibration when a thermometer is purchased. The laboratory is responsible for keeping a copy of the calibration report. Note that the *Statement of Accuracy* may not be sufficient if it does not list the calibration dates or expiration date of the calibration.

12.4 Pipettes and Liquid Handlers

Automatic pipettes are used in the laboratories to accurately transfer small liquid volumes. Glass pipettes are not highly accurate for volumes less than one milliliter, but automatic pipettes are both accurate and precise. It is up to each laboratory to determine the type of pipettes that meet their needs. The laboratory needs to take into consideration the level of accuracy and precision needed at the bench for performing their assays.

Pipettes are to be purchased with certified performance specifications (received with a calibration certificate) or be calibrated and certified before use. Pipette manufacturers do not always test every pipette that is manufactured. Instead, data from a sampling of each batch may be used to create a certificate for the entire lot. Request individual pipette certificates when placing the initial order. QA also strongly recommends users of pipettes complete an initial training and assessment followed by an annual assessment thereafter.

Pipettes are to be calibrated at least annually through a qualified external vendor. External calibrations are performed by sending pipettes to a third-party vendor or by having the vendor come into the facility to professionally perform the calibrations. When performing onsite calibration, the level of accuracy is not as high as when performed in the vendor's facilities. We primarily have used Rainin and Eppendorf for performing the calibrations.

Periodic checks are recommended between calibrations for pipettes used in critical testing. These checks may be performed by the laboratory. For volumes less than 100 µL, the Artel system is recommended.

Liquid handlers are to be calibrated at least annually. Details outlining the calibration can be found in **69790.1534** *Laboratory Equipment Preventative Maintenance Guidelines.*

Some documents available for your review are:
69790.1828 *10-tips-to-improve-your-pipetting-technique*
69790.2078 *Rainin Pipet-Lite Operating Instructions*
69790.120 *Eppendorf Pipette Standard Operating Procedure*
69790.119 *Pipetman Users Guide*
69790.1534 *Laboratory Equipment Preventative Maintenance Guidelines*
69790.1628 *Compass - End User Training & Competency Assessment*
69790.116 *Artel PCS User Guide*
69790.1826 *Liquid Handling Assessment Artel PCS System*
9790.1806 *Multichannel Verification system Procedure Guide*
69790.1831 *Multichannel Verification System, MVS*

12.5 Weights & Balances

Analytical balances must meet the level of accuracy needed for the laboratory work. Before purchasing a balance, the accuracy necessary for the laboratory work should be determined.

An analyst is to check the balance before each use or once per day, whichever is less, with at least two different reference weights. Reference weights are to be kept in a clean container and handled appropriately. Reference weights are to be checked against Class 1, Class S, or Class U weights (stored in Q1, QA Officer's office or purchased by each laboratory) referenced to NIST standards. A monthly check is required if the balance goes to three or four decimal points. If the balance goes to two decimal points or less, then the balance should be checked quarterly. All frequently used balances, i.e., used practically every day, are to be checked monthly regardless of the type.

To determine the range of reference weights used, consider the weights that are used routinely in the laboratory, the maximum weight used in the laboratory, and the maximum capacity of the balance or follow the manufacturer's recommendation.

Daily reference weight checks are to be kept in a bound laboratory notebook or a controlled sheet designed for that purpose. The date, analyst initials, and weight readings are to be legibly recorded. Use the same form to record required corrective action due to failing checks. The records are to be reviewed and initialed monthly by the responsible Supervisor. The QA Officer keeps the certification records for the class 1 weights and is responsible for sending the weights out for certification. The certification of the weights is good for five years.

12.6 Autoclaves and Sterilizers

Autoclaves use a combination of pressure, heat, and steam to sterilize media, glassware, and utensils. They are also used to destroy microorganisms on inoculated media prior to the media's disposal. WA PHL autoclaves are located in the BSL3, media preparation, and in the glassware washing area.

For each day of use, the temperature of the sterilizer is documented on a log sheet located near the autoclave. Temperatures and time cycles are monitored on charts which are dated and stored within each area. Any deviation of temperature from the validated conditions for the content type requires notification of the supervisor and/or the building maintenance supervisor. The autoclaves are on preventative maintenance schedules and all maintenance records are maintained by building maintenance.

The dry oven sterilizer is located in the glassware preparation room. The temperature is monitored by a thermometer placed in a sand container inside the oven. The thermometer is checked semi-annually for calibration using a certified thermometer. Each run is documented on a log sheet. The temperature is maintained at validated conditions for the specific content type. The run is also monitored for sterility by the use of a dated sterility indicator placed on the container to be sterilized.

All sterilizers are checked once a month, with Biological Sterility Indicators, to ensure they are functioning properly. The Sterility Indicators are to be placed in situations that are similar to regular usage, i.e., placed in mock waste containers, coffins, etc. Chemical indicators or non-biological indicators are used in conjunction with biological indicators. The advantage is that the chemical indicators or non-biological indicators can give immediate feedback that sterilization has been achieved.

Each autoclave should display in clear view the CERTIFICATE OF INSPECTION issued by the Department of Labor and Industries, Division of Building and Construction Safety Inspection Services. This is required

by RCW 70.79.320. Inspection by Washington state Labor and Industries (L&I) is required every two years.

Room	Department	Sterilizer	Indicator	Schedule
R20	Operations	Dry Heat – HAFO 1600	Paper	Monthly
R21	Operations - Media	Steam – Primus, A1	SterilAmp II	Monthly
R41	Operations - Glassware	Steam – Primus, P1	SterilAmp II	Monthly
R41	Operations - Glassware	Steam – Primus, P2	SterilAmp II	Monthly
R21	Operations - Media	Steam – Primus, A2	SterilAmp II	Monthly
C25	BSL3	Steam -	SterilAmp II	Monthly

12.7 Timers

Timers are to be checked when their function is critical to test performance. A stopwatch is located in the Facilities Office and can be used for this purpose. Documentation of the timer check should be noted on the instrument and/or in the Preventative Maintenance log for the instrument. Employees need to follow the manufacturer's product insert for checking timers. If an employee suspects that any timer is incorrect, the timer should be checked and/or replaced. The laboratory standard of practice for instruments with timing functions such as centrifuges, manual coagulation machines, anaerobic chambers, cell washers, radioactive particle counters, blood cell counters, or nucleic acid equipment, is to check yearly as a part of Preventative Maintenance.

12.8 Reagent Water Quality

All laboratory testing will utilize clinical laboratory reagent water (CLRW, previously type II) water unless otherwise specified by the individual procedure. Special reagent water (SRW, previously Type I water) is to be used for testing that requires a higher quality of reagent water. For example, the Molecular Laboratory also purchases reagent water that is RNase free.

The PHL monitors its CLRW by performing chemistry and microbiological checks. Checks are made monthly for free chlorine and Heterotrophic Plate Counts (HPC). Checks for silver, cadmium, chromium, copper, nickel, lead, and zinc are performed annually. Conductivity is monitored continually by an internal probe in the CLRW system and recorded monthly. Further details are found in **69790.1331** *Laboratory Reagent Water Quality Testing*

12.9 Glassware Washing

Glassware washing follows predetermined standards to ensure that the glassware is clean and free of contaminants/detergent before it can be used or recirculated. Appropriate documented procedures are in place for handling and cleaning glassware.

All glassware is washed with a detergent, with the final rinse in deionized water. Organic glassware is also rinsed with a solvent (EPA guidelines followed). The glassware is checked for effective detergent removal by following procedures **69790.28** *Glassware Procedure* and ***69790.211** pH Testing for Clean Glassware*. Sterilization of glassware is performed by the hot oven sterilizer or by autoclaving.

Records for detection of detergent residues resulting from improper rinsing are located in the Dish Room QA/QC Records binder located in R41. The detergent removal check is performed for each dishwasher load. Records include the test date, types of glassware tested, and test results.

12.10 Environmental Maintenance and Equipment Preventative Maintenance

Bench tops, cabinets, drawers, and sinks are to be clean and well-maintained. Preventative maintenance (PM) ensures that equipment continues to function at the level that is required for quality analysis. The majority of critical testing instruments at the PHL are under annual full-service contracts with the

instrument manufacturers that include both preventative maintenance either annually or biannually and repair services, on an as-needed basis for parts and labor. A handful of legacy systems without available service contracts due to the age of the instruments are maintained in-house with a defined maintenance schedule. All laboratory units should document the PM records including documentation from outside vendors (Spectrum, Precision Mechanical). Preventative maintenance records and records from vendors performing preventative maintenance are signed by the section supervisor.

Cleaning of the instrument and/or troubleshooting is performed either by the laboratory analysts, the laboratory maintenance department, or contracted maintenance services depending on the degree of difficulty. Records of cleaning are kept in the preventative maintenance logbook.

Biological Safety Cabinets and fume hoods are inspected annually. All personnel working in the laboratory using Biological Safety Cabinets and Fume hoods will be instructed in the use of the above by their immediate supervisor.

Instrument/equipment performance verification includes processes to verify that the instruments and equipment perform according to expectations for the intended use and within defined tolerance limits. The performance of all instruments and equipment is verified and documented prior to initial use, after major maintenance or service, and after relocation to ensure that they run according to expectations.

12.11 Monitoring of Controls

The monitoring of control is an important aspect of the analysis. If control results are not within acceptable range, the patient or sample results of the same run should not be reported. Each laboratory unit determines the frequency, concentration requirements (for quantitative testing), and the acceptable range for quality controls in a run. The results of controls must be reviewed by the laboratory unit Lead or Supervisor for acceptability before releasing result reports. Details are found in the standard operating procedure for the method.

12.12 Monitoring Analytic Performance

Performance monitoring, or quality control, is used both to detect shifts in measurement processes over time and to determine the acceptability of analysis for reporting. The parameters monitored and their associated control limits are specified in the standard operating procedure for the method, or instructions specific to the instruments and tools that are used in those methods. Typically, quality control material is used according to the manufacturer's instructions.

Please refer to the individual standard operating procedures and related guidance documents covering the method in question for a listing of quality control materials and acceptance criteria. As applicable, controls, metrics, and quality control parameters must be established during validation or verification of the method and analyte and defined in the standard operating procedure for the method or analyte.

When appropriate, statistics on test results are maintained and periodically reviewed to identify changes in test performance and detect systemic errors. See individual method or analyte standard operating procedures for methods of calculating statistics, recording statistical data, evaluation frequency, and corrective action if indicated.

12.13 Detection and Correction of Significant Clerical and Analytic Errors

It is important to detect laboratory reporting errors as quickly as possible in the testing process, monitor the causes, and reduce their impact by (1) timely intervention when errors are recognized and (2) determining the underlying reasons for reporting mistakes so that adjustments can be made to prevent recurrences. It is imperative that all laboratory staff actively watch for errors and investigate their causes when they are found. Knowledge obtained from these investigations may identify methods to improve reporting procedures so that errors do not recur.

Possible mechanisms to detect clerical errors are:

- Select random reports and review the work cards, worksheets, and final charted results for accuracy and completeness.
- Lead workers should compare results on worksheets and requisition forms when reviewing final reports.
- Review process for clerical errors regularly.
- Follow up on any quality control issues or problems.
- Make sure that satisfactory competency assessments have been completed for all personnel performing the work.
- Each unit is responsible for implementing specific steps to detect and correct possible sources of errors.

Details concerning the error correction procedure can be found in **69790.1332** *Error Correction.*

12.14 Control Charting

Quality Control Charts are normally presented graphically in the form of Levy-Jennings plots. Control limits may also be established by statistical analysis of a set of data (e.g., the mean and ± two and three standard deviations, from a set of a minimum of twenty data points). Control limits may also be based on regulatory requirements, criteria specified by a vendor, or the data quality objectives for which the method was developed. Details are further found in the individual standard operation procedure for each test.

12.15 Reagent Quality Control Testing

Reagent performance and adequacy per different lot must be verified before placing the material in service or concurrent with use. Various methods, such as direct analysis, use of reference materials, or parallel testing of old *versus* new reagents, are acceptable. The results of verification checks must be recorded.

12.16 Calibration, Analytical Measurement Range, etc.

These criteria are primarily for assays where a quantitative result is reported or where the manufacturer package insert stipulates that it be performed. Please note that this does not replace the standard method validation/verification requirements that the performance of all instruments and equipment are verified prior to initial use, after major maintenance or service, and after relocation to ensure that they run according to expectations.

Calibration

Calibration is the set of operations that establish, under specified conditions, the relationship between reagent system/instrument response and the corresponding concentration/activity values of an analyte. Calibration procedures are typically specified by a method manufacturer but may also be established by the laboratory.

Calibration Verification is the process of confirming the current calibration is still valid for a process. The frequency of verification is determined by the governing regulatory guidance and/or by the manufacturer's recommendation, whichever is more frequent. In addition, if the instrumentation undergoes a major repair or is modified or a change of critical reagents, then it is necessary to perform the calibration verification. If the calibration verification is unsuccessful, then remedial action is necessary. It is the individual laboratory unit's responsibility to establish the criteria for a successful calibration verification process.

Analytical Measurement Range (AMR)

AMR must be determined at a minimum of every six months or after a major change in the instrumentation system or a change in analytically critical reagents. The materials used for the establishment of the AMR must closely approximate the matrix characteristics of patient specimens used in the analysis. A minimum of three (low, midpoint, and high) values over a linear range is required. It is the individual laboratory unit's responsibility to establish the criteria for a successful AMR verification process.

AMR can be further expanded, either at the high or low end, when the specimen has been diluted, concentrated, or another pretreatment that can extend the AMR. The process for when to dilute or concentrate must be prescribed by a written protocol which includes the diluter or process for concentrating the specimen.

Instrument Comparability

The performance of each instrument is checked against each other at least twice a year for comparability of results when the laboratory uses more than one instrument/method to test for a given analyte. Quality control data may be used for this comparison for tests performed on the same instrument platform, with both control materials and reagents of the same manufacturer and lot number. Otherwise, the use of human samples, rather than stabilized commercial controls, is preferred to avoid potential matrix effects. The use of pooled patient samples is acceptable since there is no change in the matrix. Details procedure is found in **69790.1112** *Performance Specification & Instrument Comparison*.

12.17 Telepathology

The PHL does not provide telepathology service.

12.18 Patient and Environmental Safety Goals

The PHL's Laboratory Patient and Environmental Safety Goals are to improve:

- Specimen identification at collection, analysis, and result delivery.
- Verification and communication of life-threatening or life-altering testing as well as environmental threats to public safety.
- Identification, communication, and correction of errors.
- The coordination of the laboratory's patient and environmental safety role within the Public Health system (local health jurisdictions, nursing, administration, point of care (POC) personnel, and providers).
- Monitoring of quality indicators at the PHL.

The annual progress will be evaluated and documented in the *Effectiveness of the Quality Management Plan* report.

12.19 Reporting Quality Concerns

Laboratory personnel are always encouraged to report any quality concerns directly to their supervisor or Office Director. Personnel may also communicate with the relevant accreditation body directly if they have a concern that is not being addressed by laboratory management. Accreditation bodies hold such communications in strict confidence. Signs explaining this right are posted in the main corridor above the drinking fountain near C-wing and the main laboratory entrance to Newborn screening, outside N30. The PHL prohibits harassment or punitive action against an employee in response to a complaint or concern made to an accreditation body or other regulatory organization regarding laboratory quality or safety. The dedicated, confidential CAP telephone line for quality or safety concerns is 866-236-7212 (US, toll-free).

13 QUALITY INDICATORS

Every year, the PHL establishes quality indicators that each Office Director determines as meaningful to their office. The progress of selected quality indicators is presented during QA team meetings on a quarterly basis. For the quality indicators exhibiting persistent or substantial deterioration of performance, the respective Officer Director will present a plan for improvement. QA Officer is responsible for monitoring the progress of the proposed plan. By the end of the year, the QA Officer generates an annual report with compiled quality indicators, summaries outlining the observations, follow-up actions being taken to address any concerns, and the improvement plan, together with each Office Director. CLIA Director is responsible for the appropriate selection of quality indicators based upon SMART (Specific, Measurable, Attainable, Relevant, and Time-based) practice and responsible for the timely evaluation of the effectiveness and the improvement of quality of works through selected quality indicators. The CLIA director is the driving force of follow through on the entire cycle of quality indicator-based quality assurance practice across offices.

13.1 Turnaround Time

Turnaround time (TAT) is an important aspect of quality assurance. They provide the customer with the assurance that laboratory results will be reported in a timely manner. Since there is such a diverse amount of testing performed by the Public Health Laboratories, turnaround times are established by each Office. The listing of turnaround times for tests performed at the Washington Public Health Laboratories are listed in the Directory of Services as well on the Test Menu available on DOH's website:

http://www.doh.wa.gov/Portals/1/Documents/Pubs/301-016-PHLDirectoryServices.pdf (accessed June 2022).

Each office is responsible for monitoring and investigating TATs that indicate systemic or acute problems in laboratory operations. When the established turnaround times are expected to be exceeded by more than two working days, the laboratory will notify the submitter verbally, in writing, or both. This is done in conjunction with the supervisor, Office Director, clinical consultant, and DOH programs. Documentation of submitter notification is required, including the date, submitter institution, name of the person notified, and initial of the PHL staff member who performed the notification.

13.2 Specimen Acceptability

The acceptability of specimens received is determined by monitoring the "less than optimal" specimens received and the specimen rejection rate. This is usually done at the time of accessioning the specimen for the particular laboratory. Criteria for acceptability are located in the individual procedures and the PHL Lab Test Menu.

13.3 Proficiency Testing Performances

The evaluation of the performance on proficiency testing is crucial to determining the performance of the assays. Each Office is responsible for monitoring their performance on proficiency testing. Review for bias or trends will also be reviewed by the Supervisors and Office Directors.

13.4 Employee Quality Communication

Any employees can file quality issues with the QA Officer through IQE/MediaLab (PHL document control system). When received, the issue is reviewed, and follow-up is made until the issue is resolved. It is strictly prohibited to retaliate against the employee who reports quality issues.

14 INFORMATION SYSTEMS

The PHL Information Management Systems supports a wide range of laboratory functions at the PHL, from test ordering to result reporting. The LIMSs are managed by the agency IT division, Health Technology Solution (HTS).

14.1 Security

Physical Security
The computer equipment is located in an environmentally controlled room (the "computer room") with no exterior walls. The door to this room is secured with a key card lock that only authorized personnel can open. Access to the computer room is gained by passing through an outer room, which is occupied by Information Services staff during working hours and is locked during non-working hours.

System Security
LIMS users are assigned a unique login identification and password. Passwords are confidential. Users are required to change their password regularly; the standard is every 180 days. If a user chooses not to change their password, the system automatically denies access. Only authorized Information Services staff can allow access to the user's account. If a user tries to log in three times unsuccessfully, the system will automatically respond with an Intruder Lockout. This lockout status can only be removed by authorized Information Services staff upon the request of the user who has received this status. The File Server records all Intruder Lockout information and Information Services staff regularly monitor this server. Each PHL Office management has established the appropriate access privilege for each user when accessing information.

Data Storage and Security
All laboratory data is backed up. Local backup files are kept in a secure and environmentally controlled area. Remote backup is controlled by the agency's IT group periodically. Off-site information can be retrieved only by authorized staff.

14.2 Computer System Operations

The system is in operation 24 hours a day, seven days a week. The monthly maintenance schedule by the IT group is pre-determined to minimize workflow disruption. Unscheduled downtime (e.g., crashes, power outages) is rare. The majority of users access the computer system during the extended workday of 6:00 AM to 8:00 PM, Monday through Friday (excluding holidays). Exception users with 24/7 access require pre-approval by management.

The applications utilized by PHL staff have been pre-screened. Users are not allowed to install random software onto the system.

IT Support
During normal operation work hours (8:00 AM to 5:00 PM, M-F), the PHL Help Desk is your primary IT support. During non-normal work hours, please follow the steps as outlined in **69790.23** *IT 24 Hour Support*.

14.3 Office of Newborn Screening

Neometrics' Metabolic Screening Database System (MSDS) is currently utilized in NBS. This system consists of applications software for tracking and managing the processing of specimens through a variety of biochemical tests for newborn screening. Neometrics automates the daily workflow of testing staff and provides a mechanism for triggering follow-up activities through the Neometrics case management system (CMS).

MSDS is tightly integrated into the CMS. Presumed positives and any other specimens requiring follow-up are automatically merged from MSDS into CMS. Within CMS a case is created for each patient, and all of the patient's specimen data from MSDS are linked to the case.

The Laboratory portion of MSDS includes capabilities for demographic data capture, batch building, result entry, supervisory review, and mailer production. Instrument Merges enable the test results computed by the instruments to be automatically posted to the appropriate specimen records in the system. In addition, MSDS includes reporting and online look-up for all specimens processed through the system. Through MSDS, staff have access to complete specimen records. Additionally, MSDS provides call logs, notebook entries, and audit trails so staff can keep a record of all their activities.

The databases and related files are backed up daily and stored at an offsite location. Other files are backed up daily on PCs within the laboratory. Hard copies of the specimen forms and the birth rosters are professionally stored by Eastlake Management Inc.

14.4 Office of Public Health Microbiology

StarLIMS

The Microbiology information technology system (StarLIMS) collects demographics and test results for a large number of specimens in selected clinical areas. The system assists Microbiology staff in tracking and analyzing clinical specimens for various tests. Demographic information is entered into the LAN system by laboratory staff and CA staff. Test results are entered and verified by lead laboratory staff. Logbooks, physicians' reports, and summary information are generated and printed by laboratory staff. The entry of data and printing of reports can be done on any PC connected to the PHL intranet. All laboratory data stored on the LIMS is backed up daily and stored at an offsite location weekly.

All Microbiology PCs are Microsoft Windows-compatible and connected to the PHL intranet. Print capability within the labs has been standardized with HP-compliant printers. PCs running the Microbiology database applications are interchangeable.

The PHL Microbiology Office is in the transition of replacing its existing LIMS with a new system. The project duration is scheduled to be from 2020 through 2024.

NGS Analytical Bioinformatics Pipeline Infrastructure

Policy and procedures governing information technology (IT) infrastructure for the NGS analytical bioinformatics pipeline and its management are detailed in **69790.3152** *Bioinformatics - IT Infrastructure Manual*.

14.5 Office of Environmental Laboratory Sciences

Radiation

Applications are currently run on a variety of hardware, software, and operating systems, respective to the purchase date of, and the upgrades available for, the instruments. The logbook is run on the SQL Server in Olympia. Some counters are connected to the DECnet (instrumentation network), and other counters are connected directly to PCs. All staff are connected to the PHL intranet and have email access. Applications were developed in MS ACCESS and PASCAL. Databases are mostly ASCII files. Word processing, spreadsheets, and other PC-based applications are used by laboratory staff.

Environmental Chemistry

Instrument controllers are directly connected to laboratory equipment. Most of the instrument controllers are connected to the PHL Instrument Network, affording common printing and secure backup services. Other, older instrument controllers are stand-alone. Data from instruments and procedures not controlled via computer is collected in laboratory notebooks.

Staff use word processing, spreadsheets, and other PC-based applications on desk computers on the separate, DOH network. Information technology for ELS consists of the standard, approved software bundle from DIRM, with separate approval for specialized application software.

Shellfish
Database information is on a shared Microsoft Access platform maintained by the program in Olympia. Some instrumentation is directly connected to instrumentation but most of the laboratory activities use paper-based systems.

Chemical Incident Response
Applications are currently run on a variety of hardware, software, and operating systems. All staff computers are connected to the DOH network and have email access. Word processing, spreadsheets, and other PC-based applications are used by laboratory staff. Instrument applications are vendor specific. Individual stand-alone instrument controllers are connected to the PHL Instrument Network, where all data is backed up automatically. Reports are printed on dedicated printers not connected to the DOH network.

14.6 QA Office

QA Office
Administrative information technology consists of word processing, spreadsheets, email, project manager, and communication applications. All computers are IBM PC compatible and are connected to the PHL intranet. The print capability has been standardized with HP-compliant printers with shared access through the PHL intranet. Document control is performed using the online-based platform MediaLab, with secure access. Each user has differential access privileges.

15 EXTERNAL COMMUNICATIONS

15.1 Client Complaints

Client complaints are usually resolved by the affected office. If the complaint is not resolved by the office, the complaint and its resolution are transferred to the CLIA Director. All actions in resolving the complaint are documented in the IQE MediaLab module using **69790.793** *PHL Customer Feedback Form*.

15.2 Communication Breakdowns

Communication breakdowns, like complaints, are resolved by the affected office. If the breakdown is not resolved by the office, the problem and resolution is transferred to the QA Officer. All actions in resolving the breakdown are documented in the IQE MediaLab module using **69790.793** *PHL Customer Feedback Form*.

15.3 Critical Results and Timely Communication

Laboratory test results may require the immediate notification of a physician or other personnel when results fall within an established "alert" or "critical" range. Determination of alert or critical ranges is done in consultation with the Supervisor, Office Director, CLIA Director, Clinical Consultant, or DOH program. Each laboratory office is responsible for establishing a process for this immediate notification including how the notification is documented, this process is to be included in the test method SOP or reporting results SOP.

When any laboratory result, including critical values, is reported by telephone (including to DOH programs), the PHL staff member reporting the results must ask the receiver of the information to read back the values given to ensure that accurate communication of the results was made. This is to be documented by recording the date, to whom the result was made, laboratory specimen identifying information, verification of read-back, and initials of the employee giving the laboratory information.

- **Microbiology:** The notification procedure for critical results is documented in the test method SOP.
- **NBS:** NBS has a dedicated task group (follow-up staff) to immediately communicate with submitters regarding all abnormal results with severe clinical implications. Follow-up staff communicate those results to the clinician or providers following their protocol **69790.845** *Follow-up Protocol for Abnormal Laboratory Results*.
- **Chemical Incident Response (CIR):** CIR tests the specimens of victims of chemical exposure, all of which are reported according to CDC protocols. CIR results will go to the CDC Toxicologist/Washington Department of Health Toxicologist and/or Poison Control for review and action. Washington Environmental Biomonitoring Services, WEBS, reports all results to the Office of Non-Infectious Conditions Epidemiology of the Washington Department of Health. Critical results are reported back to the participants with recommendations according to the regulations set forth in the institutional review board (IRB) for the grant.

15.4 Securing Healthcare Information

The federal law known as HIPAA, the Health Insurance Portability and Accountability Act (HIPAA) of 1996, set standards for the protection of individually identifiable health information by covered entities, such as health care providers. All PHL employees must protect the confidentiality of healthcare information, also known as protected health information (PHI), under both federal and state law. Further details are found in **69790.1334** *Patient Healthcare Information Protection.*

15.5 Reporting of Adverse Reactions

When information reasonably suggests that any laboratory instrument, reagent, or other device has or may have caused or contributed to a patient death or serious patient injury, the FDA requires hospitals and outpatient diagnostic facilities, including independent laboratories, to report the event. If the event is death, the report must be made both to the FDA and the device manufacturer. If the event is serious patient injury, the report may be to the manufacturer only, unless the manufacturer is unknown, in which case the report must be submitted to the FDA. Individual medical device reports (MDR) must be submitted on FDA Form 3500A (or an electronic equivalent) as soon as practical but no later than 10 days from the time medical personnel become aware of the event.

Device malfunctions or problems that are reportable may relate to any aspect of a test, including hardware, labeling, reagents, or calibration; or to user error (since the latter may be related to faulty instrument instructions or design). An adverse patient event that may have resulted from inherent limitations in an analytic system (e.g., limitations of sensitivity, specificity, accuracy, precision, etc.) is not reportable.

The PHL follows FDA guidance on 1) the identification and evaluation of adverse patient events, 2) the timely submission of MDR (medical device reporting) reports, and 3) compliance with record-keeping requirements. Further details in FDA guidance are found at: https://www.fda.gov/medical-devices/postmarket-requirements-devices/mandatory-reporting-requirements-manufacturers-importers-and-device-user-facilities (accessed June 2022).

The PHL provides all laboratorians medical device safety reporting training through the Washington State Learning Center, course Medical Device Reporting Training (course code DOH_PHL_410).

The PHL will submit an annual report of device-related deaths and serious injuries to the FDA if any such event was reported during the previous year. This will be handled by the QA Officer. Annual reports will be submitted on form 3419 (or an electronic equivalent) by January 1 of each year. The PHL will keep records of MDR reports for two years in the QA Officer's Office.

Additional information is available on the FDA websites:

- https://www.fda.gov/medical-devices/medical-device-safety/medical-device-reporting-mdr-how-report-medical-device-problems
- https://www.fda.gov/medical-devices/medical-device-safety/medical-device-reporting-mdr-how-report-medical-device-problems#overview

15.6 Notifications from Vendors

Notifications may take the form of product recalls, market withdrawals, or software patches and upgrades. The PHL documents all notifications from vendors of defects or issues with supplies or software under the QA directory on the P: drive at:

\\Dohfltum01\phl\EHSPHL\PHL\QA\Vendor Notifications

Immediate action will be taken if the notifications from vendors have the potential to affect testing results or laboratory services.

16 QUALITY ASSURANCE REVIEW

16.1 Effectiveness of the Quality Management Plan

The PHL QMP is reviewed annually at the QA Meeting for its effectiveness. An annual report is compiled by the QA Officer and reviewed by PHL management including the CLIA Director. This review is to ensure that recurrent problems have been addressed and that new or redesigned activities have been evaluated. Follow-up activities taken as a result of the monitoring and evaluation of activities are documented as well. Additionally, an annual Management Review is performed for all ISO 17025-accredited laboratories. This procedure is described in document 69790.791 Management Review of the Quality System.

The annual QMP review report includes:

- Follow through on previous *Effectiveness Quality Management Plan* reports.
- Summary of reported nonconforming events (NCE)
- Quality indicator summary
- Client survey summary
- A review of the proficiency testing program
- A review of the PHL's Patient Safety Goals is to include:
  1. Improve patient and sample identification at specimen collection, analysis, and result delivery.
  2. Improve verification and communication of life-threatening or life-altering information regarding malignancies.
  3. Improve identification, communication, and correction of errors in a timely manner.
  4. Improve the coordination of the laboratory's patient safety role within healthcare organizations (nursing, administration, POC personnel, providers).
- Feedback from employees
- Lesson learned and future directions

16.2 Directorship Review

The CLIA Director will review the laboratory procedures every two years. The director will ensure that the collection of policies and technical protocols are complete, current, and have been thoroughly reviewed by a subject matter expert. Following the review, the CLIA Director will sign off on procedures and laboratory manuals that they are satisfactory. The CLIA Director may designate the Office Director of that section to review/sign off on the procedures where no major changes have occurred. If there is a change in the CLIA Director position, the laboratory procedures and manuals must undergo a review

within 90 days of the new director's appointment. Currently, controlled document review and sign-off are done electronically using the MediaLab platform.

16.3 Laboratory Reports and Forms Review

The Deputy Director or designee will review the templates and format of laboratory reports and forms at least every two years.

16.4 Root Cause Analysis

Tracing or investigating the underlying symptoms of a problem or event can be referred to as determining the root cause of the event. Determining the root cause of deficiencies is instrumental to developing effective corrective action to prevent recurrence. One model for the root cause analysis often used at the PHL is asking the 5-Why's.

All areas of the laboratory should be considered when investigating the root cause of a deficiency. KATTAR analysis can be useful in identifying the area of need so that the root cause can be addressed.

1. **Knowledge**: was the requirement to accomplish the task or item known?
2. **Assignment**: has the task or item been clearly assigned?
3. **Tools**: have procedures been developed and are they adequate?
4. **Training**: have individuals been trained and do they fully understand what they are to do?
5. **Accountability**: are procedures, training, and assignments in place?
6. **Resources**: have appropriate resources been applied to the problem?

Documentation of the root cause analysis is done by utilizing the IQE module in MediaLab following procedures **69790.945** *How to Record a New Nonconforming Event in MediaLab* and **69790.946** *Managing Non-Conforming Work with Continuous Quality Improvement*.

16.5 Material Transfer Agreements

Occasionally infectious agents need to be transferred to other institutions. Form **69790.86** *Material Transfer Agreement Pathologic* is used and communication is made to stipulate the conditions of use by the recipient. If the purpose of the transfer is other than quality control, appropriate approval such as research, surveillance, or IRB, approval is required. Copies of such requests are kept for two years in the section Supervisor's files.

17 MEMBERSHIP ORGANIZATIONS

The PHL maintains memberships of the following organizations:

- Clinical Laboratory Standards Institute (formerly NCCLS)
- American Society of Testing Materials (ASTM)
- Association of Official Analytical Collaboration AOAC International (formerly Association of Official Analytical Chemists)
- Association of Public Health Laboratories (APHL)

Clinical Laboratory Standards Institute

The PHL is an active member of the Clinical Laboratory Standards Institute. The Institute has published some 200 standards, guidelines, and committee reports. Document topics include safety, chemistry, microbiology, and toxicology. The membership includes a report of their guidelines and standards. Many of the guidelines, though directed at clinical laboratories, are appropriate to the laboratories as a whole. All guidelines, upon receipt, are routed to the laboratories that may have an interest. The guidelines are maintained in office R1.

American Society of Testing Materials

Organized in 1898, ASTM International is one of the largest voluntary standards development organizations in the world. ASTM Intl. is a not-for-profit organization that provides a forum for the development and publication of voluntary consensus standards for materials, products, systems, and services. More than 20,000 members representing producers, users, ultimate consumers, and representatives of government and academia develop documents that serve as a basis for manufacturing, procurement, and regulatory activities.

Association of Official Analytical Collaboration
AOAC INTERNATIONAL is a 120-year-old not-for-profit scientific association committed to worldwide confidence in analytical results. AOAC serves the communities of analytical sciences by providing the tools and processes necessary for community stakeholders to collaborate and, through consensus building, develop fit-for-purpose methods and services for assuring quality measurements. AOAC has a number of communities in various stages of development including food safety and security, agricultural materials, marine and freshwater toxins, dietary supplements, food allergens, and disinfectants.

Association of Public Health Laboratories
The APHL is a membership organization in the United States representing the laboratories that protect the health and safety of the public. In collaboration with members, APHL advances laboratory systems and practices and promotes policies that support healthy communities. APHL serves as a liaison between laboratories and federal and international agencies and ensures that the network of laboratories has current and consistent scientific information to be ready for outbreaks and other public health emergencies. Membership consists of local, territorial, county, and state public health laboratories; environmental, agricultural, and veterinary laboratories; and corporations and individuals with an interest in public health and laboratory science.

18 DEBRIEFING PROCESS FOR REVIEW OF RESPONSE TO PUBLIC HEALTH THREATS OR DISEASE OUTBREAKS

A debriefing process for review of response to public health threats or disease outbreaks has been established. The regular Office Director meetings will be used or if needed, additional meetings will be added to supplement the debriefing process. This includes the consideration of surveillance, staff roles, investigation procedures, and communications. When a public health threat or outbreak exists, the PHL Laboratory Director or the PHL Deputy Laboratory Director will coordinate with the laboratory management, Office of Epidemiology, Risk Management, and other appropriate offices for the response of the public health laboratory.

19 METHOD EVALUATION PARAMETERS

Many methods are developed by PHL personnel both for clinical and environmental analysis. Guidance on method development is offered by various organizations. The PHL has utilized a variety of resources in developing method evaluation parameters some of which include: the EPA, the National Committee for Clinical Laboratory Standards (NCCLS), FDA, AOAC, published literature, etc.

Method evaluation parameters include but are not limited to, precision (intra- and inter-assay), accuracy, sensitivity, specificity, reference range (normal range), reportable range, limit of detection, clinical cut-off, and/or linearity. Further details are found in **69790.959** *Method Validation Verification* and **69790.1112** *Performance Specification & Instrument Comparison.*

Some laboratory units may apply different method evaluation parameters, called data quality objectives (DQO). DQO must be established for bias and precision for each test. Analytical testing is performed on

instrumentation of differing capabilities and with different performance standards. Therefore, the details for each DQO are method-dependent. DQO must be realistic compared to method expectations.

Each laboratory must set accuracy and precision goals either by client-specific requests or by reference to applicable regulations or statutes. The point of contact of each laboratory shall solicit written documentation of the client's DQO for the laboratory work prior to accepting samples or specimens for analysis. Each of the laboratories must meet or exceed the client's DQO. If the client's DQO cannot be met by the laboratory, the point of contact of the laboratory should notify the client and consult on the client's options.

Method validation prior to the introduction of new analytic methods yields an estimation of the method bias and precision. Analysis of matrix-matched Quality Control samples with each batch provides an ongoing check of the method bias and precision.

20 LISTING OF PROCEDURES AT THE PUBLIC HEALTH LABORATORIES

As part of the PHL's CAP accreditation, PHL is required to document a listing of laboratory-developed tests (LDT). This listing is kept in the PHL's document control system, **69790.39** *LDT Test List*. The listing is reviewed at least every two years. For tests that have been retired over two years, the test will be deleted from the listing since it will be available in previous listings in document control.

21 MEASUREMENT OF CLIENT SATISFACTION OF THE LABORATORY SERVICE

The input from the clients of the PHL is essential for the continual improvement of the services that PHL provides. Since 2011, DOH has mandated that all sections of the DOH perform a survey of the clients they serve. This will also serve to meet the needs of the PHL accreditation requirement. Every two years, the PHL will survey clients from the Office of Microbiology, Environmental Laboratory Sciences and Operations. Newborn Screening does a quarterly client screening. The questions are designed by the Office Directors. Survey recipients are selected randomly. Survey documentation can be found at P:\EHSPHL\PHL\QA\Client Survey's

22 REFERENCES

Cited MediaLab Documents

69790.3152	Bioinformatics - IT Infrastructure
69790.958	CAP Accreditation
69790.957	CAP Internal Inspection
69790.465	Central Accessioning Data Entry
69790.466	Central Accessioning Reporting Send Out Results
69790.20	Delegation Form IT Hardware
69790.125	Delegation Form Lead
69790.21	Delegation Form Office Director
69790.22	Delegation Form Supervisor
69790.1057	Document Control
69790.1332	Error Correction
69790.845	Follow-up Protocol for Abnormal Laboratory Results
69790.28	Glassware Procedure
69790.945	How to Record a New Nonconformance Event in MediaLab
69790.2077	Ishihara's Online Test for Color-Deficiency

69790.23	IT 24 Hour Support
69790.1229	Laboratory Contamination Prevention and Monitoring: Nucleic Acid Testing
69790.1331	Laboratory Reagent Water Quality Testing
69790.39	LDT Test List
69790.1862	Liquid Handling Assessment Artel PCS System
69790.946	Managing Non-Conforming Work with Continuous Quality Improvement
69790.86	Material Transfer Agreement Pathologic
69790.959	Method Validation Verification
69790.67	New Test & Instrument Checklist
69790.70	Non-results Generating PHL Procedure Template
69790.1334	Patient Healthcare Information Protection
69790.1112	Performance Specification & Instrument Comparison
69790.965	Personnel Training and Competency
69790.211	pH Testing for Clean Glassware
69790.793	PHL Customer Feedback Form
69790.1330	Proficiency Testing
69790.14	PT Quality Improvement Assessment Sheet
69790.12	Quality Management Plan
69790.1038	Reagent Handling
69790.1308	Record and Specimen Retention Policy
69790.107	Results Generating PHL Procedure Template
69790.468	Specimen Handling in Central Accessioning
69790.46	Specimen Label Deficiency Authorization Form
69790.368	Specimen Processing
69790.501	Specimen Rejection Log
69790.81	Temperature Monitoring System

CLIA Brochures | CMS

23 APPENDIX

Appendix A Abbreviations and Definitions

Definitions – See **69790.1335** *Terms and Definitions*

Abbreviations

Alternative PT	Inter-laboratory comparison
AMR	Analytical measurement range
ANAB	ANSI National Accreditation Board
AOAC	Association of Official Analytical Collaboration International
APHL	Association of Public Health Laboratories
ASTM	American Society of Testing Materials
CAP	College of American Pathologists
CIR	Chemical incident response
CLIA	Clinical Laboratory Improvement Amendments
CLRW	Clinical laboratory reagent water, previously type II
CMS	Neometrics case management system
DOE	Department of Ecology
DOT	Department of Transportation
DQO	Data quality objectives

EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FEMA	Federal Emergency Management Administration
HIPAA	Health Insurance Portability and Accountability Act
HTS	Health Technology Solution, previously IT
IEC	International Electrotechnical Commission
IQCP	Individual Quality Control Program
IQE	Intelligent quotient engine (MediaLab)
IRB	Institutional Review Board
ISO	International Organization for Standardization
L&I	Labor and Industries, Washington State
LDT	Laboratory developed test
LIMS	Laboratory information management system
LRN	Laboratory response network
MAPEP	Mixed Analyte Proficiency Evaluation Program
MDR	Medical device reports
MPEP	Model Performance Evaluation Program
MSDS	Metabolic screening database system
NCCLS	National Committee for Clinical Laboratory Standards
NGS	Next generation sequencing
NIST	National Institutes of Standards
OSHA	Occupational Safety and Health Administration
PHI	Protected Health Information
PM	Preventative maintenance
POC	Point of care
PT	Proficiency testing
QMP	Quality Management Program
REML	Laboratory proficiency
RESL	Radiological and Environmental Measures Laboratory
SA	CDC Select Agent Program
SOP	Standard operating procedure
SRW	Special reagent water, previously type I
TAT	Turnaround time
TQCP	Total Quality Control Program
WSLH	Wisconsin State Laboratory of Hygiene
WAC	Washington Administrative Code

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative
Submitter	Shelley Lankford,
Affiliation	WA State DOH Public Health Laboratories , Office of Enviornmental Laboratory Sciences Biotoxin Laboratory
Address Line 1	1610 NE 150th Street
Address Line 2	
City, State, Zip	Shoreline, WA 98155-9701
Phone	(206) 418-5520
Fax	
Email	Shelley.Lankford@doh.wa.gov
Proposal Subject	**Method Modification Request: Receptor Binding Assay for the Detection of Paralytic Shellfish Poisoning Toxins: porcine tissue option for use as a receptor source.**
Specific NSSP Guide Reference	**2023 NSSP Guide for the Control of Molluscan Shellfish** Section IV Guidance Documents – Chapter IV. Chapter III. Laboratory Page | 266 SHELLFISH LABORATORY EVALUATION CHECKLIST Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP) https://www.issc.org/sites/default/files/uploads/-2023/2023-guide-docs/11-rba-psp-checklist-with-comments.pdf Part I Quality Assurance 1.5 Reference Solution Reagent Storage, Preparation and Security 1.5.8 Commercial porcine brain homogenate is stored according to the manufactures specification and is properly labeled with received date and expiration date. 1.7 ~~Rat Brain~~ Membrane Tissue Preparation 1.7.1 The protein/receptor concentration of the rat or porcine membrane tissue preparation is determined for each new commercial lot or batch or using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube method) or equivalent. 1.7.2 The dilution of the protein/receptor concentration of the rat or porcine brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined by either titration to meet the required 'top' value or by generating a tissue linearity curve. 1.7.3 Dilutions of the protein/receptor concentration of the rat or porcine brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous

Text of Proposal/ Requested Action	This submission presents the Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination for consideration to offer an optional tissue type as a receptor source. We propose that the Interstate Shellfish Sanitation Conference (ISSC) approve a modification to the Receptor Binding Assay (RBA) method [SLV (2023;Proposal13-114] for the detection of Paralytic Shellfish Poisoning (PSP) toxins. The proposed modification would allow the use of porcine brain tissue as an alternative source of receptorsto rat brain tissue for the RBA. This modification would provide an additional tissue source for preparation of the receptor material while maintaining the established principles and intended analytical application of the RBA method. The proposed modification would be supported by appropriate method validation data demonstrating that the use of porcine brain tissue provides comparable analytical performance to the currently specified rat brain tissue.
Public Health Significance	The Receptor Binding Assay (RBA) provides a sensitive and efficient method for detecting Paralytic Shellfish Poisoning (PSP) toxins in shellfish and supports timely public health decisions related to shellfish safety and harvest closures. Maintaining reliable access to the tissue required for the assay is important to ensure continued laboratory capacity for routine monitoring and response to PSP events. Allowing the use of porcine brain tissue as an alternative to rat brain tissue would provide laboratories with a commercially accessible source of receptors, which reduces the cost associated with obtaining and preparing the receptor material. This modification could therefore reduce the overall cost and ease of performing the RBA while supporting the sustainability and availability of the method. The proposed modification would not change the intended use of the RBA for detection of PSP toxins. Use of porcine brain tissue would be supported by appropriate validation demonstrating comparable analytical performance to the established rat brain tissue source. Providing laboratories with an additional, readily available and cost effective receptor source would strengthen the long term sustainability of the RBA and support continued, timely public health monitoring of shellfish for PSP toxins.
Cost Information	The estimated cost per 96-well plate using porcine is ~$235. Including standards and samples with triplicate measurements (as well as three dilutions per sample [ranging from 3.5-600 µg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$35. In contrast, the estimated cost for a 96-well plate using rat brain is ~$300 with a cost per sample for quantitation of ~$40. For a potential savings of ~$5 per sample while using porcine. If running multiple plates or in screening mode, sample costs would be reduced for both receptor protein sources." In 2025, the Washington State Public Health Laboratories (WA PHL) analyzed approximately 3,000 PSP samples. The use of less expensive porcine tissue in the Receptor Binding Assay would have resulted in an estimated cost savings of approximately $15,000 that year.

ISSC Method Application Format for Biotoxin Methods: Method Modification

Critical Information:
Applicants shall attach all procedures, with materials, methods, calibrations and interpretations of data with the request for review and potential approval by the ISSC. The ISSC also recommends that submitters include peer-reviewed articles of the procedure (or similar procedures from which the submitting procedure has been derived) published in technical journals with their submittals. Methods submitted to the ISSC Laboratory Committee for acceptance will require, at a minimum, 6 months for review from the date of submission.

Note:
The applicant should provide all information and data identified above as well as the following material, if applicable:

Method Overview
Name of the New Method: Modified Application of Receptor Binding Assay to Mussels Monitoring for Paralytic Shellfish Poison using Porcine Brain Membrane Preparation
Specify the Type of Method (e.g., Chemical, Molecular, or Culture): Molecular. The RBA is a functional assay, whereby toxins present in the standard/sample bind to sodium channel preparations in the assay. Radiolabeled toxins are added to solution to compete with toxins present in the standard/sample for binding sites, and thus a decrease in signal from radiolabeled toxins represents a higher standard/sample concentration. This competitive RBA allows for quantitation that directly relates to the composite toxicity of the sample.
Name of Method Developer / Submitter: Shelley Lankford, Washington State Department of Health Public Health Laboratories, Biotoxin Laboratory and WA DOH Shellfish Program Tod Leighfield & Kelly Cusick, NOAA, National Ocean Service, Hollings Marine Lab
Developer / Submitter Contact Information [e.g., Address and Phone Number(s)]: Shelley Lankford, Washington State Department of Health Public Health Laboratories, Biotoxin Laboratory and WA DOH Shellfish Program Tod Leighfield: 331 Fort Johnson Rd, Charleston, SC 29412 and 843-580-8154
Date of Submission: September 26th, 2026
Purpose and Intended Use of the Method: The receptor binding assay (RBA) is intended for use as an NSSP Approved Method for the quantitative assessment of paralytic shellfish poison (PSP) in mussels using porcine brain membrane preparation. The application of this project is specifically intended for use in

regulatory laboratory settings. The RBA as described herein serves as an alternative to the rat brain membrane preparation in these applications by utilizing commercially available porcine brain, which is a readily available byproduct of the food industry. The assay achieves a reliable measure of integrated toxicity with high throughput, while successfully eliminating the need for dedicated sourcing of rat brains and the need for local laboratory animal euthanasia, this reduces preparation effort and costs.

Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods:
There is an acknowledged need for this method in the NSSP. Even though the MBA, PCOX, and RBA methods have been respectively NSSP Approved and Approved for Limited Use, there remains a need for the proposed method. The RBA would provide an alternative to (1) the MBA, which uses live animals, (2) the PCOX method, which requires costly equipment and skilled personnel and offers low throughput, (3) the RBA method, which requires direct laboratory animal euthanasia to prepare rat brain receptors and also results in higher preparation costs.

Method Limitations and Potential Indications of Cases Where the Method May Not Be Applicable:
n/a

Other Comments

Method Documentation

Method Title:
Modified Application of a Receptor Binding Assay to Mussels Monitoring for Paralytic Shellfish Poison using Porcine Brain Membrane Preparation

Method Scope:
The RBA provides a high throughput, sensitive, accurate, quantitative assay for PSP toxins in shellfish. The method is being submitted for consideration as an NSSP Approved Method for the purposes of screening for PSP toxicity using porcine brain homogenate as the receptor source instead of rat brain homogenate.

References:
Van Dolah et al. 2009. Single-laboratory validation of the microplate receptor binding assay for paralytic
shellfish toxins in shellfish. Journal of AOAC International 92(6): 1705-1713. See Appendix II.

Van Dolah et al. 2012. Determination of paralytic shellfish poisoning toxins in shellfish by receptor binding assay: Collaborative study. Journal of AOAC International 95(3): 795-812. See Appendix III.

OMA 2011.27. AOAC Official Method 2011.27 Paralytic shellfish toxins (PSTs) in shellfish, receptor binding assay. In Official Methods of Analysis of AOAC International. http://www.eoma.aoac.org. See Appendix IV.

Principle:
This PSP receptor binding assay is based on the interaction between the toxins and their native receptor, the voltage-gated sodium channel. All paralytic shellfish toxins (PSTs) bind to site 1 of the voltage-gated sodium channels relative to their affinity to the channel (receptor). These affinities are a primary driver of in vivo toxicity and the read-out of the RBA, results in a measure of integrated potency (independent of knowing which toxin congeners are present) similar to mouse intraperitoneal potency. In the RBA, tritiated saxitoxin (3H-STX) competes with unlabeled PSTs in a shellfish extract for a finite number of available receptor sites in a brain membrane preparation. After a binding equilibrium is reached, unbound 3H-STX is removed by filtration and the remaining 3H-STX is measured with a scintillation counter (measured as counts per minute or CPM). The amount of 3H-STX present is indirectly related to the quantity of unlabeled PSTs in the sample. This output is compared to a standard curve prepared from saxitoxin dihydrochloride and is reported at saxitoxin dihydrochloride equivalents agnostic to the specific PSP congener composition of the extract.

Analytes/Measurands:
The target analytes are paralytic shellfish toxins (PSTs), with NIST Saxitoxin Dihydrochloride (RM 8642a) serving as the certified reference standard for generating calibration curves and spikes for shellfish samples.

The target measurand includes mussels, including blue mussels (*Mytilus edulis*) and California mussels (*Mytilus californianus*)

Proprietary Aspects:
None. All reagents can be prepared or purchased.

Equipment:
(Provide a list of specialized equipment needed to perform the method.)
The following list identifies the equipment and supplies needed for conducting the RBA.

<u>For the assay:</u>
(a) Microplate Scintillation counter suitable for measuring low counts of tritium (<1000dpm) (e.g. MicroBeta from Revvity)
(b) An 8-channel pipettor (5-200 µl variable volume and disposable tips)
(c) Micropipettors (1-1000 µl variable volumes and disposable tips)
(d) 96-well microtitre filter plate (1 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (MilliporeSigma; Cat. No. MSFB N6B 50)
(e) MultiScreen vacuum manifold (MilliporeSigma; Cat. No. NSVMHTS00)
(f) Vacuum pump
(g) Centrifuge tubes (15 and 50 ml, conical, plastic)
(h) Mini dilution tubes in 96-tube array
(i) Reagent reservoirs
(j) Ice bucket and ice
(k) Vortex mixer
(l) Sealing tape (Millipore; Cat. No. MATA HCL00)
(m) Volumetric flask or graduated beaker (1 L) (for buffer preparation)

(n) -80 °C freezer
(o) Refrigerator
(p) pH meter (for buffer preparation)

For sample extraction:
(q) Blender or homogenizer for sample homogenization
(r) Pipets
(s) Centrifuge tubes (15 ml, conical, plastic)
(t) pH meter or pH paper
(u) Hot plate or water bath
(v) Graduated centrifuge tubes (15 ml)
(w) Centrifuge and rotor for 15 ml tubes

Reagents:
(Provide a list of specialized chemicals, reagents, etc. needed to perform the method.)
For the assay:
(a) STX diHCl standards (NIST RM 8642a; National Institute of Standards and Technology; www.nist.gov) [This is the same standard used for the MBA]
(b) 3H-STX (0.05 mCi per ml, ≥10 Ci per mmol, ≥90% radiochemical purity; American Radiolabeled Chemicals, St. Louis, MO)
(c) 3-Morpholinopropanesulfonic acid (MOPS; Sigma; St. Louis, MO; Cat. No. M3183-500G [or equivalent])
(d) Choline chloride (Sigma; Cat. No. C7527-500G [or equivalent])
(e) ultrapure water (MilliQ or equivalent)

For microplate counter only:
(f) Ultima Gold liquid scintillation cocktail (Revvity.; Waltham, MA; Cat. No. 6013321 [or equivalent])

For sample extraction:
(g) Hydrochloric acid (HCl; 1.0 and 0.1 N)
(h) Sodium hydroxide (0.1 N)
(i) Water (distilled or deionized [18 μΩ])

Media:
None.

Matrix or Matrices of Interest:
Mussels: Blue mussel (*Mytilus edulis*) and California mussel (*Mytilus californianus*)

Sample Collection, Preservation, Preparation, Storage, Cleanup, etc.
A representative shellfish sample should include 12 market size organisms pooled together (should be at least 100 g). Clean the outside of shellfish with running tap water. Open the shell by cutting into the adductor muscle, being careful to not cut or damage the viscera.
Rinse the inside to remove sand and dirt and remove tissue from ~12 organisms. Collect the tissue on a number 10 sieve and allow to drain for ~5 minutes. Remove any obvious pieces of

shell or debris. Transfer meat to blender or homogenizer and blend until homogeneous. This homogenate is then extracted for toxins. For the detailed sample extraction procedure see Sample Extraction in Appendix A. Shellfish homogenates must be tested immediately or stored frozen prior to analysis. Saxitoxin standards must be stored refrigerated and 3H-STX must be stored at -70 °C. The rat brain preparation can be produced in bulk, partitioned into aliquots, and stored long-term at -70 °C until use.

Safety Requirements:
(Describe the safety measures, beyond those of routine laboratory practices, required to perform the method, including personal protective equipment, fume hoods, etc.)
General safety requirements (e.g., personal protective equipment including gloves, safety glasses, and laboratory coat) for working with toxins, biological reagents, and low-level radioactive material must be followed. Users must be trained in and follow all in-house safety procedures for working with toxins and radiolabeled materials. Although low levels of radiation are used for this assay, sometimes under an NRC exemption clause for the ARC sourced 3HSTX, users must follow all local, state and federal laws and procedures regarding the receipt, use, and disposal of radioisotopes. Please see Appendix B for further safety requirements.

Other Information (Cost of the Method, Special Technical Skills Required to Perform the Method, Special Equipment Required and Associated Cost, Abbreviations and Acronyms Defined and Details of Turn Around Times [Time Involved to Complete the Method]):

The estimated cost per 96-well plate assay is ~$235. Including standards and samples with triplicate measurements (as well as three dilutions per sample [ranging from 3.5-600 µg STX eq 100 g-1] to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitation would be ~$35. If running multiple plates or in screening mode, sample costs would be reduced. A microplate scintillation counter is needed and the cost is ~$70-250K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$13K.

Microplate scintillation counting provides the ability to test multiple samples simultaneously with a turnaround time for data in approximately 3 hours. Up to four plates per analyst are possible in one day, yielding a throughput of at least 36 samples per day.

Test Procedures, (Be Specific and Provide Easy-to-Follow Step-by-Step Procedures and indicate critical steps.):

The protocol is very clear and easy to follow. Please see the detailed protocol below in Appendix A.

Quality Control (Provide Specific Steps):
Quality control steps are in place to determine if assay results are acceptable:

(a) The slope of the standard curve must be between - 0.8 and -1.2 (theoretical slope is -1). If the slope of a standard curve from a given assay falls outside of this range, the data should be considered unacceptable and the assay must be rerun.

(b) The RSDs of triplicate counts per minute (CPMs) for the standards must be below 30%.

(c) If the IC50 (inhibitory concentration at which CPM is 50% max) is out of the acceptable range (1.75 nM ± 30%), the data should be considered unacceptable and the assay should be rerun.

(d) A QC sample should always be included on each plate and found to be in range. Typically, a 1.8 x 10-8 M STX concentration (3 nM STX in-well concentration) is run as a QC and the measured value should be within 30% of the expected concentration. Results outside of this range should trigger consideration of assay acceptance. The following criteria must be met to accept sample measurement:

(e) For sample measurement, quantitation should only be done on sample dilutions that fall within the linear range. As such, binding (B, measured as counts per minute) scaled by the maximum binding (B_0) should be between 0.2-0.7 for sample quantitation to be performed (any sample falling outside of this range is considered out of the dynamic range). If $B/B_0 > 0.7$, the concentration is too low to be quantified and should be reported as below the limit of detection (LOD). If $B/B_0 < 0.2$, the sample should be diluted and rerun if quantitation is needed.

(f) The RSDs for the sample CPMs should be ≤ 30%. These quality control criteria are also stated in section H in Appendix IV.

(g) The calculated 'top' value (0 nM STX in-well concentration) must be greater than 1,000 CPM. This threshold is required to maintain an adequate signal-to-noise ratio and a sufficient dynamic range, both of which are essential for accurate curve fitting and reliable IC_{50} determination.

Validation Criteria (Include Accuracy / Trueness, Measurement Uncertainty, Precision [Repeatability and Reproducibility], Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, Matrix Effects and Comparability (if intended as a substitute for an established method accepted by the NSSP).

Please see below for Specificity, Ruggedness, and Comparability results.

Data and Statistical Analyses Performed for Each Validation Criterion Tested (Be Specific and Provide Clear Easy-to-Follow Step-by-Step Procedures.).

Please see below for SOP 1, 2, and 3 for data and statistical analyses completed for Specificity, Ruggedness, and Comparability, respectively.

Calculations and Formulas Used for Each Validation Criterion Tested.

Please see below for calculations and formulas used for Specificity, Ruggedness, and Comparability criterions.

Results for Each Validation Criterion Tested. Please see below for results for Specificity, Ruggedness, and Comparability criterions.
Discussion of Each Validation Criterion Tested. Please see below for discussion for Specificity, Ruggedness, and Comparability criterions.
Summary of Results. This validation study evaluates replacing the standard rat brain membrane preparation with a commercially prepared porcine brain membrane preparation in the Receptor Binding Assay (RBA) for Paralytic Shellfish Toxins (PSTs). Across 120 paired naturally incurred mussel samples, the porcine preparation demonstrated near-perfect correlation with the rat preparation ($R^2 = 0.99$). Although a standard paired t-test detected a minor systematic offset where the rat preparation read 5.7% higher on average (+0.024 log units), a Two One-Sided Test (TOST) confirmed that this difference falls cleanly within the predefined biological equivalence bounds of ±20%. Furthermore, the assay proved highly rugged against routine batch-to-batch reagent variation ($t(9)= -0.254$, $p > 0.05$) and demonstrated high specificity, showing no significant interference in the presence of co-occurring domoic acid ($SI_{avg} = 1.01$, $p > 0.05$).

Additional Requirement:
If a laboratory method is found acceptable for use in the National Shellfish Sanitation Program and adopted by the Interstate Shellfish Sanitation Conference, the method submitter will draft a laboratory checklist that can be used to evaluate laboratories performing their procedure. The checklist will be submitted to the ISSC and reviewed by the Laboratory Quality Assurance Committee for Conference approval as a modified version of the existing checklist.

(For guidance: refer to the checklists in the National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish 2017, Guidance Documents, Chapter II – Growing Areas, .15 Evaluation of Laboratories by State Laboratory Evaluation Officers Including Laboratory Evaluation
Checklists.)

Background:

This submission follows the proposal for extending the Receptor Binding Assay (RBA) to multiple shellfish matrices. The current RBA method uses a rat brain membrane preparation; the proposed method modification replaces the rat brain membrane with a porcine brain membrane preparation. The use of porcine brain membrane homogenate also offers both economic and operational advantages over the current rat brain preparation, provided the porcine preparation demonstrates comparable assay performance. Based on the current testing volume of approximately 1,500 samples over six months at the Washington State Department of Health (WADoH), the porcine preparation costs approximately $0.50 per sample compared with approximately $1.00 per sample for the rat preparation, resulting in an estimated direct savings of $750 over six months. Importantly, the $1.00 per-sample cost for the rat preparation accounts only for direct material costs, including MOPS, rat brains, cryovials, PMSF, and choline chloride, and does not include additional labor, equipment use, consumables, or time required to prepare the rat brain homogenate. In contrast, the commercially prepared porcine brain membrane homogenate substantially reduces the preparation burden and has the potential to provide greater consistency and standardization while reducing dependence on animal sourcing and in-house tissue preparation. Therefore, if the porcine preparation demonstrates comparable receptor-binding performance, sensitivity, precision, and QC results to the rat preparation, its use represents a more cost-effective, deployable, and operationally efficient approach for routine PSP testing while maintaining the analytical purpose and performance of the RBA.

A total of 641 mussel samples were collected for this project, including both samples with little to no detectable PSP-toxins and with PSP-toxins detected using the mouse bioassay (MBA). The ‘blank’ materials used in the study contained very low, but still detectable, PSP-like activity, ranging from less than 2 µg STX/100 g of shellfish tissue to approximately 10 µg STX/100 g of shellfish tissue. Initial screening using the MBA (limit of detection ~40 µg STX/100g of shellfish tissue) identified negative samples, which were subsequently re-screened using a modified RBA with a lower limit of detection of ~2 µg STX/100 g. To achieve the required quantity for the validation study, the identified ‘blank’ materials were combined to produce the 11 homogenates with 41 sub-aliquots needed for spikes at 1/2x 1x, and 2x the regulatory limit (80 ug STX/100 g of shellfish tissue) for the shellfish matrix as shown in the study design (Figure 1). The ‘blank’ sample for each homogenate served as a reference blank to be subtracted from the spiked sub-aliquots after performing RBA to ensure accuracy in measured spiked samples (RBA-Blank)

In total, 403 of 641 PSP samples were below the MBA’s limit of detection from Washington state and were re-screened using a modified extraction procedure (0.5 g sample + 0.5 mL 0.1 N HCl) (Figure 2). For the study, 17 samples were selected for inclusion. While 396 samples were not used for the study, but they are currently being stored in the NOAA Hollings Marine Laboratory’s freezer inventory for future studies (Charleston, SC).

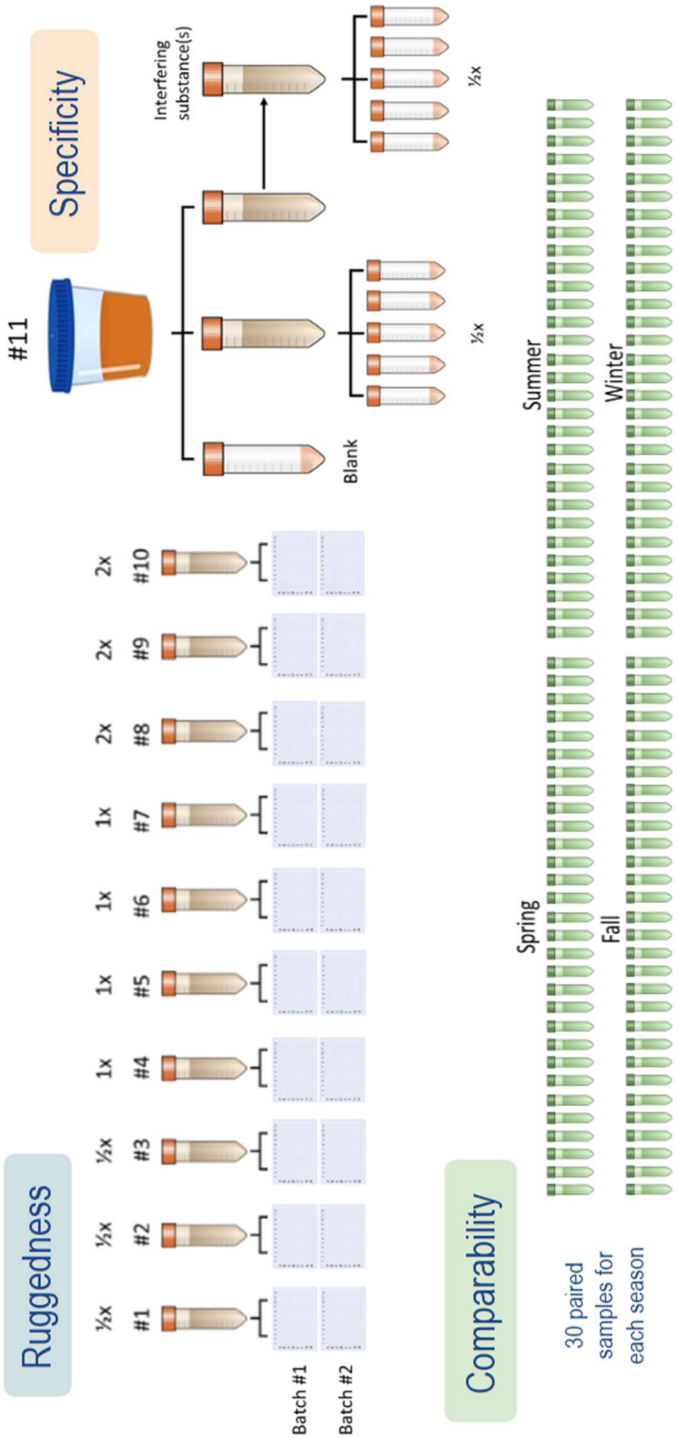


Figure 1. Method Modification study design showing allocation of 121 samples for mussels to meet three validation data criteria: ruggedness (blue), specificity (orange), and comparability (green).

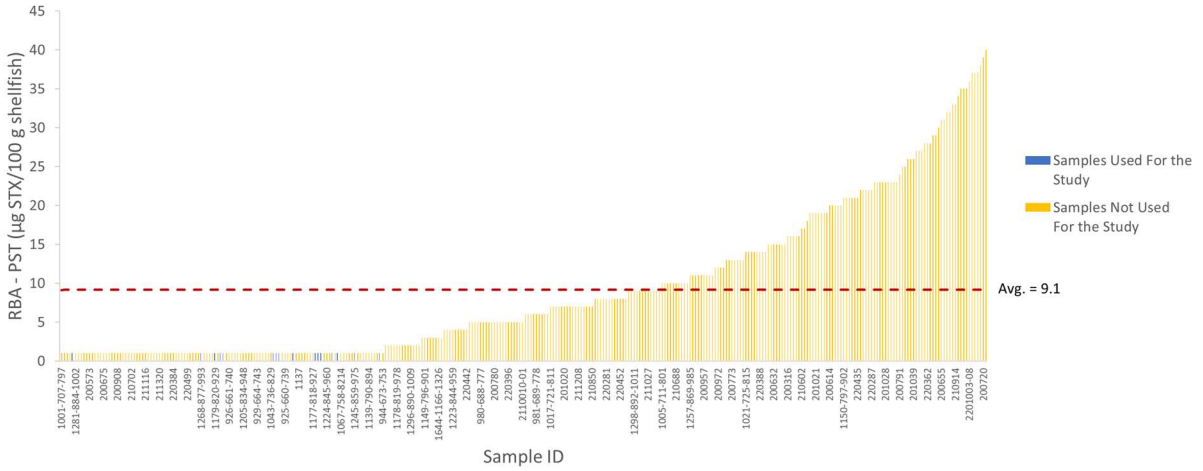


Figure 2. A total of 403 samples from mussel matrix that tested negative for toxins using the MBA (limit = 45 µg STX/100 g shellfish tissue) were re-screened with a modified RBA to identify "blank" samples for spiking in homogenates for the Method Modification study. Average PST values (indicated by the red dashed line) for mussels is 9.1 µg STX/100 g shellfish tissue. Only 17 samples were selected and used for the study (blue), while the remaining samples (n= 396; yellow) are stored at the Hollings Marine Laboratory, Charleston, SC.

Samples were collected across Washington State over various harvest days, locations, and calendar seasons. Seasons were defined as follows: Spring (March 20–June 20), Summer (June 21–September 21), Fall (September 22–December 20), and Winter (December 21–March 19). To obtain the required tissue weight for each homogenate, multiple individual samples collected within 30-days in the same season were combined prior to spiking. Meeting the requested sample size for each season to satisfy the comparability criteria for naturally incurred PSP positive samples was challenging because PSP levels during the winter season were consistently below the MBA limit of detection and therefore unavailable to use for this study. For this study, 120 samples were collected and analyzed, including 87 summer, 20 fall, 13 spring, and 0 winter samples. Although the target of 30 paired samples per season was not achieved, the overall sample-size requirement was met. The seasonal imbalance was attributable to the naturally occurring distribution of PSP and the associated limitations in obtaining spring, fall, and winter samples, rather than insufficient sample effort. The available samples provide broad representation across harvest dates, location, and seasons and are considered adequate to evaluate the conditions relevant to the comparability criteria (Table 1).

Table 1. Summary of mussel tissue samples (blue mussels and California mussels) collected across Washington sites from 2019 to 2025, categorized by group, location, and season. Group letter represents the spiked samples while group number represents the naturally incurred samples.

Group Letter/ Number	Shellfish Matrix	Location	Collection Date	Season
A	blue mussel	South King County, King	6/23/2023	Summer
B	blue mussel	Port Ludlow, Jefferson	5 & 6/2023	Spring
C	blue mussel	Possession Sound, Snohomish	5 & 6/2023	Spring
D	blue mussel	Nisqually Reach, Thurston	5 & 6/2023	Spring
E	blue mussel	Narrows, Pierce	6/1//2023	Spring
F	blue mussel	West Bainbridge, Kitsap	6/22/2023	Summer
G	blue mussel	Sinclair Inlet, Kitsap	6/22/2023	Summer
H	blue mussel	Budd Inlet, Thurston	6/23//2023	Summer
I	blue mussel	North King County, King	6/23/2023	Summer
J	blue mussel	Liberty Bay, Kitsap	6/21/2023	Summer
K	blue mussel	Case Inlet, Mason	5-6/2023	Spring
21-1	blue mussel	Kingston Mariana, Kitsap	8/4/2025	Summer
62-1	blue mussel	Discovery Bay, Jefferson	7/30/2025	Summer
67-1	blue mussel	Ediz Hook, Clallam	7/16/2025	Summer
71-1	blue mussel	Fossil Bay, San Juan	8/7/2024	Summer
72-1	blue mussel	Semiahmoo Mariana, Whatcom	5/21/2024	Spring
73-1	California mussel	Freshwater Bay, Clallam	8/6/2024	Summer
74-1	California mussel	Grays Harbor Coast, Grays Harbor	5/22/2024	Spring
75-1	blue mussel	Fossil Bay, San Juan	7/23/2024	Summer
76-1	California mussel	Clallam Bay, Clallam	7/8/2024	Summer
77-1	blue mussel	Port Ludlow, Jefferson	7/1/2024	Summer
78-1	blue mussel	Sequim Bay, Clallam	8/26/2024	Summer
79-1	blue mussel	Cape George, Clallam	6/25/2024	Summer
80-1	blue mussel	Anacortes Marina, Skagit	7/17/2024	Summer
81-1	blue mussel	Bellingham Bay, Whatcom	6/19/2024	Spring
82-1	California mussel	Long Beach, Pacific	6/10/2024	Spring
83-1	blue mussel	Silverdale, Kitsap	10/21/2024	Fall
84-1	California mussel	South Jetty, Grays Harbor	6/6/2024	Spring
85-1	California mussel	Mocrocks Area, Grays Harbor	6/2/2024	Spring
86-1	blue mussel	Squalicum Harbor, Whatcom	10/21/2024	Fall
87-1	blue mussel	Sequim Bay, Clallam	10/28/2024	Fall
88-1	blue mussel	Ediz Hook, Clallam	11/6/2024	Fall
89-1	California mussel	Neah Bay, Clallam	10/28/2024	Fall
90-1	California mussel	South Jetty, Grays Harbor	5/30/2024	Spring
91-1	blue mussel	Sequim Bay, Clallam	11/4/2024	Fall
92-1	California mussel	Willapa Bay, Pacific	6/5/2024	Spring
93-1	California mussel	Makah Bay, Clallam	6/11/2024	Spring

94-1	blue mussel	Rosario Point, San Juan	10/16/2024	Fall
95-1	California mussel	Neah Bay, Clallam	11/12/2024	Fall
96-1	blue mussel	Rosario Point, San Juan	12/2/2024	Fall
97-1	blue mussel	Squalicum Harbor, Whatcom	6/10/2024	Spring
98-1	blue mussel	Poulsbo Marina, Kitsap	8/4/2025	Summer
99-1	blue mussel	EPA Lab Dock, Kitsap	8/4/2025	Summer
100-1	blue mussel	Penn Cove, Island	7/28/2025	Summer
101-1	blue mussel	Utsalady Bay, Island	7/27/2025	Summer
102-1	blue mussel	Brownsville, Kitsap	7/14/2025	Summer
103-1	blue mussel	South Egler Bay, Island	7/16/2025	Summer
104-1	blue mussel	Dockton, King	7/28/2025	Summer
105-1	blue mussel	Ediz Hook, Clallam	7/31/2025	Summer
106-1	blue mussel	Dockton, King	7/2/2025	Summer
107-1	blue mussel	Port Orchard Marina, Kitsap	8/11/2025	Summer
108-1	blue mussel	Silverdale, Kitsap	8/4/2025	Summer
109-1	blue mussel	Utsalady Bay, Island	8/6/2025	Summer
110-1	blue mussel	Quartermaster Harbor, King	8/4/2025	Summer
111-1	blue mussel	Port Washington Marina, Kitsap	8/11/2025	Summer
112-1	blue mussel	Kingston Marina, Kitsap	7/15/2025	Summer
113-1	blue mussel	Gig Harbor, Pierce	8/7/2025	Summer
114-1	blue mussel	Poulsbo Marina, Kitsap	7/14/2025	Summer
115-1	blue mussel	Poulsbo Marina, Kitsap	7/28/2025	Summer
116-1	blue mussel	South Egler Bay, Island	7/28/2025	Summer
117-1	blue mussel	Brownsville, Kitsap	7/28/2025	Summer
118-1	blue mussel	Brownsville, Kitsap	8/4/2025	Summer
119-1	blue mussel	Penn Cove, Island	8/3/2025	Summer
120-1	California mussel	Bay Center Dock, Pacific	7/2/2025	Summer
121-1	blue mussel	Discovery Bay, Jefferson	8/4/2025	Summer
122-1	blue mussel	Kingston Marina, Kitsap	7/28/2025	Summer
124-1	blue mussel	Penn Cove, Island	7/24/2025	Summer
126-1	blue mussel	South Lopez, San Juan	8/9/2024	Summer
127-1	blue mussel	President Channel, San Juan	8/9/2024	Summer
128-1	blue mussel	President Channel, San Juan	8/23/2024	Summer
129-1	blue mussel	Kingston, Kitsap	9/12/2023	Summer
130-1	California mussel	East Straits, Clallam	8/21/2024	Summer
131-1	blue mussel	Sequim Bay, Clallam	9/18/2024	Summer
132-1	blue mussel	Port Susan, Island	8/22/2024	Summer
133-1	blue mussel	East Bainbridge, Kitsap	6/18/2024	Spring
134-1	blue mussel	West Fidalgo Island, Skagit	7/25/2024	Summer
135-1	California mussel	Oregon	6/25/2024	Summer
136-1	blue mussel	Port Townsend, Jefferson	8/28/2024	Summer
138-1	blue mussel	Sequim Bay, Clallam	9/8/2023	Summer
139-1	blue mussel	Lopez Sound, San Juan	8/23/2024	Summer

140-1	blue mussel	West Sound, San Juan	8/23/2024	Summer
141-1	blue mussel	Kilisut Harbor, Jefferson	7/26/2024	Summer
142-1	blue mussel	Kilisut Harbor, Jefferson	8/28/2024	Summer
143-1	blue mussel	Hood Canal #3, Jefferson	8/27/2024	Summer
144-1	blue mussel	Penn Cove, Island	8/19/2024	Summer
145-1	blue mussel	Birch Bay, Whatcom	8/7/2024	Summer
146-1	blue mussel	Saratoga Passage, Island	8/29/2024	Summer
147-1	blue mussel	Eagle Harbor, Kitsap	7/14/2025	Summer
148-1	blue mussel	Sequim Bay, Clallam	9/7/2022	Summer
149-1	blue mussel	Kingston, Kitsap	8/19/2024	Summer
150-1	blue mussel	Sequim Bay, Clallam	10/25/2022	Fall
151-1	blue mussel	Sequim Bay, Clallam	8/27/2024	Summer
152-1	California mussel	Clallam Coast, Clallam	10/1/2019	Fall
153-1	blue mussel	Sequim Bay, Clallam	10/18/2022	Fall
154-1	blue mussel	Port Susan, Island	8/1/2024	Summer
155-1	blue mussel	Saratoga Passage, Island	8/5/2024	Summer
156-1	blue mussel	Sequim Bay, Clallam	9/11/2023	Summer
157-1	blue mussel	Saratoga Passage, Island	8/14/2024	Summer
158-1	blue mussel	Liberty Bay, Kitsap	9/30/2019	Fall
159-1	blue mussel	Bellingham Bay, Whatcom	6/19/2024	Spring
160-1	blue mussel	Port Ludlow, Jefferson	8/30/2021	Summer
161-1	blue mussel	Sequim Bay, Clallam	10/25/2022	Fall
162-1	blue mussel	Sequim Bay, Clallam	8/23/2021	Summer
163-1	blue mussel	Discovery Bay, Jefferson	7/30/2024	Summer
164-1	blue mussel	Penn Cove, Island	7/17/2025	Summer
165-1	blue mussel	Saratoga Passage, Island	7/23/2024	Summer
166-1	blue mussel	Lopez Sound, San Juan	7/23/2024	Summer
167-1	blue mussel	Sequim Bay, Clallam	9/15/2022	Summer
168-1	blue mussel	Kingston, Kitsap	9/18/2023	Summer
169-1	blue mussel	San Juan Channel, San Juan	8/22/2024	Summer
170-1	blue mussel	Kilisut Harbor, Jefferson	8/19/2024	Summer
171-1	blue mussel	West Fidalgo Island, Skagit	9/3/2024	Summer
172-1	blue mussel	Kingston, Kitsap	9/13/2021	Summer
173-1	blue mussel	Kingston, Kitsap	8/12/2024	Summer
176-1	blue mussel	Sequim Bay, Clallam	10/1/2019	Fall
177-1	blue mussel	Liberty Bay, Kitsap	8/14/2023	Summer
178-1	blue mussel	Sequim Bay, Clallam	10/2/2019	Fall
179-1	blue mussel	Bellingham Bay, Whatcom	8/29/2024	Summer
180-1	California mussel	Grays Harbor Coast, Grays Harbor	8/3/2023	Summer
181-1	blue mussel	Discovery Bay, Jefferson	10/4/2021	Fall
182-1	blue mussel	Port Ludlow, Jefferson	6/5/2023	Spring
183-1	blue mussel	Sequim Bay, Clallam	9/23/2024	Fall
184-1	blue mussel	Port Ludlow, Jefferson	8/16/2021	Summer

185-1	California mussel	West Straits, Clallam	10/6/2021	Fall
186-1	blue mussel	East Sound, San Juan	8/9/2022	Summer
187-1	blue mussel	East Straits, Clallam	9/11/2024	Summer
188-1	blue mussel	East Straits, Clallam	8/17/2021	Summer
189-1	blue mussel	Sequim Bay, Clallam	11/1/2022	Fall
190-1	blue mussel	Drayton Harbor, Whatcom	9/7/2021	Summer
191-1	blue mussel	Sequim Bay, Clallam	9/6/2022	Summer
192-1	blue mussel	Bellingham Bay, Whatcom	7/28/2022	Summer

IX. #1 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Comparing Methods

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:
New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method: This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data:
Sample type _____

Date	Sample/Station #	Conc. Recognized method	Conc. Alternative Method
	1		
	2		
	3		
	4		
	5		
	6		
	7		
	8		
	9		
	10		
	n		

n is the last sample in the comparison

For shellfish samples, repeat for each tissue type of interest

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be

used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:

Value for the test of symmetry for the distribution of the data generated by the officially recognized method __RBA with **Rat** Brain Membrane Preparation = 0.55_____

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method _____RBA with **Porcine** Brain Membrane Preparation = 0.44_____

Variance of the data generated from the officially recognized analytical method __RBA with **Rat** Brain Membrane Preparation = 0.23_____

Variance of the data generated from the proposed alternative analytical method __RBA with **Porcine** Brain Membrane Preparation = 0.22_____

Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____1.02_____

Is there a significant difference between the analytical methods Y/N → **paired t-test results: t = 3.74, p = 0.00028, df = 119**

Following the data handling procedure and utilizing log-transformed data, the initial paired t-test confirmed a significant difference between porcine and rat brain membrane preparations ($t(119) = 3.74$, $p<0.001$). This result is primarily an artifact of high statistical power from the large sample size (n=120) rather than a meaningful biological discrepancy. In null hypothesis significance testing (NHST), a paired t-test evaluates whether the mean difference between two methods is zero. Consequently, as sample size increases, the standard error decreases, resulting in large a t-value and a correspondingly small p-value; therefore, biologically irrelevant differences will likely reject the null hypothesis. Because a t-test evaluates difference rather than functional equivalence, relying on it alone risks an inappropriate rejection of a method substitution due to the test's over-sensitivity to irrelevant differences. To overcome this limitation, three complementary analyses such as linear regression, Bland-Altman agreement, and Two One-Sided Test (TOST), were performed to assess relative affinity, bias magnitude, and true analytical equivalence against predefined regulatory thresholds.

Linear regression analysis was conducted to evaluate how PST binding values vary between the two tissue types (porcine versus rat). The model demonstrated a near-perfect linear relationship ($R^2 = 0.99$), confirming that PSTs binding tightly to rat voltage-gated sodium channels exhibit the same relative affinity towards porcine sodium channels (Figure 3). However, a high correlation can mask proportional bias, so a Bland-Altman agreement analysis was performed. The analysis revealed a minor mean systematic bias of +0.024 log units (~ +5.7%), indicating that the assay using rat brain membrane preparation results in values on average 0.024 log units higher than the assay using porcine brain membrane preparation (Figure 4). In routine RBA for PST detection, inter-assay variability of ±10-20% is standard practice; thus, a systematic shift of 5.7% has a minimal effect on analytical accuracy and regulatory decision-making.

A TOST was performed to assess biological equivalence between assays using porcine versus rat brain membrane preparations. This test determines whether the mean difference between methods falls within a predefined, biologically acceptable interval. An equivalence margin of ± 0.097 log units (approximately 20% difference) was predefined for the analysis. The observed mean difference (+0.024 log units) and its 90% confidence interval fell within the upper and lower equivalence bounds, demonstrating a statistically significant equivalence ($t(119) = -11.0$, $p <0.01$; Figure 5). While the porcine brain membrane preparation yielded slightly lower average results than the rat brain membrane preparation (+5.7%), however, this minor variation represents a predictable baseline offset rather than a biological divergence. Because the relative compound affinities remained nearly identical ($R^2 = 0.99$) and absolute discrepancies fell within established equivalence bounds, porcine brain membrane preparation is a validated, functionally equivalent, scalable, and ethically favorable alternative to rat brain tissue for routine RBA to detect PSTs in mussels.

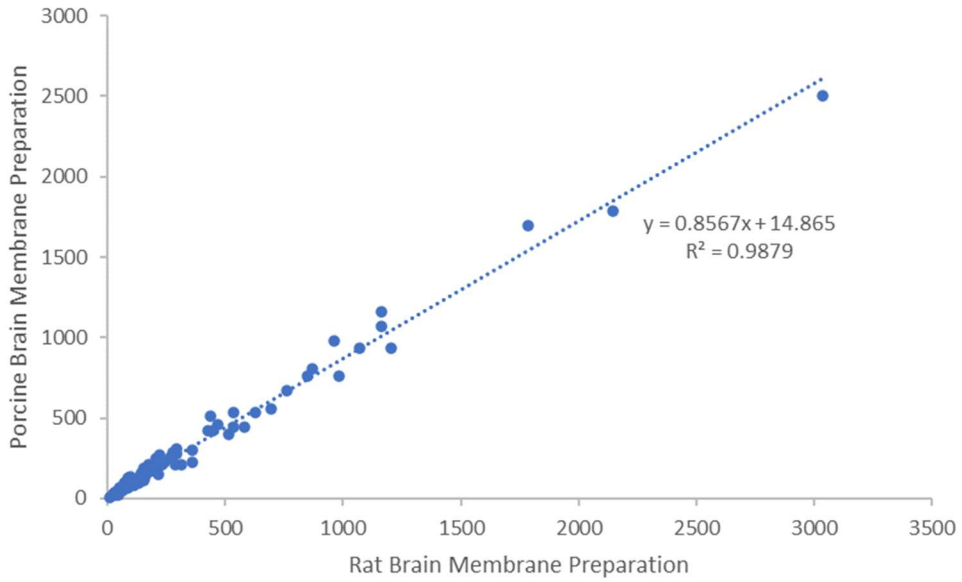


Figure 3. Scatterplot of comparing saxitoxin dihydrochloride equivalents (µg STX diHCl equiv./100 g shellfish) in naturally incurred mussel samples measured across seasons, harvest areas, and dates between the officially recognized method (rat brain membrane preparation) and the proposed alternative analytical method (porcine brain membrane preparation). The linear regression line (y = 0.86x + 14.87) and correlation coefficient (R^2 = 0.99) are shown.

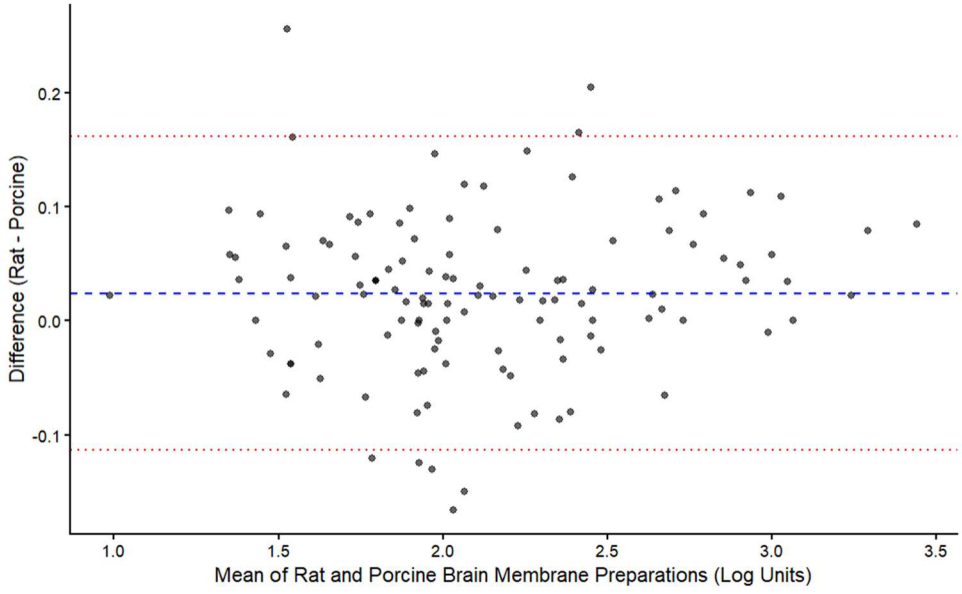


Figure 4. Bland-Altman plot assessing agreement between rat and porcine brain membrane preparations for Paralytic Shellfish Toxins (PSTs). Differences between paired log-transformed measurements (Rat - Porcine) are plotted against their paired mean values. The blue dashed line represents the mean systematic bias (+0.024 log units) and the red dashed lines represent the 95% limits of agreement (-0.113 to +0.161 log units).

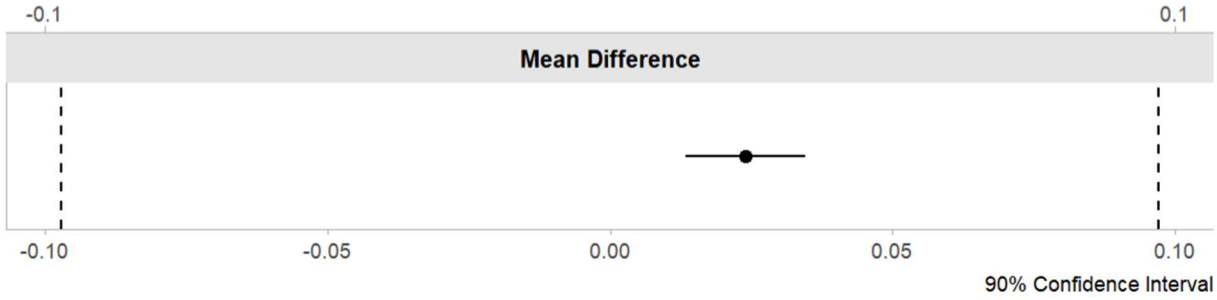


Figure 5. A Two One-Sided Test (TOST) equivalence plot for rat vs. porcine brain membrane preparations in the RBA for PSTs. The black circle represents the mean difference (+0.024 log units), and the horizontal line represents the 90% confidence interval (+0.011 to +0.037 log units). Vertical dashed lines denote the predefined biological equivalence bounds ± 0.10 log units, corresponding to approximately ± 20% variance.

Table 2. PST values reported for each sample using two methods for RBA: rat brain membrane preparation and porcine brain membrane preparation (µg STX diHCl equiv./100 g shellfish).

Sample ID	Rat Brain Membrane Preparation	Porcine Brain Membrane Preparation
71-1	61	50
72-1	74	69.5
73-1	469	458
74-1	447	424
75-1	72	65
76-1	133	101
77-1	82.5	98
78-1	438	509
79-1	112	103
80-1	65	60
81-1	268	259
82-1	277	286
83-1	33	36
84-1	581	447
85-1	960	983
86-1	152	170
87-1	65	60
88-1	188	170
89-1	117	115
90-1	1206	938
91-1	81.5	67
92-1	143	152
93-1	223	214
94-1	92	89
95-1	105	101.5
96-1	76	91.5

97-1	89	86
98-1	223	241
99-1	31	25
100-1	357	304
101-1	80	108
102-1	197	197
103-1	98	107
104-1	223	268
105-1	161	134
106-1	29	31
107-1	36	31
108-1	54	63
109-1	65	60
110-1	80	89
111-1	58	51
112-1	152	188
113-1	95	99
114-1	89	71
115-1	286	214
116-1	232	214
117-1	107	98
118-1	357	223
119-1	871	804
120-1	10	9.5
121-1	514	402
122-1	205	197
124-1	286	286
126-1	83	92
127-1	58	47
128-1	112	98
129-1	3037	2501
130-1	1072	938
131-1	42	29
132-1	116	94.5
133-1	73.5	98
134-1	27	27
135-1	1161	1072
136-1	84.5	84.5
138-1	983	759
139-1	98	138.5
140-1	80	71
141-1	36	33
142-1	58	54
143-1	42	40

144-1	214	152
145-1	49	42
146-1	45	25
147-1	89	75.5
62-1	89	130.5
67-1	152	116
21-1	131.5	125
148-1	79	76
149-1	536	447
150-1	313	214
151-1	536	536
152-1	146	139
153-1	103	103
154-1	41	43
155-1	424	422
156-1	759	670
157-1	625	536
158-1	84	84.5
159-1	175	168
160-1	92	97.5
161-1	145	160
162-1	25	23
163-1	59	56
164-1	112	80
165-1	75	75
166-1	242	223
167-1	95.5	86.5
168-1	2144	1787
169-1	173	209
170-1	293	311
171-1	53	70
172-1	692	558
173-1	849	759
176-1	1787	1697
177-1	47	40
178-1	1161	1161
179-1	40	45
180-1	25	20
181-1	89	85
182-1	31	36
183-1	24	21
184-1	25	22
185-1	223	232
186-1	134	125

187-1	94	96
188-1	67	69
189-1	295	277
190-1	33	36
191-1	205	250
192-1	67	54

IX. #2 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Ruggedness

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must be sufficiently rugged to withstand the relatively minor day to day changes likely to occur in routine use. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Ruggedness of the new or modified method is the ability of the new or modified method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure for testing the ruggedness of new or modified methods: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals. For each sample take two aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine new method/modified method concentration for the target analyte/measurand/ organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the new method or modified method's intended application to spike sample aliquots. Both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days if possible.

Data for demonstrating the ruggedness of the new or modified method:

Sample type _____

Sample	Conc. "Batch or lot 1" Media and/or Reagents	Conc. "Batch or lot 2" Media and/or Reagents
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		

For shellfish samples, repeat for each tissue type of interest.

Data handling to demonstrate the ruggedness of the new or modified method
In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the new or modified method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

To determine whether the new or modified method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 greater than 2 indicates a lack of homogeneity of variance.

5. Use either the paired t-test or Welch's t-test for the statistical analysis based on the following considerations.
   - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the statistical analysis.
   - If the distributions of the data from batch/lot1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the statistical analysis.
   - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the statistical analysis.
   - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both; but, the data lacks homogeneity of variance, use Welch's t-test to statistically analyze the data.

Data summary for demonstrating the ruggedness of the new or modified method:

Value for the test of symmetry of the distribution of batch/lot 1 __-0.021_____

Value for the test of symmetry of the distribution of batch/lot 2 ____-0.039_____

Variance of batch/lot 1 data ___0.054_____

Variance of batch/lot 2 data ____0.055_____

Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 ___1.03____

Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples Y/N → **paired t-test results: t = -0.254, df = 9, p = 0.805**

To evaluate assay ruggedness under SOP #2 criteria, two distinct multi-well microplates were analyzed as Batch 1 and Batch 2 to test the inter-plate variability and consistency of saxitoxin dihydrochloride (µg STX diHCl equiv./100 g shellfish) detection in spiked mussel samples (n = 10). Assay values across three target spike concentrations (40, 80, and 160 (µg STX diHCl equiv./100 g shellfish) demonstrated strong inter-batch agreement, exhibiting a linear regression relationship of $y = 0.94x + 7.33$ and a correlation coefficient of $R^2 = 0.87$ between Batch 1 and Batch 2 (Figure 6, Table 3). This close alignment between distinct assay plates confirms that the receptor binding assay maintains reliable operational performance and robust analytical consistency despite normal inter-plate execution variables.

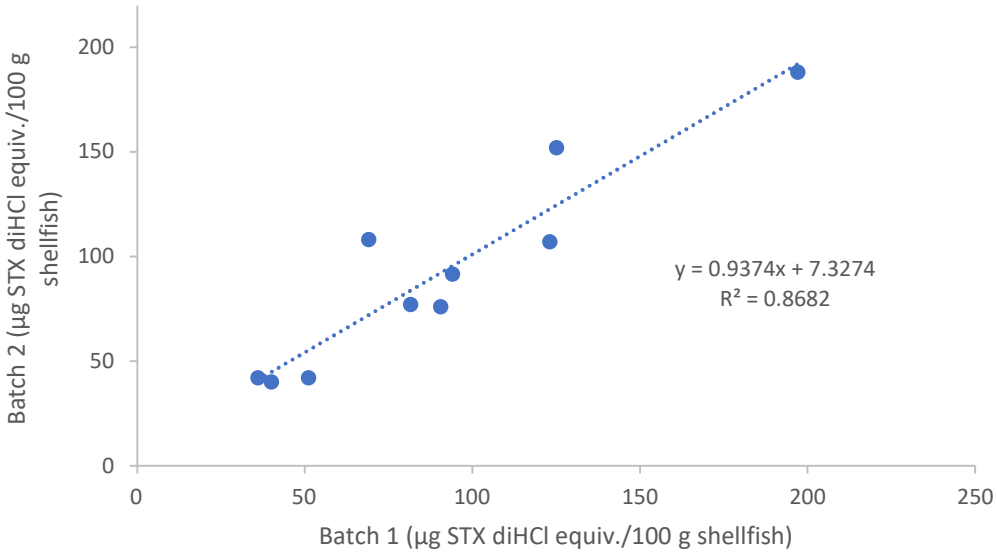


Figure 6. Scatterplot of comparing saxitoxin dihydrochloride (µg STX diHCl equiv./100 g shellfish) in spiked mussel samples measured across two batches (Batch 1 vs. Batch 2), showing the linear regression line ($y = 0.94x + 7.33$) and correlation coefficient ($R^2 = 0.87$).

Table 3. Comparison of target spike concentrations (µg STX diHCl equiv./ 100 g shellfish) and receptor binding assay values in spiked mussel samples across two assay batches (Batch 1 vs. Batch 2).

Sample	Expected Concentration (µg STX diHCl equiv./ 100 g shellfish)	Batch 1 (µg STX diHCl equiv./ 100 g shellfish)	Batch 2 (µg STX diHCl equiv./ 100 g shellfish)
1	40	40	40
2	40	51	42
3	40	36	42
4	80	90.5	76
5	80	81.5	77
6	80	69	108

7	80	94	91.5
8	160	197	188
9	160	123	107
10	160	125	152

IX. #3 SOP for the Single Laboratory Validation of New or Modified Analytical Methods Intended as Alternatives to Officially Recognized NSSP Methods – Specificity

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable, the new or modified method must be specific for the analyte/measurand/organism of interest. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Specificity of the new or modified method is the ability of this new or modified method to measure only what it is intended to measure. To determine the specificity of new or modified methods, samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/target organism of interest.

Procedure for demonstrating the specificity of the new or modified method: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method/modified method under study) of the target analyte/measurand/organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank, the aliquot spiked with the target analyte/measurand/organism of interest and the aliquot spiked with the target analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method/modified method concentration for the target analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one (1) sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

Data for demonstrating the specificity of the new or modified method:
Name of suspected interfering organism/compound/toxin #1 **domoic acid**

Sample type **blue mussel**

Sample blank concentration for the target analyte/measurand/organism of interest **NIST Saxitoxin Dihydrochloride (RM 8642a)**

	Conc. of aliquot spiked with target analyte/measurand/organism of interest interest	Conc. of aliquot spiked with target analyte/measurand/organism of of interest in the presence of suspected interfering organism/compound/toxin
Replicate 1		
2		
3		
4		
5		

Repeat for each suspected interfering organism/compound/toxin tested.

Data handling for demonstrating specificity of the new or modified method
The specificity index will be used to test the specificity of the new or modified method in the presence of suspected interfering organisms/compounds/toxins. The specificity index (SI) is calculated as indicated below:

$$\text{Specificity index (SI)} = \frac{\text{Sample spiked with only target of interest}}{\text{Sample spiked with target in presence if suspected interferences}}$$

All microbiological count data must be converted to logs before statistical analysis. Samples spiked with both the target analyte/measurand/organism of interest and the target analyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any correction that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index (SI) should equal one (1) in the absence of interferences. To test the significance of a Specificity index (SI) other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test at the .05 significance level is used. For each suspected interfering organism/compound/toxin calculate the average Specificity index (SIavg) for the five (5) replicates analyzed for each sample by obtaining the average concentration for both the aliquot containing the target analyte/measurand/organism of interest only and the aliquot containing the target analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

$$SI_{avg} = \frac{\text{Avg. conc. of sample spiked only with target of interest}}{\text{Avg. conc. of sample spiked with target in the presence of suspected interferences}}$$

Perform the t-test to determine if the average Specificity index (SI) obtained from the five (5) replicates from each analysis differs from one (1). Repeat for all the suspected interfering organisms/compounds/toxins tested.

Data summary for testing the specificity of the new or modified method:

Interfering organism/compound/toxin #1- domoic acid $SI_{avg} = 1.01$

SI different from 1 $p = 0.70$

Specificity was evaluated to determine whether the co-occurrence of domoic acid (DA), a non-target marine toxin, interferes with the quantitation of saxitoxin dihydrochloride (STX diHCl) in the RBA using porcine brain membrane preparation. Across five experimental replications comparing STX diHCl alone versus STX diHCl in combination with DA, the mean Specificity Index SI_{avg} was 1.01, showing no statistically significant deviation from the ideal value of 1.00 ($p > 0.05$, Table 4). Further validation in blue mussel samples spiked at 40 µg STX diHCl equiv./100 g shellfish demonstrated consistent toxin recovery regardless of matrix co-fortification with DA (0.02 µg DA equiv./g shellfish). Specifically, samples fortified with STX diHCl alone an average RBA concentration of 38.4 µg STX diHCl equiv./100 g shellfish, while samples co-fortified with both STX diHCl and DA yielded an equivalent average of 36.2 µg STX diHCl equiv./100 g shellfish (Table 5). The blank homogenate (MBk-00) produced zero detectable signal, confirming no baseline matrix interference. Together, these data demonstrate

high method specificity, proving that DA does not cross-react or cause analytical interference during PST quantitation.

Table 4. Specificity index (SI) values derived from log-transformed toxicity concentrations of saxitoxin dihydrochloride (STX diHCl) alone and in combination with domoic acid (DA) across five experimental replications.

Replication	log (STX diHCl)	log (STX diHCl & DA)	Specificity Index (SI)
1	1.60	1.60	1.00
2	1.68	1.59	1.06
3	1.62	1.46	1.11
4	1.45	1.60	0.90
5	1.53	1.52	1.01
Average	1.58	1.56	1.01

Table 5. Blue mussel homogenate samples collected from Case Inlet, Mason County, Washington, in May–June 2023 (spring season), and spiked with saxitoxin (STX diHCl) and/or domoic acid (DA) standards. Samples MBk-01 through MBk-05 were fortified with NIST Saxitoxin Dihydrochloride (RM 8642a) at 40 µg STX diHCl equiv./100 g shellfish, while samples MBk-06 through MBk-10 were fortified with both Biosense Laboratories AS domoic acid standards (#A31300401-06) at 0.02 µg DA equiv./g shellfish and NIST Saxitoxin Dihydrochloride (RM 8462a) at 40 µg STX diHCl equiv./100 g shellfish. MBk-00 was not spiked with either toxin and served as a blank to correct for any matrix effects. The table reports the final concentration of ug STX diHCl equiv/100 g shellfish using RBA.

Sample ID	STX diHCl Spike (µg STX diHCl equiv./ 100 g shellfish)	DA Spike (µg DA equiv./ g shellfish)	RBA (µg STX diHCl equiv./ 100 g shellfish)
MBk-00	0	0	0
MBk-01	40	0	40
MBk-02	40	0	48
MBk-03	40	0	42
MBk-04	40	0	28
MBk-05	40	0	34
MBk-06	40	0.02	40
MBk-07	40	0.02	39
MBk-08	40	0.02	29
MBk-09	40	0.02	40
MBk-10	40	0.02	33

Standard Operating Procedure (SOP)
Appendix I

Method Modification: Step-by-Step Procedure

A. Sample Homogenate
    a. Each sample that was below the detection limit from the MBA (~40 µg STX/100g of shellfish) underwent mini saxitoxin extraction to be pre-screened by the RBA to detect true negative samples (<10µg/100g).
        i. Mini saxitoxin extraction was performed using the same protocol as Appendix A (Section A. Sample Extraction), by performing a 1:1 ratio of 0.5 g of tissue and 0.5 mL of 0.1 N HCl.
    b. Once the negative samples were identified, samples were combined to produce 30 sub-aliquots (~130 g of tissue) needed for spiking five homogenates for each shellfish matrix.
        1. We doubled the number of tissue samples for this study to maintain a backup supply, which safeguards against potential future issues such as lost, broken, or spilled samples.
        ii. To combine samples for one homogenate, it followed these criteria:
            1. The sample collection dates must be within a 30-day range and occur within the same season.
                a. Due to the limited availability of cockle tissue samples, collection dates must be within a 90-day window and fall within the same season.
            2. The samples must be harvested from the same water system.
        iii. Homogenates were distinct, reflecting differences based on the season of collection, harvest area, or year.
    c. The positive samples that have MBA data (>40 µg STX/100g of shellfish) were distinct, reflecting differences based on the season of collection, harvest area, or year.
    d. All homogenates were blended by a blender and/or a homogenizer to liquify the homogenate for spikes.
    e. Once the homogenate is liquified, the designated amount of tissue is aliquoted into 50 mL falcon tubes for each spike level:
        i. 40 µg STX/100g of shellfish: 12 ± 0.3 grams of tissue
        ii. 80 µg STX/100g of shellfish: 12 ± 0.3 grams of tissue
        iii. 160 µg STX/100g of shellfish: 12 ± 0.3 grams of tissue
B. Spike Procedure
    a. Every homogenate was spiked with RM 8642a FDA Saxitoxin Dihydrochloride Solution (103 µg/g ± 4 µg/g)
    b. Prior to spiking the pre-weighed tissue, the laboratory technician gravimetrically verified the dispensed volume of water to ensure the spiking accuracy was within 3% of the designated volume.
    c. The volume of the RM 8642a FDA saxitoxin dihydrochloride solution was calculated based on the desired concentration and tissue weight available.
        i. 40 µg STX/100g of shellfish: 47 µL

ii. 80 µg STX/100g of shellfish: 93 µL
iii. 160 µg STX/100g of shellfish: 186 µL

d. Once spiked, each aliquoted homogenate was vortexed for approximately 2 minutes to ensure complete mixture of the spike and tissue.
e. Following the mixing of the sample, each sample was again aliquoted into 5 ± 0.3 grams, thereby achieving a minimum of three replicates for each spike level.
    i. Blank samples were not spiked and aliquoted into 5 ± 0.3 grams.
f. At least 16 samples for each homogenate (5) were frozen at -20°C (total of 80 aliquots).
    i. In addition,10 more homogenates from different seasons, harvest areas, and years were spiked to a concentration of 80 µg STX/100g of shellfish and aliquoted into 5 ± 0.3 grams (20 aliquots).
    ii. 20 naturally incurred samples were aliquoted into 5 ± 0.3 grams.
g. All samples, except for backups, were shipped to Washington State Department of Health for RBA using the protocol listed in Appendix A.

C. Spike Procedure for Interfering Toxin
a. Domoic acid and STX diHCl spike
    i. Once the homogenate is liquified, the designated amount of tissue is aliquoted into 50 mL falcon tubes with the weight of 28.8 grams.
    ii. Using the domoic acid standard at 0.02 ppm, 7.2 mL of the domic acid standard was added to the tissue sample.
    iii. The addition of 7.2 mL of domoic acid standard resulted in a total sample mass/volume basis of approximately 36 grams and provided the target domoic acid concentration of 0.02 ug/g.
    iv. 140 uL of the STX diHCl standard was added to the sample.
    v. The sample was mixed thoroughly.
    vi. The final sample contained 0.02 ug/g domoic acid and 40 ug STX diHCl/100g shellfish.
b. STX diHCl Only Spike
    i. Once the homogenate is liquified, the designated amount of tissue is aliquoted into 50 mL falcon tubes with the weight of 28.8 grams.
    ii. 7.2 mL of 10% acetonitrile in water was added to the sample in place of the domoic acid standard.
    iii. The sample was mixed thoroughly.
    iv. 140 uL of the STXdiHCl standard was added to the sample.
    v. The sample was mixed thoroughly to ensure uniform distribution of the spike.
    vi. The resulting sample contained a total of 36 grams and is targeted to contain 40 ug STX diHCl/100 g shellfish.
c. Following the mixing of the sample, each sample was again aliquoted into 5 ± 0.3 grams, thereby achieving a minimum of five replicates.
    i. Blank samples contained 7.2 mL of 10% acetonitrile in water and aliquoted into 5 ± 0.3 grams.

Appendix A: RBA Step-by-Step Procedure

A. Sample Extraction
    a. The extraction detailed below represents a small scale MBA procedure. The actual MBA extraction could be used instead of the small scale version described here.
    b. Accurately weigh 5.0 g of tissue homogenate into a tared, labeled 15 ml conical tube.
    c. Add 5.0 ml of 0.1 M HCl, vortex, and check pH.
        i. If necessary, adjust pH to 3.0-4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing.
    d. Place the tube in a beaker of boiling water on hot plate (or in a water bath) for 5 min with the caps loosened.
    e. Remove and cool to room temperature.
    f. Check pH and, if necessary, adjust cooled mixture to 3.0-4.0 as described above.
    g. Transfer entire contents to a labeled, graduated centrifuge tube and dilute volumetrically to 10 ml.
    h. Gently stir contents to homogeneity and then allow to settle until a portion of supernatant is translucent and can be decanted free of solids.
    i. Pour 5-7 ml of the translucent supernatant into a labeled centrifuge tube.
    j. Centrifuge at 3000 x g for 10 min.
    k. Retain clarified supernatant and transfer to a clean, labeled centrifuge tube.
    l. Store extracts at -20 °C until tested in RBA.

B. Preparation of Stock Solutions and Standards
    a. Assay buffer: 100 mM MOPS/100 mM choline chloride, pH 7.4
        i. Weigh 20.9 g MOPS and 13.96 g choline chloride and add to 900 ml distilled or milli-Q water.
        ii. Adjust pH to 7.4 with NaOH while stirring.
        iii. Bring to a final volume of 1 L with distilled or milli-Q water.
        iv. Store at 4 °C.
    b. Radioligand solution: 3H-STX
        i. Calculate the concentration of 3H-STX stock provided by the supplier. Suppliers generally provide specific activity in Ci/mmol (~10-30 Ci/mmol) and activity in mCi/ml (~0.05-0.1 mCi/ml), from which the molar concentration can be calculated.
        ii. Prepare 4 ml of a 15 nM working stock of 3H-STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate.
        iii. Measure total counts of each working stock prior to running an assay. Add 36 µl of working stock 3H-STX in buffer to a liquid scintillation counter vial with 4 ml scintillant and count on a traditional liquid scintillation counter to confirm correct dilution. The CPM should be consistent and within 15% of expected value.

c. Unlabeled STX standard working solution: The STX diHCl standard (NIST RM 8642 STX diHCl) is provided at a concentration of 268.8 µM (100 µg/ml).
    i. A bulk standard curve can be made up in advance and stored at 4 °C for up to one month. The use of a bulk standard curve minimizes time needed for routine analyses and improves repeatability.
    ii. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µl in 50 ml) and use for the serial dilutions.
    iii. Serial dilutions should result in the following stock concentrations (M):
        1. 6 x 10-6 [100 µl 268.8 µM STX + 4.38 ml 0.003 M HCl]
        2. 6 x 10-7 [500 µl 6 x 10-6 M STX + 4.5 ml 0.003 M HCl]
        3. 1.8 x 10-7 [1.5 ml 6 x 10-7 M STX + 3.5 ml 0.003 M HCl]
        4. 6 x 10-8 [500 µl 6 x 10-7 M STX + 4.5 ml 0.003 M HCl]
        5. 1.8 x 10-8 [500 µl 1.8 x 10-7 M STX + 4.5 ml 0.003 M HCl]
        6. 6 x 10-9 [500 µl 6 x 10-8 M STX + 4.5 ml 0.003 M HCl]
        7. 6 x 10-10 [500 µl 6 x 10-9 M STX + 4.5 ml 0.003 M HCl]
        8. 5 ml 0.003 M HCl.

d. Interassay calibration standard (QC check): Reference standard STX (1.8 x 10-8 M STX) in 3 mM HCl. For long-term storage keep at -80 °C; for routine use (up to one month), store at 4 °C.

e. Porcine brain membrane preparation: Store bulk rat brain membrane preparations at -80 °C.
    i. Thaw an aliquot of porcine brain preparation on ice.
    ii. Dilute membrane preparation with cold (4 °C) 100 mM MOPS/100 mM choline chloride, pH 7.4 to yield a working stock with a protein concentration of 1.0 mg/ml.
    iii. Vortex vigorously to achieve a visibly homogeneous suspension.
    iv. Keep the diluted membrane preparation on ice.

C. Performing the Assay

a. Plate setup: When possible use a multichannel pipet to minimize effort and increase consistency.
    i. Run standards, samples, and QC check in triplicate.
    ii. For quantitation, multiple dilutions per extract should be analyzed in order to obtain a value that falls within the dynamic range of the assay. A minimum sample extract dilution of 1:10 is recommended to minimize potential matrix effects.
    iii. Use of a standard plate layout (Figure 1) is recommended. This will improve ease of analysis and can help maximize the number of samples/standards that can be analyzed per plate.

b. Addition of samples/standards: Add in the following order to each well
    i. 35 µl assay buffer
    ii. 35 µl STX standard/QC check/sample extract
    iii. 35 µl 3H-STX
    iv. 105 µl membrane preparation (ensure solution is homogeneous)
    v. Cover the plate and incubate at 4 °C for 1 h.

c. Assay filtration: Use the vacuum manifold attached to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process.

i. Set the vacuum pressure gauge on the pump or manifold to ~4-8" Hg (~135-270 millibar).
ii. Place the 96-well plate on the vacuum manifold.
iii. Fill any empty wells with 200 µl MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate.
iv. Turn on vacuum. Optimum vacuum will pull the wells dry in 2-5 s.
v. With vacuum pump running, quickly rinse each well twice with 200 µl ice cold MOPS/choline chloride buffer using a multichannel pipet. Maintain vacuum until liquid is removed.

d. Preparation of the assay for counting: Remove the plastic bottom from the plate and blot the plate bottom once on absorbent towel.
   i. For counting in microplate scintillation counter:
      1. Seal the bottom of a counting cassette with sealing tape.
      2. Place the microplate in the counting cassette.
      3. Add 50 µl scintillation cocktail per well using multichannel pipet.
      4. Seal the top of the plate with sealing tape.
      5. Incubate for 30 min at room temperature.
      6. Place the plate in the scintillation counter and count for 1 min per well.
   ii. For counting in traditional scintillation counter:
      1. Place the microplate in the MultiScreen punch system apparatus and place the disposable punch tips on top of the microplate.
      2. Punch the filters from the wells into scintillation vials and fill with 4 ml scintillation cocktail.
      3. Place caps on the vials and vortex.
      4. Allow vials to sit overnight in the dark.
      5. Count using a tritium window in a traditional scintillation counter.

D. Analysis of Data
   a. Curve fitting: Perform curve fitting using a four-parameter logistic fit (sigmoidal dose response curve with variable slope).
      i. $y = \text{min} + (\text{max-min})/1+10^{(\text{x-log } IC_{50})\text{Hill slope}}$
      ii. where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX (also known as B_0); min is the bottom plateau, equal to nonspecific binding in CPM in the presence of saturating nonradiolabeled STX; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is the total igand binding in CPM (B/B_0).
   b. Sample quantification: Sample quantification is only carried out on dilutions having a B/B_0 in the range of 0.2-0.7.
      i. Where B represents the bound 3H-STX in CPM in the sample and B_0 represents the max bound 3H-STX in the sample.

ii. Sample concentration is calculated in µg STX diHCl equivalents (eq)/kg shellfish as described below:

(nM STX eq) x (sample dilution) x [(210 µl total volume)/35 µl sample]
= nM STX eq in extract

(nM STX diHCl eq in extract) x (1 L/1000 ml) x (372 ng/nmol) X (1 µg/1000 ng)
= g STX diHCl eq/ml

µg STX diCHl eq/ml x (ml extract/g shellfish) x (1000g/kg)
= µg STX diHCl eq/kg

Figure 1. Example plate layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U4 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

Concentrations indicate those of the STX standard curve; REF = reference; QC = quality control; U = unknown sample (with dilutions indicated). The same standard curve made be used for additional plates run on the same day using the same reagents (i.e., 11 samples can be run on subsequent plates).

Appendix B: Radiation Safety Requirements

A. All users must follow all local, state, and federal laws and procedures regarding receipt, use and disposal of isotopes.
B. All users must be trained in and follow all in-house safety procedures for working with radiolabeled materials.
C. All isotopes and work stations where isotopes are used should be controlled access areas. Any one with access to the area must also receive radiation safety training.
D. Freezers where the isotopes are stored must be locked.
E. Personal protective equipment must include lab coats (designated specifically for use with radioactive materials), safety glasses, and gloves.
F. Radioactive materials will only be handled and manipulated in designated areas, which have been clearly identified and labeled accordingly.
G. Work with source radiation material must be conducted in a fume hood.
H. Radioactive materials will be stored and/or carried in secondary containment.
I. When possible, disposable supplies such as pipet tips, absorbent paper, and kim wipes will be used so that contaminated supplies can be readily disposed of as radioactive waste.
J. Wipe surveys will be conducted at the end of each experiment as well as monthly to ensure that there is no contamination in the laboratory.
K. The filter plates used in the assay will be designated as solid radioactive waste, while the washes from the filter plates (containing buffer and unbound 3H-STX) will be handled as liquid radioactive waste. There will be a dry active waste container to hold contaminated items such as the plates, gloves, absorbent paper and kim wipes. There will be a liquid waste jug to hold the contaminated liquid radioactive waste.
L. All wastes must be disposed of according to state and local laws.

Appendix C. Abbreviations and Acronyms

3H-STX	Tritiated saxitoxin
AOAC	Association of Analytical Communities
ARC	American Radiolabeled Chemicals
B	Bound CPM
B_0	Maximum bound CPM
CFSAN	Center for Food Safety & Applied Nutrition
CPM	Counts per minute
diHCl	Dihydrochloride
Eq	Equivalents
HCl	Hydrochloric acid
IC_{50}	Inhibitory concentration at which CPMs are at 50% max
LC-FD	Liquid chromatography with fluorescence detection
LOD	Limit of detection
LOQ	Limit of quantitation
MBA	Mouse bioassay
MOPS	3-Morpholinopropanesulfonic acid
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NSSP	National Shellfish Sanitation Program
OMA	Official method of analysis
PMSF	Phenyl methylsulfonyl fluoride
PCOX	Post-column oxidation liquid chromatography with fluorescence detection
Pre-COX	Pre-column oxidation liquid chromatography with fluorescence detection
PSP	Paralytic shellfish poisoning
PSTs	Paralytic shellfish toxins
QA	Quality assurance
QC	Quality control
QS	Quality System
RBA	Receptor binding assay
RSD	Relative standard deviation
SLV	Single laboratory validation
STX	Saxitoxin

Appendix II: Van Dolah et al. 2009

FOOD CHEMICAL CONTAMINANTS

Appendix II

Single-Laboratory Validation of the Microplate Receptor Binding Assay for Paralytic Shellfish Toxins in Shellfish

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A single-laboratory validation (SLV) study was conducted for the microplate receptor binding assay (RBA) for paralytic shellfish poisoning (PSP) toxins in shellfish. The basis of the assay is the competition between [^{3}H]saxitoxin (STX) and STX in a standard or sample for binding to the voltage dependent sodium channel. A calibration curve is generated by the addition of 0.01–1000 nM STX, which results in the concentration dependent decrease in [^{3}H]STX-receptor complexes formed and serves to quantify STX in unknown samples. This study established the LOQ, linearity, recovery, accuracy, and precision of the assay for determining PSP toxicity in shellfish extracts, as performed by a single analyst on multiple days. The standard curve obtained on 5 independent days resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%) and a dynamic range of 1.2–10.0 nM. The LOQ was 5.3 μg STX equivalents/100 g shellfish. Linearity, established by quantification of three levels of purified STX (1.5, 3, and 6 nM), yielded an r^2 of 0.97. Recovery from mussels spiked with three levels (40, 80, and 120 μg STX/100 g) averaged 121%. Repeatability (RSD_r), determined on six naturally contaminated shellfish samples on 5 independent days, was 17.7%. A method comparison with the AOAC mouse bioassay yielded r^2 = 0.98 (slope = 1.29) in the SLV study. The effects of the extraction method on RBA-based toxicity values were assessed on shellfish extracted for PSP toxins using the AOAC mouse bioassay method (0.1 M HCl) compared to that for the precolumn oxidation HPLC method (0.1% acetic acid). The two extraction methods showed linear correlation (r^2 = 0.99), with the HCl extraction method yielding slightly higher toxicity values (slope = 1.23). A similar relationship was observed between HPLC quantification of the HCl- and acetic acid-extracted samples (r^2 = 0.98, slope 1.19). The RBA also had excellent linear correlation with HPLC analyses (r^2 = 0.98 for HCl, r^2 = 0.99 for acetic acid), but gave somewhat higher values than HPLC using either extraction method (slope = 1.39 for HCl extracts, slope = 1.32 for acetic acid). Overall, the excellent linear correlations with the both mouse bioassay and HPLC method and sufficient interassay repeatability suggest that the RBA can be effective as a high throughput screen for estimating PSP toxicity in shellfish.

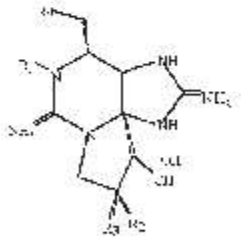
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Paralytic shellfish poisoning (PSP) is a seafood intoxication caused by the consumption of shellfish tainted with saxitoxins (STXs) produced by certain species of harmful algae. Saxitoxins are a suite of heterocyclic guanidinium toxins, of which currently more than 21 congeners are known (Figure 1). These congeners occur in varying proportions in the dinoflagellates that produce them and are further metabolized in shellfish that accumulate them, making analytical determination of PSP toxins in shellfish complex. The long-standing regulatory method for PSP toxins is the AOAC mouse bioassay (1), with a regulatory limit of 80 μg/100 g shellfish generally applied. Increasing resistance to whole animal testing has driven the need to develop alternative methods suitable for use in a high throughput monitoring or regulatory setting. In the past decade, several alternatives to the mouse bioassay have been developed and validated to various degrees. The precolumn oxidation HPLC method (2) has received First Action approval by AOAC as an Official Method for PSP (**2005.06**; 3) and has been accepted into the European Food Hygiene Regulations as an alternative to the mouse bioassay and further refined to optimize its use in the United Kingdom Official Control monitoring of PSP toxins in mussels (4). However, although the HPLC method performs well quantitatively, it is quite time consuming for high throughput screening needed by many monitoring programs. A qualitative lateral flow antibody test for PSP toxins with a detection limit of 40 μg/100 g, developed by

Jellett Rapid Testing Ltd (Chester Basin, NS, Canada), has been approved in the United States by the Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration (FDA) as a screening method. This method performed well in a comparison study with the mouse bioassay, with a false-positive rate of 6% and a false-negative rate of <0.1% (5), but it has not been put through a full AOAC collaborative trial, and does not provide quantitative analysis. To date, a suitable quantitative, high throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The current study establishes the single laboratory performance characteristics of the microplate receptor binding assay (RBA) for PSP toxins in shellfish and identifies it as a candidate for fulfilling the requirements of high throughput, quantitative analysis that measures a composite toxic potency in a manner analogous to the mouse bioassay.

STX elicit their paralytic effects by binding to site 1 on the voltage dependent sodium channel, thereby blocking the transmission of neuronal and muscular action potentials. Because all STX congeners bind to site 1 with affinities proportional to their mouse intraperitoneal (IP) toxicity (6), a receptor binding competition assay can be used to measure the integrated toxic potency of STX congeners in a sample, independent of which toxin congeners are present. Moreover, any toxin metabolites originating in the shellfish matrix will also be detected by the assay according to their affinity for the sodium channel receptor. In this binding competition assay, [^{3}H]STX competes with unlabeled STX and/or its derivatives for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H]STX is removed by filtration and bound [^{3}H]STX is quantified by liquid scintillation counting. The percent reduction in [^{3}H]STX binding in the presence of unlabeled toxin is directly proportional to the amount of unlabeled toxin present. A standard curve is established using increasing concentrations of unlabeled STX, and the concentration of PSP toxins in an unknown sample is quantified using this standard curve.

The assay tested in this single laboratory trial is a modification of the method of Doucette et al. (7) to a 96-well microplate format described by Van Dolah et al. (8). Application of microplate scintillation counting to the PSP assay was first reported by Powell and Doucette (9), who applied it to phytoplankton analysis. The use of the microplate format, in conjunction with microplate scintillation counting, makes the assay suitable for use in a high throughput monitoring or regulatory setting. Several versions of the PSP receptor binding assay have undergone method comparisons in different laboratories with favorable correlations to the mouse bioassay and/or other assays for PSP toxins in shellfish. Suarez-Isla and Valez (10) showed excellent linear correlation ($r^2 = 0.97$) between the RBA and mouse bioassay of 41 shellfish extracts between 40 and 10 000 µg STX equivalents/100 g. Llewellyn et al. (11) found that the sodium channel receptor assay compared well to three other methods of analysis for PSP toxins in shellfish (HPLC, mouse bioassay, and N2A cytotoxicity assay). Ruberu et al. (12) optimized the microplate format assay for use in the Packard Top Count microplate scintillation counter (a single channel counter; GMI, Inc., Ramsey, MN), compared results with the same assay performed on the Wallac microplate counter (a two-channel coincidence counter; Perkin Elmer Wallace, Gaithersburg, MD), and provided further correlation data with



		R1	R2	R3	R4	MU/µmole
Carbamate	STX	H	H	H	$OCONH_2$	2182
	Neo STX	OH	H	H	$OCONH_2$	2285
	GTX1	OH	OSO_3^-	H	$OCONH_2$	2766
	GTX2	H	OSO_3^-	H	$OCONH_2$	792
	GTX3	H	H	OSO_3^-	$OCONH_2$	1684
	GTX4	OH	H	OSO_3^-	$OCONH_2$	1938
N-sulfocarbamoyl	GTX5 (B1)	H	H	H	$OCONHSO_3^-$	180
	GTX6 (B2)	OH	H	H	$OCONHSO_3^-$	-
	C1	H	OSO_3^-	H	$OCONHSO_3^-$	16
	C2	H	H	OSO_3^-	$OCONHSO_3^-$	225
	C3	OH	OSO_3^-	H	$OCONHSO_3^-$	33
	C4	OH	H	OSO_3^-	$OCONHSO_3^-$	143
Decarbamoyl	dcSTX	H	H	H	OH	1274
	dcNeoSTX	OH	H	H	OH	
	dcGTX1	OH	OSO_3^-	H	OH	-
	dcGTX2	H	OSO_3^-	H	OH	16.7
	dcGTX3	H	H	OSO_3^-	OH	18.2
	dcGTX4	OH	H	OSO_3^-	OH	
Deoxydecarbamoyl	doSTX	H	H	H	H	-
	doGTX2	H	H	OSO_3^-	H	
	doGTX3	H	OSO_3^-	H	H	

Figure 1. Structures and toxic potency of 21 saxitoxin congeners. Toxic potency is listed as mouse units (MU)/µmole, where a mouse unit is defined as the minimum amount required to kill a 20 g mouse in 15 min when administered by IP injection. The table is modified from ref. 15.

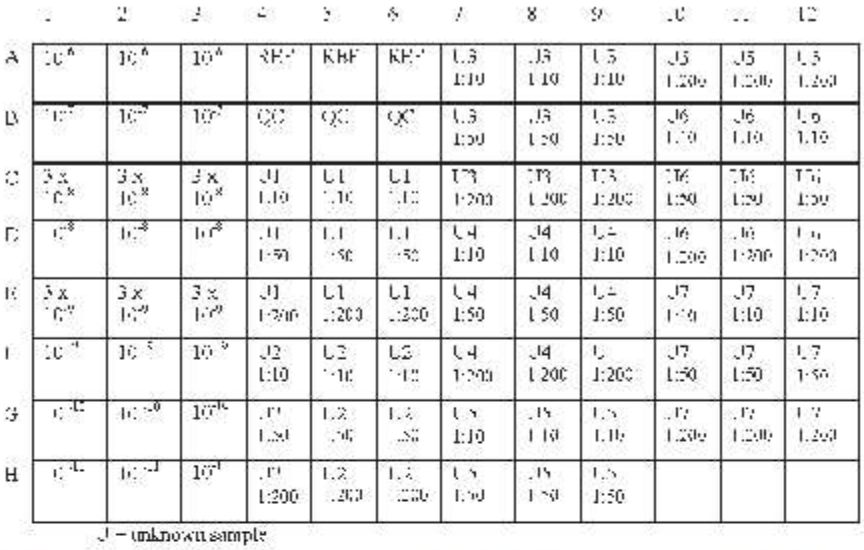


Figure 2. Standardized plate layout recommended for the microplate RBA for PSP toxins in shellfish extracts. U = unknown sample.

the mouse bioassay. Usup et al. (13) utilized the microplate RBA method to compare predicted toxicity values in samples spiked with different STX congeners as assayed by the mouse bioassay and the RBA. Llewellyn (14) defined the competitive behavior of PSP toxin mixtures in receptor binding assays, using both the sodium channel and saxiphilin receptors, which explains their composite toxicity. However, none of these previous studies fully characterized assay performance according to AOAC single-laboratory validation (SLV) criteria that are the underpinning required for proceeding with an AOAC collaborative trial. Therefore, the current study was carried out to fulfill those requirements.

Experimental

Apparatus

(a) *Microplate scintillation counter.*—Wallac Microbeta, GMI Inc. (Ramsey, MN).

(b) *Microplate filtration manifold.*—Millipore (Bedford, MA).

(c) *Hot plate.*—Fisher Scientific (Suwannee, GA).

(d) *Countertop centrifuge.*—For 15 mL tubes, capable of 3000 × *g* (Fisher Scientific).

(e) *Microtiter filter plates (96 well) with 1.0 µm pore size type FB glass fiber filter/0.65 µm pore size Durapore support membrane.*—Cat. No. MSFB N6B 50 (Millipore Corp., Billerica, MA).

(f) *Microplate sealing tape.*—Cat. No. MATA HCL00 (Millipore Corp.).

(g) *Vortex mixer.*—Daigger Vortex Genie II (Daigger Scientific, Vernon Hills, IL).

(h) *Teflon/glass tissue homogenizer.*—Wheaton (Millville, NJ).

(i) *Polytron homogenizer.*—Brinkmann Instruments (Westbury, NY).

Reagents

(a) *Hydrochloric acid (HCl).*—0.1 M.

(b) *[^{3}H]STX.*—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (International Isotopes Clearinghouse, Leawood, KS).

(c) *STX diHCl.*—FDA reference standard (Office of Seafood, Laurel, MD) or National Research Council (NRC) of Canada Institute of Marine Biosciences (Halifax, NS, Canada).

(d) *Assay buffer.*—75 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Cat. No. H9136]/140 mM NaCl, pH 7.5 (Sigma, St. Louis, MO).

(e) *Liquid scintillation cocktail.*—Optiphase (PerkinElmer Life Sciences, Downers Grove, IL).

Preparation of Samples (0.1 M HCl Extraction)

Shellfish samples were shucked and homogenized according to the AOAC mouse bioassay protocol (1). For the HCl extraction method, 5.0 (±0.1) g of tissue homogenate was transferred to a tared 15 mL conical polypropylene centrifuge tube. A 5.0 mL volume of 0.1 M HCl was added, and the sample was mixed on a Vortex mixer. The pH was checked to

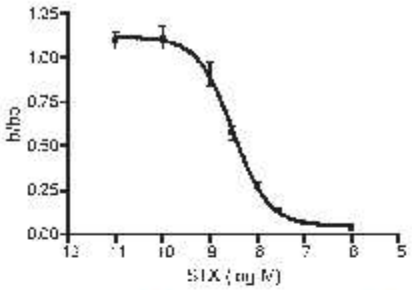


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

confirm it was between 3.0 and 4.0 in order to avoid alkalinization and destruction of the toxin, and adjusted with 1 M HCl or 0.1 M NaOH as needed. Tubes were placed in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Following removal from the boiling water bath, samples were allowed to cool to room temperature, and the pH was again confirmed to be between 3.0 and 4.0. The entire contents were then transferred to a graduated cylinder, diluted volumetrically to 10 mL, and centrifuged for 5 min at 1000 × *g*. The supernatant was transferred to a clean tube.

Preparation of Samples (Acetic Acid Extraction Method)

In a 50 mL plastic centrifuge tube, 5.0 ± 0.1 g homogenate was mixed with 3.0 mL 1% acetic acid on a vortex mixer. Tubes were capped loosely to avoid pressure buildup and placed in a boiling water bath for 5 min. Following removal from the water bath, samples were cooled in a beaker of cold water for 5 min, and then centrifuged for 10 min at 3000 × *g*. The supernatant was transferred to a 15 mL graduated conical test tube. A 3 mL amount of 1% acetic acid was added to the original tube with solid residue, mixed well on a vortex mixer, and centrifuged again for 10 min at 3000 × *g*. The second supernatant was combined with the first and diluted to 10 mL with water.

Preparation of Stock Solutions, Standards, and Reagents for Assay

(a) *Radioligand solution.*—[^{3}H]STX stock is provided in 50 µCi ampules, 24 Ci/mmol, 0.1 mCi/mL (4.17 µM). A 15 nM working stock of [^{3}H] STX was prepared fresh daily in 75 mM HEPES/140 mM NaCl (for 2.5 nM final in-well concentration).

(b) *STX standard curve.*—FDA STX dihydrochloride reference standard (100 µg/mL or 268.8 µM) used to prepare a bulk standard curve made up in advance and stored at 4°C for up to 1 month. The stock standard curve was made consisted of eight concentrations of STX in 0.003 M HCl [6×10^{-6}, 6×10^{-7}, 1.8×10^{-7}, 6×10^{-8}, 1.8×10^{-8}, 6×10^{-9}, 6×10^{-10}, 6×10^{-11}, and 0.003 M only HCl (reference)], which when diluted 1:6 in the assay, resulted in a standard curve of 0.01 nM–1000 nM STX. The reference provided a measure of total [^{3}H]STX binding in the absence of unlabeled STX.

(c) *Calibration standard (QC check).*—A reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-9} M STX in assay) was prepared in 0.003 M hydrochloric acid, aliquotted in 1 mL volumes, and stored at 4°C for routine use (stable up to 1 month). On the day of the assay, 200 µL of each standard were pipetted into mini-dilution tubes for ease of pipetting into the microplate using an eight-channel pipettor.

(d) *Rat brain membrane homogenate.*—Cerebral cortices from 6-week-old male Holzman rats (Harlan Bioproducts, Indianapolis, IN) were homogenized on ice in a glass/Teflon tissue homogenizer in 75 mM HEPES/140 mM NaCl, pH 7.5, containing 0.1 mM PMSF (phenylmethanesulfonylfluoride;12.5 mL/brain) at 385 rpm for 10 strokes. Pooled homogenates were centrifuged at 20 000 × *g* for 15 min at 4°C and the pellet was resuspended in HEPES buffer (12.5 mL/brain) and rehomogenized on ice using a Polytron homogenizer set at 70% power for 20 s to ensure a fine suspension. The brain homogenate was aliquotted 2 mL/tube in cryovials and stored at –80°C. The protein concentration of the brain homogenate was determined using the Micro bicinchoninic acid (BCA) Assay (Pierce, Rockford, IL). For each assay, an aliquot of brain homogenate was thawed on ice and diluted with ice cold 75 nM HEPES/150 mM NaCl, pH 7.5, to yield a final protein concentration of 0.5 mg/mL in the assay.

Table 1. RBA measurements of calibration standards for assay linearity assessment (nM STX; *n* = 5)

Nominal	Mean	SD	RSD
1.5	1.7	0.16	10
3.0	3.0	0.52	17
6.0	6.0	0.34	6

Table 2. Recovery of analyte from spiked samples (µg STX equiv./100 g)

Nominal	Mean	SD	Measured RSD,	Recovery, %
0	<dl*			
40	47	8.6	18.7	115
80	103.7	21.8	21	129
120	145.5	15.2	10.5	121

* <dl = Less than LOQ (5 µg STX equiv./100 g).

Table 3. Comparison of receptor binding assay (RBA; $n = 5$) with AOAC mouse bioassay (MBA) of naturally contaminated shellfish (μg STX equiv./100 g)

Sample	MBA	RBA mean	SD	RSD
LP1	340	438	74	17
LP2	534	715	96	13
LP3	1158	1533	329	21
LP4	65	91	7	9
LP5	350	608	150	25
LP6	462	518	114	22

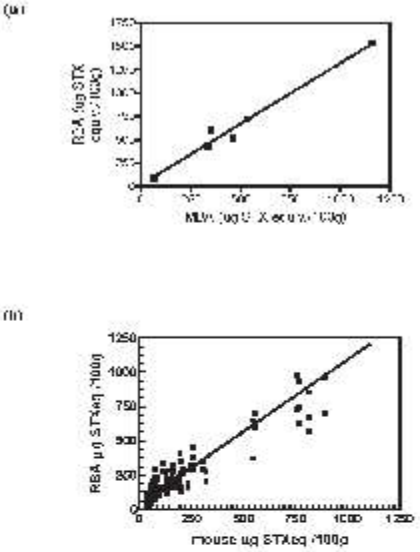


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days ($r^2 = 0.98$, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with a slope of 1.32.

Assay Procedure

(a) *Plate setup and incubation.*—A standardized plate layout was used for all assays (Figure 2). All standards, reference, QC check, and shellfish extracts were run in triplicate wells. For shellfish extracts, a standardized dilution series was run for each sample (1:10, 1:50, and 1:200), which ensured that at least one dilution would fall on the linear part of the competition curve for shellfish that contains between approximately 5 and 1500 μg STX equiv./100 g. Reagents were added in the following order: 35 μL STX standard or sample, then 35 μL [^{3}H]STX, followed by 140 μL brain homogenate. The addition of brain homogenate was carried out with sufficient force to ensure mixing of the well contents, but without risk of splashing. The plate was then covered and incubated at 4°C for 1 h.

(b) *Assay filtration and counting.*—The plate was filtered using a microplate vacuum filtration manifold, and each well rinsed twice with 200 μL ice-cold HEPES buffer at a filtration rate that ensured all wells were dry within 2–5 s. The microplate was then placed in a microplate scintillation counter cassette, and the bottom was sealed with plate sealing tape. Lastly, 50 μL scintillation cocktail was added to each well, and the top of the plate was sealed with sealing tape. The plate was allowed to sit for 30 min to ensure impregnation of the filters with scintillant prior to counting for 1 min/well in the microplate scintillation counter.

Data Analysis

Curve fitting was performed using a four-parameter logistic curve fitting model for a one-site receptor binding using Wallac Multicalc software. The software reports the in-well sample concentration in nM equiv. STX. Sample concentration was then calculated in μg STX equialents/100 g shellfish using the following formulas:

$$(\text{nM equiv. STX}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM equiv. STX in extract}$$

$$(\text{nm equiv. STX in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX equiv./mL}$$

$$\mu\text{g STX equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish extracted}} \times 100 = \mu\text{g STX equiv./100 g shellfish}$$

Critical Control Points

(*1*) For a ligand that interacts specifically at one receptor site, the slope of the resulting competition curve should theoretically be 1.0. If the slope of the curve for a given assay is outside of the acceptable range of 0.8–1.2, linearity of the assay will be compromised, and quantification of the unknowns will be incorrect. Therefore, the assay should be re-run.

(*2*) The QC check standard should fall within ±30% of the stated value (3.0 nM). If the QC check standard does not fall within acceptable limits, the assay should be re-run.

Table 4. RBA-determined toxicities of nine naturally contaminated shellfish homogenates extracted using the 0.1 M HCl extraction method or the 1% acetic acid extraction method (μg STX equiv./100 g)

	HCl			Acetic acid		
Sample	Mean	SD	RSD	Mean	SD	RSD
1	11	4	36	19	7	39
2	600	143	24	488	104	21
3	690	142	21	584	167	29
4	136	8	6	131	41	31
5	152	27	18	167	21	13
6	302	87	29	270	72	27
7	340	88	26	264	63	24
8	262	79	30	252	48	19
9	63	26	41	54	19	34

(*3*) Sample quantification should be done only on dilutions that on the linear part of the curve [b/b_o = 0.2–0.7, where B is the bound counts/min (CPM) in the sample and B_o is the maximum CPM)]. The RSD of the CPM must be <30%.

(*4*) For a given sample, if none of the sample dilutions falls within the linear range (i.e., the concentration is too high, $b/b_o < 0.2$), further dilutions must be made and the sample reanalyzed if a quantitative value is desired. If the sample concentration is too low to be quantified (i.e., $b/b_o > 0.7$) at sample dilution 1:10, the sample must be reported as below the LOQ.

Mouse Bioassay and HPLC Procedures

Shellfish samples extracted in parallel using the HCl and acetic acid extraction methods described above were analyzed using the standard protocols prescribed by the AOAC methods for mouse bioassay (1) or precolumn oxidation HPLC method (2).

Results and Discussion

Calibration Curve

To establish the dynamic range and repeatability of the calibration curve, five assays were performed by one analyst on separate days. The composite curve (Figure 3) resulted in a half-maximal inhibition (IC_{50}) of 2.3 nM STX ± 0.3 (RSD = 10.8%) with a slope of 0.96 ± 0.06 (RSD = 6.3%). Using the linear part of the curve (0.2–0.7 b/b_0) for quantification, a dynamic range of approximately one order of magnitude, 1.2–10.0 nM STX, was observed, as expected for a one-site binding assay. A QC check sample (3.0 nM STX) run in each assay averaged 3.0 ± 0.5 nM (RSD_r = 17.3%), with a recovery of 99.3%.

LOQ

Shellfish extracts were diluted a minimum of 10-fold prior to analysis to minimize matrix effects that can result in false positives. The LOQ was empirically determined as the concentration, in a 10-fold diluted sample, that results in a b/b_o of 0.7. This is a more conservative cutoff than the 0.8 b/b_o frequently used in receptor assays and was used because quantification was unacceptably variable above this b/b_o cutoff. This results in an LOQ of approximately 5 μg equiv. STX/100 g shellfish, which provides a more than one order of magnitude margin relative to the regulatory limit of 80 μg/100 g.

Linearity

Linearity was assessed by five independent assays of three calibration standards that were expected to fall on the curve between 0.2 and 0.7 b/b_o: 1.5, 3.0, and 6.0 nM STX prepared from FDA STX diHCl standard. Expected and measured values are listed in Table 1. Linear regression yielded a slope of 0.98 and an r^2 of 0.97.

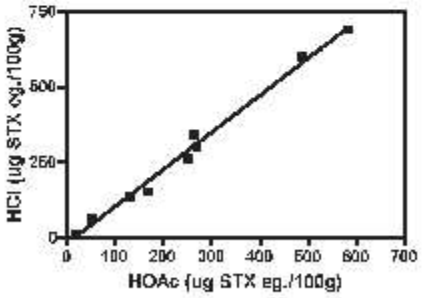


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

Table 5. HPLC analysis of nine naturally contaminated samples (1–9) extracted using 0.1 M HCl[a]

Sample	STX	NEO[b]	GTX1,4[c]	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HCl-1	3.5	0.0	0.0	7.3	0.0	0.0	10.8	6
HCl-2	231.6	23.9	42.6	324.5	28.3	249.7	900.4	412
HCl-3	220.8	53.7	74.9	436.3	43.4	338.1	1167.2	494
HCl-4	48.3	2.7	8.6	85.1	10.7	17.1	172.5	90
HCl-5	86.5	1.1	0.0	64.7	14.9	11.3	178.5	113
HCl-6	114.5	0.0	0.0	166.6	15.1	36.8	333.0	180
HCl-7	96.4	10.1	72.9	398.7	9.3	36.1	623.5	304
HCl-8	84.6	6.0	32.8	225.7	4.9	18.5	372.5	197
HCl-9	11.2	0.0	6.1	47.9	0.0	0.0	65.2	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.
[b] NEO = Neosaxitoxin.
[c] GTX = Gonyautoxin.

Recovery

Mussel tissue homogenates obtained from a local market were spiked with FDA STX diHCl standard at four levels bracketing the regulatory limit (0, 40, 80, and 120 μg/100 g) followed by thorough homogenization using a Polytron blender. Aliquots of spiked homogenate were stored at –80°C until extraction in 0.1 M HCl according to the protocol in the *Experimental* section. Extracts were analyzed in five assays performed on independent days. The mean recovery was 121% (Table 2).

Comparison of RBA-Reported Toxicity with the AOAC Mouse Bioassay

Six naturally contaminated shellfish samples were extracted in 0.1 M HCl according to the protocol in the *Experimental* section, and analyzed in five assays on independent days (Table 3). Three shellfish species were represented: clam *Mya arenaria* (whole) LP1, LP4; mussel *Mytilus edulis* (whole) LP2, LP3; and scallop *Placopecten magellanicus* (viscera) LP5, LP6. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 of 0.98 was obtained relative to the mouse bioassay, with a slope of 1.29 (Figure 4a).

A separate study of 110 naturally contaminated shellfish samples, extracted using the 0.1 M HCl method, and analyzed by RBA and mouse bioassay, yielded similar results with an r^2 of 0.88 and a slope of 1.32 (Figure 4b).

Effect of Extraction Method on RBA-Reported Toxicities

The recent approval of the precolumn oxidation HPLC method for PSP toxins as AOAC Official Method **2005.06** (3) and its potential recognition as a reference method for PSP

Table 6. HPLC analysis of the same nine naturally contaminated samples (1–9) extracted using 1% acetic acid[a]

Sample	STX	NEO	GTX1,4	GTX2,3	B1	C1,2	Total PSP	As STX equivalent
HOAc-1	3.4	0.0	0.0	7.3	0.0	0.0	10.7	6
HOAc-2	187.6	13.1	21.7	280.7	25.1	248.9	777.1	329
HOAc-3	175.2	35.6	79.2	335.9	37.2	237.7	900.9	393
HOAc-4	33.4	3.1	11.3	61.8	6.0	15.5	131.1	68
HOAc-5	59.3	3.1	0.0	67.6	10.8	19.3	160.0	89
HOAc-6	100.8	0.0	0.0	158.0	11.8	28.4	299.0	162
HOAc-7	67.4	11.2	42.7	228.4	5.2	15.6	370.5	192
HOAc-8	71.0	8.3	34.4	190.3	4.3	12.6	320.8	173
HOAc-9	11.2	0.0	11.7	38.1	0.0	61.0	122.1	33

[a] Values are in μg/100 g, as specific PSP congener or its STX equivalents, as indicated by the column headers.

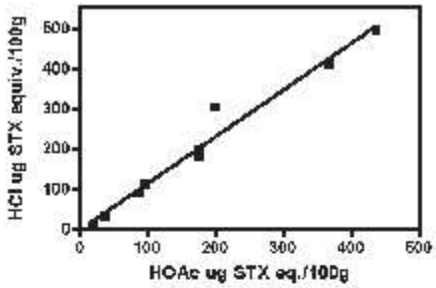


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

toxins prompted an investigation of the effects of extraction method on toxicity values reported by the RBA. Whereas the AOAC mouse bioassay prescribes shellfish extraction in 0.1 M HCl, the HPLC method uses extraction in 1% acetic acid. The 0.1 M HCl extraction procedure is known to result in the partial conversion of certain low-toxicity sulfocarbamoyl congeners to more highly toxic congeners in shellfish extracts, especially gonyautoxins, GTX5 and GTX6, to STX and neoSTX, and, thus, may result in somewhat higher toxicity values. To assess the effects of extraction procedure on RBA-reported toxicity, nine naturally contaminated shellfish samples (six blue mussel and three scallop) were homogenized and extracted independently using 0.1 M HCl and 1% acetic acid as described in the *Experimental* section. PSP toxicity in the extracts was then determined in four RBA assays run on independent days (Table 4). The between-assay RSD did not differ for samples prepared using the two extraction methods (25.8 and 26.3%, respectively). In general, the HCl extraction method resulted in slightly higher total toxicity values than reported for the acetic acid extracts (slope 1.23, r^2 = 0.99; Figure 5). The higher values reported for the HCl extracts are not explained by the conversion of sulfocarbamoyl toxins to more potent congeners in the HCl extracts, as can be seen in the toxin profiles determined by HPLC (Tables 5 and 6). Rather, the recovery of most congeners appears to be higher in the HCl extract. The higher concentrations reported in the HCl extract may reflect differences in the method by which volume is adjusted in the two extraction procedures. In the HCl method, final extract volume adjustment is made with the shellfish matrix present. In the acetic acid extraction, the matrix is first removed, the pellet re-extracted, the two extracts pooled, and then the final volume adjusted. HPLC analysis of the same samples showed a similar relationship between values reported for the HCl and acetic acid extracts (slope = 1.16, r^2 = 0.97; Figure 6) as seen in the RBA, with the HCl extracts containing greater STX equivalent/100 g.

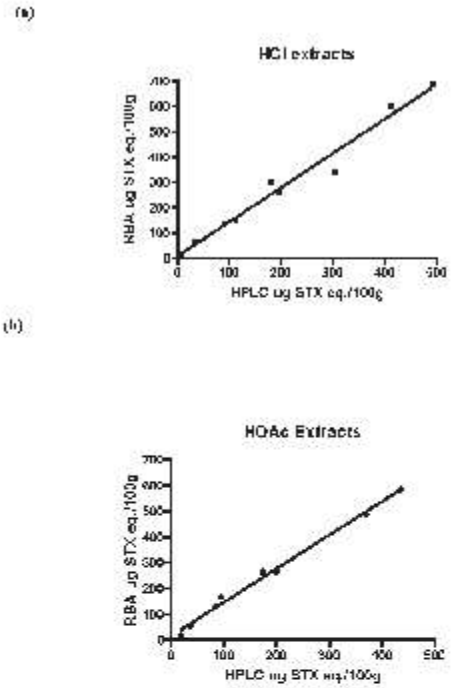


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

Comparison of RBA with HPLC

The RBA showed good linear correlation with HPLC analysis of both HCl (r^2 = 0.98, slope = 1.39) and acetic acid (r^2 = 0.99, slope = 1.32) extracts, in both cases giving somewhat higher toxicities than the HPLC method (Figure 7). A number of factors may contribute to the difference in results for total toxic potencies by these two methods. The higher toxicity values given by the RBA may result in part from the fact that the HPLC method uses the STX free base molecular weight (300 Da), whereas the receptor assay (and mouse bioassay) uses the STX dihydrochloride molecular weight (372 Da) to calculate concentration, which would result in approximately 20% higher values in the RBA. Additional differences may result from the use of FDA as compared to the NRC saxitoxin standards in the RBA and HPLC methods, respectively. Higher RBA results may also result from the dominance of the more potent PSP congeners over the weaker congeners in mixtures competing for binding to the receptor, as detailed in ref. 13, which reflects their binding affinities. In

contrast to this complex behavior, the HPLC method adds linearly the concentrations of each congener based on toxic potencies determined by mouse bioassay for isolated congeners. In some cases, e.g., 11-hydroxysulfate epimers, the concentrations of separate epimers pairs are not resolved by HPLC, although their potencies differ widely as do their ratios in shellfish samples. Lastly, higher toxicity values reported by the RBA may reflect the presence of congeners or metabolites not reported by the HPLC method.

Ruggedness

Although formal ruggedness testing was not carried out during this SLV study, several steps in the procedure might be noted that can affect the precision and accuracy of the results. First, it is important to clarify shellfish extracts by centrifugation prior to running the assay, particularly if extracts are stored refrigerated or frozen before analysis, as precipitates in the extract may cause nonspecific binding that may result in overestimates of PSP toxin concentrations. Second, since the rat brain homogenate is a suspension, it is important to ensure that it remains evenly suspended by frequent vortex mixing or pipetting prior to and during its addition to the plate. The rate of assay plate filtration should ensure that the wells clear in 2–5 s, and the rinse buffer should be ice cold in order to minimize the rate of toxin release from the receptor. Lastly, following addition of liquid scintillant to the microplate wells, it is essential to allow a minimum of 30 min for the scintillant to penetrate the filters before counting. Counting prematurely can result in increased variability between wells and lower counts/well, thus increasing RSD. A count time of 1 min/well was chosen for this study as a compromise between optimum RSD and assay throughput. Increasing the count time to 5 min/well has been shown to improve the between-well RSD in this assay when using the Packard Top Count scintillation counter, a single detector instrument with somewhat lower efficiency than the Wallac Microbeta used in the current study (11).

Summary

This SLV and method comparison study demonstrates excellent linear correlation ($r^2 > 0.98$) between the microplate receptor binding assay and both the mouse bioassay and the precolumn oxidation HPLC method for the determination of PSP toxins in shellfish. The microplate format of the assay, when coupled with microplate scintillation counting, provides a quantitative high throughput screening tool for PSP toxin testing in shellfish. The tendency of the RBA to overestimate PSP toxicity relative to the reference methods minimizes the chance of returning false negatives. Where RBA-measured toxicity results in STX equivalent values close to the regulatory limit, confirmation with a reference method is necessary if a regulatory decision is being made. Nonetheless, application of the assay as a high throughput screen can alleviate the unnecessarily large numbers of animals used for the mouse bioassay on negative samples and, similarly, alleviate the lengthy analysis of samples by HPLC at very high or very low concentrations. We propose that this method be collaboratively tested to establish if it is robust enough to be used in monitoring and regulatory laboratories.

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Appendix III: Van Dolah et al., 2012

FOOD CHEMICAL CONTAMINANTS

Appendix III

Determination of Paralytic Shellfish Toxins in Shellfish by Receptor Binding Assay: Collaborative Study

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A collaborative study was conducted on a microplate format receptor binding assay (RBA) for paralytic shellfish toxins (PST). The assay quantifies the composite PST toxicity in shellfish samples based on the ability of sample extracts to compete with ^{3}H saxitoxin (STX) diHCl for binding to voltage-gated sodium channels in a rat brain membrane preparation. Quantification of binding can be carried out using either a microplate or traditional scintillation counter; both end points were included in this study. Nine laboratories from six countries completed the study. One laboratory analyzed the samples using the precolumn oxidation HPLC method (AOAC Method 2005.06) to determine the STX congener composition. Three laboratories performed the mouse bioassay (AOAC Method 959.08). The study focused on the ability of the assay to measure the PST toxicity of samples below, near, or slightly above the regulatory limit of 800 (µg STX diHCl equiv./kg). A total of 21 shellfish homogenates were extracted in 0.1 M HCl, and the extracts were analyzed by RBA in three assays on separate days. Samples included naturally contaminated shellfish samples of different species collected from several geographic regions, which contained varying STX congener profiles due to their exposure to different PST-producing dinoflagellate species or differences in toxin metabolism: blue mussel (*Mytilus edulis*) from the U.S. east and west coasts, California mussel (*Mytilus californianus*) from the U.S. west coast, chorito mussel (*Mytilus chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the west coast of the United States, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, from which only the hepatopancreas was homogenized. Among the naturally contaminated samples, five were blind duplicates used for calculation of RSD_r. The interlaboratory RSD_R of the assay for 21 samples tested in nine laboratories was 33.1%, yielding a HorRat value of 2.0. Removal of results for one laboratory that reported systematically low values resulted in an average RSD_R of 28.7% and average HorRat value of 1.8. Intralaboratory RSD_r, based on five blind duplicate samples tested in separate assays, was 25.1%. RSD_r obtained by individual laboratories ranged from 11.8 to 34.9%. Laboratories that are routine users of the assay performed better than nonroutine users, with an average RSD_r of 17.1%. Recovery of STX from spiked shellfish homogenates was 88.1–93.3%. Correlation with the mouse bioassay yielded a slope of 1.64 and correlation coefficient (r^2) of 0.84, while correlation with the precolumn oxidation HPLC method yielded a slope of 1.20 and an r^2 of 0.92. When samples were sorted according to increasing toxin concentration (µg STX diHCl equiv./kg) as assessed by the mouse bioassay, the RBA returned no false negatives relative to the 800 µg STX diHCl equiv./kg regulatory limit for shellfish. Currently, no validated methods other than the mouse bioassay directly measure a composite toxic potency for PST in shellfish. The results of this interlaboratory study demonstrate that the RBA is suitable for the routine determination of PST in shellfish in appropriately equipped laboratories.

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Paralytic shellfish poisoning (PSP) is caused by a suite of heterocyclic guanidinium toxins collectively called saxitoxins (STXs). Currently more than 21 congeners of STX are known; they occur in varying proportions in the dinoflagellates that produce them and may be further

Table 1. Shellfish homogenate samples analyzed for PSTs in the collaborative study[a]

Sample No.	Sample ID	Shellfish species and origin	Blind duplicate
1	MLV05	Atlantic sea scallop (*Placlopecten magellanicus*) from the U.S. east coast	x
2	MLV06	California mussel (*Mytilus californianus*) from the U.S. west coast	x
3	MLV08	Green mussel (*Perna canaliculus*) from New Zealand	
4	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
5	MLV12	Blue mussel (*M. edulis*) east coast U.S., spiked with 200 µg/kg STX diHCl	
6	MLV14	Blue mussel (*M. edulis*) east coast U.S., spiked with 1200 µg/kg STX diHCl	
7	MLV16	Almeja clam (*Venus antique*) from Chile	
8	MLV01	Surf clam (*Spisula solidissima*) from the U.S. east coast	
9	MLV02	Chorito mussel (*M. chilensis*) from Chile	
10	MLV04	Scallop (*Placlopecten magellanicus*) from the U.S. east coast	
11	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
12	MLV09	Blue mussel (*M. edulis*) from the U.S. west coast	x
13	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
14	MLV13	Blue mussel (*M. edulis*) east coast U.S., spiked with 500 µg/kg STX diHCl	
15	MLV03	Chorito mussel (*M. chilensis*) from Chile	
16	MLV05	Atlantic sea scallop (*Placlopecten magellanicus*) from the U.S. east coast	x
17	MLV06	California mussel (*M. californianus*) from the U.S. west coast	x
18	MLV07	Blue mussel (*M. edulis*) east coast U.S.	x
19	MLV10	Butterclam (*Saxidomus gigantea*) from the U.S. west coast	
20	MLV11	Almeja clam (*Venus antique*) from Chile clam	x
21	MLV15	Blue mussel (*M. edulis*) negative control, east coast U.S.	

[a] Sample number identifies the individual samples analyzed in the assays, with 1–7 analyzed in assay 1, 8–14 in assay 2, and 15–21 in assay 3. Sample identification (MLV for multilaboratory validation) describes the 16 unique samples, among which five were assayed as blind duplicates, to make a total of 21 samples. Blind duplicates, run in different assays, are identified by an "x."

metabolized in shellfish that accumulate them, making analytical determination of paralytic shellfish toxins (PST) in shellfish complex. The long-standing regulatory method for PST is the AOAC mouse bioassay (1; AOAC Method **959.08**), with a regulatory limit of 800 µg STX di HCl equiv./kg shellfish generally applied, but established at 400 µg STX diHCl equiv./kg in certain countries (e.g., the Philippines). However, at concentrations near the regulatory limit, the mouse bioassay can significantly underestimate PST in shellfish (2). This, in addition to increasing resistance to live animal testing in both the United States and the European Union (EU), has increased the need to develop alternative methods suitable for use in a high-throughput monitoring or regulatory setting.

In the past decade, several alternatives to the mouse bioassay have been developed. In the EU, the mouse bioassay remains the reference method for PST in shellfish, but European Commission (EC) Regulation 1664/2006 specifies that other internationally recognized methods may be used. Two HPLC methods, a precolumn oxidation method (3, 4; AOAC Method **2005.06**) and a postcolumn oxidation method (5; AOAC Method **2011.02**), have been approved by AOAC as *Official Methods*℠ for PSP toxin analysis. The EC directive recognizes the precolumn oxidation HPLC method (AOAC Method **2005.06**) as an alternative to the mouse bioassay, but retains the mouse bioassay as the reference method in instances where results are challenged. HPLC methods separate and quantify individual STX congeners, which are then recombined according to their toxic equivalencies to yield a composite PST toxicity value. Although the HPLC methods perform well quantitatively, a high-throughput screening method capable of reporting toxic potency directly is still desirable for monitoring programs that often screen large numbers of negative samples. A qualitative lateral flow antibody test for PST with a reported detection limit of 400 µg STX equiv./kg was developed by Jellett Rapid Testing Ltd (Chester Basin, NS, Canada) and approved by the U.S. Interstate Shellfish Sanitation Conference and the U.S. Food and Drug Administration as a screening method in specific circumstances. This method performed well in a comparison study with the mouse bioassay (6), but is not fully quantitative and has not been subjected to a full AOAC collaborative trial. To date, a suitable quantitative, high-throughput alternative to the mouse bioassay has not been validated through the AOAC Official Methods Program. The receptor binding assay (RBA) for PST is an excellent candidate for fulfilling the requirements of a high-throughput, quantitative assay that directly reports a composite toxic potency.

The basis of the RBA is the interaction between the toxins and their pharmacological target. All STX congeners bind to site 1 on the alpha subunit of the voltage-gated sodium channel with binding affinities proportional to their toxic potency (7). Therefore, an RBA can quantitatively measure the combined toxic potency of mixtures of STX congeners in a sample,

independent of the toxin congeners present (8). In the RBA for PST, tritiated STX ([^{3}H] STX) competes with unlabeled STX and/or its congeners for a finite number of available receptor sites in a rat brain membrane preparation. Following establishment of binding equilibrium, unbound [^{3}H] STX is removed by filtration and receptor bound [^{3}H] STX quantified by liquid scintillation counting. The reduction in [^{3}H] STX binding is directly proportional to the amount of unlabeled toxin present. A standard curve is generated using increasing concentrations of nonradiolabeled STX standard from 10^{-10} to 10^{-6} M STX. The concentration of toxin in samples is determined in reference to the standard curve.

The assay being tested in this collaborative trial is a modification of the method of Doucette et al. (9) to incorporate a 96-well microtiter plate format, which increases sample throughput and minimizes error by reducing sample handling and pipetting steps. This microplate PST RBA was evaluated in a single-laboratory validation (SLV) study (10), which established an interassay repeatability (RSD_r) of 17.7% and good correlation with the mouse bioassay and precolumn oxidation HPLC methods. The toxin concentrations in shellfish tested in the SLV study ranged from near to well above the regulatory limit (approximately 900–15 000 μg STX diHCl equiv./kg). The current study focuses more specifically on the performance of the RBA in the critical range of shellfish toxicities below, near, and slightly above the regulatory limit (approximately 150–2400 μg STX diHCl equiv./kg).

The results of the collaborative study suggest that the RBA for PST is a suitable high-throughput screen for PST in shellfish. Although HPLC methods offer quantitative information on congener composition of samples, often the desired information is composite toxic potency, which requires the summation of individual congeners, corrected for their individual toxic equivalencies. The RBA provides a single integrated toxic potency value that reflects activity of all known and potential unknown congeners present in the sample. Use of the microtiter plate format, in conjunction with microplate scintillation counting, provides the ability to screen multiple samples simultaneously in a total assay time of less than 3 h. The assay format described in the current study provides for the quantitative determination of composite PST toxicity in seven shellfish extracts per 96-well microplate, each run in triplicate at three dilutions, covering toxicity ranges of approximately 35–6000 μg STX diHCl equiv./kg. In a high-throughput assay setting, multiple plates can be set up simultaneously, so that six assay plates can easily be accommodated each day by a single analyst, for a throughput of 42 samples/day. This compares favorably to an estimated throughput of 20–25 samples a day by the precolumn HPLC method (B. Niedzwiadek, Health Canada, personal communication) or 30–35 by mouse bioassay (B. Suarez, University of Chile, personal communication).

Collaborative Study

The focus of this study was to assess the performance of the RBA to determine PST toxicity in samples of commercially important shellfish at a range of concentrations below and above the regulatory limit. Twenty-one shellfish homogenates were included in the study, which represented 16 unique samples (Table 1). The homogenates included 12 naturally contaminated shellfish samples of different species collected from several geographic regions: blue mussel (*M. edulis*) from the U.S. east and west coasts, California mussel (*M. californianus*) from the U.S. west coast, chorito mussel (*M. chiliensis*) from Chile, green mussel (*Perna canaliculus*) from New Zealand, Atlantic surf clam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from U.S. west coast, almeja clam (*Venus antigua*) from Chile, and Atlantic sea scallop (*Plactopecten magellanicus*) from the U.S. east coast. All samples were provided as whole animal homogenates, except Atlantic sea scallop and green mussel, which included hepatopancreas only. Among the naturally contaminated samples, five were blind duplicates tested on separate days that were used for calculation of RSD_r. Samples run as duplicates are indicated in Table 1. Three samples consisting of STX-spiked mussel homogenate (*M. edulis*) at levels that bracketed the regulatory limits of 800 μg/kg (500 and 1200 μg/kg spike) and 400 μg/kg (200 μg/kg spike) were included to calculate recovery. One sample was the negative control homogenate of *M. edulis* to which the STX spikes were added. All homogenates were extracted by the study participants and the extracts analyzed by RBA in three assays on separate days.

Study Participants

Ten laboratories from seven countries agreed to carry out RBAs for this study, including the United States, Italy, Australia, New Zealand, Thailand, the Philippines, and South Africa. Participants included laboratories from regulatory authorities, as well as government and academic laboratories with monitoring needs. Five of the participating laboratories (Laboratories 1–5) have this method well established and may be considered routine users. Two laboratories had previous experience running this format of the PST RBA, but have not implemented it routinely. One laboratory had previous experience with receptor assays, but had not used the microplate filtration format of the assay. One laboratory had no previous experience with RBAs. Three laboratories from different countries, United States, Chile, and Thailand, carried out the AOAC official mouse bioassay method (AOAC Method **959.08**) on the same set of samples. All mouse bioassay laboratories were experienced regulatory authorities with monitoring responsibilities. One laboratory (Health Canada) performed the precolumn oxidation HPLC method for PST (AOAC Method **2005.06**).

Preparation of Homogenates

All shellfish samples were thoroughly homogenized using a polytron blender. For spiked samples, saxitoxin standard reference material (STX diHCl) was added to the specified concentration, and the sample was thoroughly rehomogenized to ensure homogeneity. The toxin congener profiles and concentrations of all samples were determined by the precolumn oxidation HPLC method (performed by Health Canada). STX equivalents were determined by mouse bioassay (performed by Maine Department of Marine Resources). Subsamples of each homogenate (12 g) were packaged in polycarbonate tubes and stored at –80°C until shipment to collaborating laboratories by courier. All samples were coded prior to distributing to collaborating laboratories, with the codes to each laboratory being unique, and provided blind. Coding consisted of two letters followed by a number in the form X A1-7, X B1-7, and

X C1-7, where the X indicated the laboratory, the second letter indicated the three assays to be conducted, and the numerical code indicated sample number within that assay. Three practice homogenates were similarly produced.

Shipment of Study Material

The following reagents were provided to the collaborating laboratories in a single shipment containing enough dry ice to keep the contents frozen for 5 days: [^{3}H] STX; STX diHCl standard; rat brain membrane preparation; 21 coded shellfish homogenates; three practice homogenates; and a QC check sample consisting of 18 nM STX diHCl. Sufficient homogenate (12 g) was provided to ensure an accurate weight of material could be removed from the storage vial if an additional extraction were necessary due to unexpected circumstances. The identity of the samples was not released to collaborators. All reagents were received frozen and in good condition. Each participant received electronically a detailed assay protocol, comprehensive instructions for conducting the study and data reporting, and data reporting forms.

Analysis

Participants extracted all homogenates using a modification of the 0.1 M HCl extraction method used in the AOAC standard mouse bioassay protocol (modified only by scale). They were asked to perform three RBAs, each on separate days. Each assay consisted of one 96-well plate that included a standard curve, QC check sample, and seven shellfish extracts. All samples and standards were tested in triplicate wells. All shellfish extracts were run at three dilutions (1/10, 1/50, and 1/200), which ensured that at least one dilution would fall on the linear part of the standard curve. Participants were instructed to analyze samples coded A, B, or C in the first, second, or third assay, respectively, in numerical order. The five blind duplicate samples were coded so that they were tested in two independent assays, with the combination of assays differing between duplicates. Before performing the official study, participants were asked to run a practice assay that included three shellfish homogenates in the same format to ensure that any unexpected problems were encountered and addressed prior to the official study. The practice samples consisted of a negative control mussel homogenate (MLV15), and two naturally contaminated samples that were also included in the full study (MLV05 and MLV11). The identity of the practice samples was not made known to participants. Results of the practice run were submitted by e-mail to the coordinating laboratory for review before proceeding with the full study.

For the mouse bioassay, participants followed the AOAC official mouse bioassay method (AOAC Method 959.08), with the exception of a modified 0.1 M HCl extraction protocol used in the RBA protocol, which was modified only by scale so that 5 mL 0.1 M HCl was added to 5 g of shellfish homogenate, with all other aspects of the extraction protocol being identical. The HPLC laboratory followed the precolumn oxidation HPLC method for PST (AOAC Method 2005.06); however, final concentrations in μg/kg and μg STX equiv./kg were calculated using the formula weight of STX diHCl [372 daltons (da)], as opposed to the free base (299.3 da) in the standard HPLC protocol, to more directly compare with the RBA.

Data Analysis and Reporting

Participants were asked to report whether they used a standard or microplate scintillation counter for the study and, if a microplate counter was used, which model, because of differences in inherent counting efficiency between current commercially available counters. For data analysis, participants were instructed to use GraphPad Prism software (La Jolla, CA) or the on-board curve-fitting software provided with their microplate scintillation counter e.g., PerkinElmer Wallac MultiCalc (Gaithersburg, MD) or Packard Top Count software (Packard Instrument Co., Meriden, CT), and to report what software was used. For analysis, a four parameter logistic fit, also known as a sigmoidal dose response with variable slope, or Hill equation, was prescribed. Participants presented their analyzed data on the spreadsheet template provided, including assay quality parameters (slope, IC_{50}, and quantification of the QC check sample), between-well *CV*s for each sample dilution that fell within the linear part of the standard curve (0.2–0.7 B/B_o), and calculated values for these samples in the well (nM), in the extract (μg STX equiv./mL), and in the shellfish tissue (μg STX equiv./kg). Participants were also asked to report all raw count data so that all results could be analyzed by the coordinating laboratory using identical software (GraphPad Prism 4.0) to assess whether systematic differences in quantification arose from using different curve-fitting software. All data were reported via e-mail to the coordinating laboratory.

The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in dilutions and calculations and for use of the prescribed curve-fitting model. Obvious errors were corrected and the participant laboratory was consulted for concurrence. The reviewed results were then used for evaluation in the collaborative study.

Statistical Evaluation of the Collaborative Study

For each sample analyzed, outliers were first determined using the Grubbs test at a probability value of 1% (www.graphpad.com), with no more than one outlier removed, so that valid data remained from a minimum of eight laboratories. The mean, S_R, and RSD_R, and HorRat values were then calculated for each sample. For blind duplicates, the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0, was used to further evaluate for outliers and determine S_r and RSD_r. GraphPad Prism was used to determine correlation among the RBA, mouse bioassay, and HPLC results.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as μg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 μg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 μg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration.

All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(a) *Traditional or microplate scintillation counter.*

(b) *Micropipettors.*—1–1000 µL variable volumes and disposable tips.

(c) *Eight channel pipettor.*—5–200 µL variable volume and disposable tips.

(d) *96-Well microtiter filter plate.*—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA; Cat. No. MSFB N6B 50).

(e) *MultiScreen vacuum manifold.*—Millipore; Cat. No. NSVMHTS00.

(f) *Vacuum pump.*

(g) *Centrifuge tubes.*—15 and 50 mL, conical, plastic.

(h) *Mini dilution tubes in 96-tube array.*

(i) *Reagent reservoirs.*

(j) *Ice bucket and ice.*

(k) *Vortex mixer.*

(l) *Sealing tape.*—Millipore; Cat. No. MATA HCL00.

(m) *Volumetric flask.*—1 L.

(n) *–80°C freezer.*

(o) *Refrigerator.*

For traditional scintillation counter only:

(p) *MultiScreen punch device.*—Millipore; Cat No. MAMP 096 08.

(q) *MultiScreen disposable punch tips.*—Millipore; Cat. No. MADP 196 10.

(r) *MultiScreen punch kit B for 4 mL vials.*—Millipore; Cat. No. MAPK 896 0B.

(s) *Scintillation vials.*—4 mL.

For sample extraction:

(t) *Pipets.*

(u) *Centrifuge tubes.*—15 mL, conical, plastic.

(v) *Vacuum pump or house vacuum.*

(w) *pH meter or pH paper.*

(x) *Hot plate.*

(y) *Graduated centrifuge tubes.*—15 mL.

(z) *Centrifuge and rotor for 15 mL tubes.*

C. Reagents

(a) *[^{3}H] STX.*—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, or International Isotopes Clearinghouse, Leawood, KS).

(b) *STX diHCl.*—NIST RM 8642 (www.nist.gov).

(c) *3-Morpholinopropanesulfonic acid (MOPS).*—Sigma (St. Louis, MO; Cat. No. M3183-500G), or equivalent.

(d) *Choline chloride.*—Sigma (Cat. No. C7527-500G), or equivalent.

(e) *Rat brain membrane preparation.*—See *Appendix*.

For traditional counter:

(f) *Scintiverse BD liquid scintillation cocktail.*—Fisher Scientific (Waltham, MA; Cat. No. SX-18), or equivalent.

For microplate counter:

(g) *Optiphase liquid scintillation cocktail.*—PerkinElmer Life Sciences (Downers Grove, IL; Cat. No. 1200-139), or equivalent.

For sample extraction:

(h) *Hydrochloric acid (HCl).*—1.0 and 0.1 M.

(i) *Sodium hydroxide.*—0.1 M.

(j) *Water.*—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on a hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in receptor assay.

E. Preparation of Stock Solutions and Standards

(a) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(b) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride

Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230*	1149*	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R,%		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

* Outlier; not used in calculation.

buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(c) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table **2011.27F**) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table **2011.27F**).

(d) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-9} M STX standard (3.0×10^{-9} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(e) *Rat brain membrane preparation.*—Prepare rat brain membrane preparation in bulk (*see Appendix: Rat Brain Membrane Preparation*) and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(a) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_0 on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table **2011.27G**).

(b) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(c) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8" Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to

Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Lab	ID	Day 1	Day 2	Mean	s_r	RSD_r, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230[a]	1150[a]			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

[a] Outlier; not used in calculations.

ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(**d**) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure **2011.27**), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log \mathrm{IC}_{50}) \times \text{Hill slope}}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_0; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure **2011.27**); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is total ligand binding in CPM (here represented as B/B_0, or bound/max bound). A curve fitting package such as Prism (GraphPad Software, Inc.) is recommended. For the microplate counter users, receptor

Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{70}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	–0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD).

(a) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl equiv./kg shellfish, from the in-well nM concentration obtained from the curve fitting software using the following formulas:

$$(\text{nM STX equiv}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCl equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	950	940	1060	1130	1040	750	1460	1320	810	1051	618	485
7	660	930	1080	870	840	1320	1490	2420	490	960	685	528
2	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	931	595
17	1290	1520	1460	970	1800	2520	1470	870	1250	1460	931	595
4	860	680	950	870	980	1120	1460	820	590	926	1070	653
12	810	1150	1130	810	1630	1390	1880	1120	870	1203	1070	653
11	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	965	714
18	1010	1600	1390	1000	1720	1860	1520	2150	890	1452	965	714
8	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	894	752
9	830	1180	1130	1150	1130	1780	1340	980	690	1134	802	792
19	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	2000	1027
10	2440	2840	2910	1740	2150	1800	2690	2490	1210	2252	1890	1080

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

(a) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(b) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(c) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(d) QC check should be 3 nM STX ± 30% (in-well concentration). Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(a) Sample quantification should be done only on dilutions that fall within B/B_0 of 0.2–0.7. In the event that all sample dilutions fall below B/B_0 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_0 > 0.7$), the sample is reported as below LOD. If more

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

Microplate row	Microplate column											
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-6}	3×10^{-6}	3×10^{-6}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-6}	10^{-6}	10^{-6}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [Note: The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(b) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int.* **95**, 795(2012)

Results and Discussion

Sample Characterization

All shellfish homogenates (MLV1–16) were analyzed by HPLC using the precolumn oxidation method (AOAC Method **2005.06**) to determine toxin congener profiles and quantify total PST as μg STX diHCl equiv./kg prior to initiation of the study (Table 2). It is noteworthy that the clear majority of samples, irrespective of shellfish species and location, were dominated largely by STX and GTX2,3 whereas the N1-hydroxylated congeners NEO and GTX1,4 were virtually absent, except in blue mussel from the U.S. west coast. The most unusual profile was observed in green mussel, which was dominated by the weakly toxic N-sulfo-carbamoyl congeners C1,2. The samples were analyzed by the AOAC mouse bioassay (AOAC Method **959.08**) by three laboratories that routinely perform the mouse bioassay for regulatory purposes (Table 3). The mouse bioassay detection limit is approximately 400 μg STX diHCl equiv./kg (one laboratory reported values as low as 290 μg STX equiv./kg). Because the study design included samples that bracketed the lower regulatory limit of 400 μg STX diHCl equiv./kg, several samples were reported as being below the mouse bioassay detection limit. For samples in which all values were above the detection threshold, the between-laboratory RSD_R of the mouse bioassay was 18.9%.

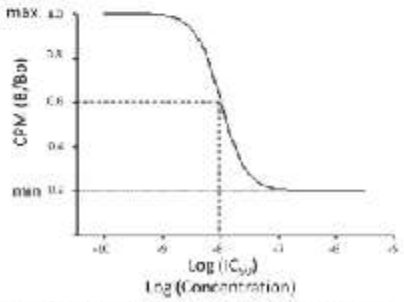


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

Data Reporting and Initial RBA Data Review

Nine of the 10 laboratories that received the study materials completed the study and reported results. All nine carried out the practice assay and reported results to the coordinating laboratory, which evaluated the results and provided feedback to the participating laboratories before initiating the full study. Following completion of the full study, the participating laboratories provided all raw and calculated data for each of

the three assays performed via e-mail to the coordinating laboratory. The calculated results sheets were reviewed by the coordinating laboratory for obvious errors in sample dilutions and calculations, and for the use of the prescribed curve-fitting model. One laboratory used a sigmoidal curve-fitting model with the slope set to 1 (one-site binding curve in Prism), rather than the prescribed four-parameter logistic fit. In this case, the raw data were reanalyzed by the coordinating laboratory using the prescribed method. Obvious errors in calculation were corrected, such as accounting for the two-fold sample dilution resulting from the extraction process. In some cases, the participating laboratory carried out a fourth assay due to variability or inconsistency among dilutions for selected samples. In these cases, the value reported from the repeat (fourth) assay was used. One laboratory had consistent disagreement between the 1/50 and 1/200 dilutions when both fell within B/B_0 0.2–0.7. In all cases the 1/200 dilution overestimated almost two-fold relative to the 1/50 dilution, suggesting a systematic dilution error. In standard practice, these samples should be rerun. However, the instructions did not direct the participants to do so. Therefore, where there was corroborative evidence for the value reported by the 1/50 dilution, based on the 1/10 dilution, the 1/200 dilution was omitted. Where there was no basis on which to exclude the 1/200 value, an average value was calculated. This tended to result in an overestimate, and in two cases resulted in statistical outliers.

Overall Performance of the Method: Reproducibility

Table **2011.27A** summarizes the results obtained for 21 individual shellfish samples analyzed in three RBAs, determined by nine participating laboratories. Samples 1–7 were analyzed in the first assay, samples 8–14 in the second assay, and samples 15–21 in the third assay. Among these samples were five blind duplicates, treated here as individual unknown samples. One sample (marked by an footnote a in Table **2011.27A**) had a high variability in CPM between wells that was not attributable to any known cause, and was, therefore, omitted from analysis. Outliers identified by Grubbs test ($P < 0.01$) were excluded from the analysis (marked by footnote b in Table **2011.27A**). The overall RSD_R among all 21 independent samples was 33.2%, resulting in an average HorRat value of 2.0 (Table **2011.27A**). The HorRat values on individual samples ranged from 1.4 to 3.3, with a median value of 1.8. There was no apparent trend in reproducibility according to sample concentration or among shellfish species. If only the laboratories that are routine users of the RBA for PST (Laboratories 1–5) are included in the analysis, the average RSD_R is 23.1%, resulting in an average HorRat value of 1.4. Laboratory 9 tended to report the lowest values among the participating laboratories (14 of 21 samples), and although its individual sample values were not found to be statistical outliers, removing the results of this laboratory reduces all but one HorRat value (which remains unchanged), yielding an average HorRat value of 1.8 (range 1.0–2.8; Table **2011.27A**). Removal of any other single laboratory's results does not appreciably change the overall study performance. The reason for the systematically low values reported by Laboratory 9 is not clear, since the assay parameters fall well within those reported by the other laboratories. Given that assay parameters are within normal range, one possible source of systematic error could be incomplete extraction or pH adjustment of extracts, either of which would result in lower toxicity values.

A comparison of the RBA reproducibility with that of existing AOAC *Official Methods* is instructive. The AOAC collaborative study of the mouse bioassay (11), which entailed the analysis of seven samples representing three levels of STX-spiked shellfish by 11 participating laboratories, yielded a similar average RSD_R of 22%. More recent proficiency tests of the mouse bioassay performed in European regulatory laboratories report RSD_R of 2.3–38.3% on three samples run by eight laboratories (2) and RSD_R of 18.1–44.8% on two samples run by 20 laboratories (12). The mouse bioassay RSD_R values obtained in the current study ranged from 1.1 to 46.3% (average 19%) for three laboratories. The collaborative studies of the HPLC methods report reproducibility values for individual PST congeners, but do not report reproducibility of the composite toxic potency values. Collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an average RSD_R of 27.0% and HorRat value of 1.3 (range 0.8–2.1) for STX following C18 cleanup, but the reproducibility of other congeners varied considerably, with the maximum HorRat value (4.7), exceeding the highest HorRat value obtained by RBA (3.3).

Because composite toxic potency values were not reported in the studies of the HPLC methods, it is uncertain how this variability influences the composite toxic potency calculated from these methods. The average and ranges of HorRat values obtained for different congeners were: neoSTX–1.7 (range 1.2–2.5); dcSTX–1.1 (range 0.6–2.1); GTX1,4–1.9 (range 1.1–4.2), GTX2,3–1.4 (range 0.8–1.9); B1–1.1 (range 0.7–1.9); and C1,2–1.6 (range 0.9–4.5). Because of the variability obtained in neoSTX, GTX1,4, C3,4, and B2, AOAC Method **2005.06** calls for a second SPE-COOH cleanup of samples suspected of containing these congeners, after which reproducibility improved somewhat: neoSTX–1.8 (range 1.3–2.1); GTX1,4–1.3 (range 1.0–2.1); and C3,4–1.2 (range 0.8–1.8). The postcolumn oxidation HPLC method (AOAC Method **2011.02**) reported an average HorRat value of 0.6 for STX. In this method, neoSTX with an average HorRat of 1.9 (range 0.6–4.0) and GTX4 with an average HorRat of 1.6 (range 1.0–2.9) had reproducibility values that may affect the overall composite potency values. The maximum HorRat value (4.0) reported in this study also exceeded the maximum value reported in the RBA.

In summary, with the removal of Laboratory 9, the overall reproducibility of the RBA falls within the performance measures achieved by the established AOAC *Official Methods* for PST. The difference in reproducibility achieved by the laboratories that are routine users of the assay and participants who are not routine users of the method highlights the importance of training if this method were to be implemented in a regulatory setting.

Within-Laboratory Repeatability

Within-laboratory variability (RSD_r) was determined on five samples that were provided as blind duplicates. Participants were unaware that blind duplicates were included among the coded samples received. The duplicate samples were coded so that they were analyzed in separate assays, with different duplicate pairs falling into different assays (Table 1). One outlier was found among the results of the blind duplicates by Cochran's

test, $P < 0.025$ (Laboratory 7, sample MLV11) using the AOAC INTERNATIONAL Interlaboratory Study Workbook for Blind Duplicates, v2.0. An overall RSD_r of 25.1% was observed, with an RSD_R of 32.9%, yielding a HorRat value of 2.0, similar to that of the overall study (Table **2011.27B**). When the performance of individual laboratories was evaluated separately, the average RSD_r was 22.2%, with individual laboratories varying from 11.8 to 34.4% (Table **2011.27C**). Routine users of the microplate format of the PST RBA (Laboratories 1–5) obtained an average RSD_r of 17.1%, which is similar to that obtained in the SLV study (10), and lower than that obtained by nonroutine users (Laboratories 6–9), which averaged 26.1% and ranged as high as 34.4%. The AOAC collaborative study of the mouse bioassay (11) did not report RSD_r; however, analysis of the data from that study using AOAC INTERNATIONAL's Interlaboratory Study Workbook for Blind Duplicates results in an average RSD_r of 16.5% for three STX-spiked samples. Proficiency testing of the mouse bioassay performed in eight French laboratories reported an average RSD_r of 8.3% on three samples (2). The analysis of blind duplicates in the collaborative study of the precolumn oxidation HPLC method (AOAC Method **2005.06**) resulted in an RSD_r of 15.2% for STX following SPE C18 cleanup and an average RSD_r of 16.4% across all congeners, which ranged from 6.0 to 31.7%. Following SPE–COOH cleanup, repeatability was similar, with RSD_r of 17.2% across all congeners. The intralaboratory repeatability values obtained in the postcolumn oxidation HPLC method (AOAC Method **2011.02**) averaged 6.4% for STX; most other congeners were similar, with neoSTX being the only congener that showed a somewhat higher RSD_r of 23.3%.

In summary, the within-laboratory repeatability of the RBA was found to be acceptable, with all but two laboratories achieving an RSD_r of 23.3% or less, and the routine users of the assay achieving an average RSD_r of 17.1%.

Spike Recovery

Three samples included in the study were homogenates of blue mussel spiked with STX diHCl at concentrations intended to bracket the regulatory limits of 800 μg STX equiv./kg used by most countries and 400 μg STX equiv./kg imposed in the Philippines. Nominal concentrations in the spiked samples were 200, 500, and 1200 μg STX equiv./kg. Also included in the study was the blue mussel homogenate to which the STX spikes had been added, which was determined to be negative for STX by the precolumn oxidation HPLC method. The negative control homogenate was reported as nondetectable by eight of nine laboratories. Recovery of spiked STX by the RBA was 84.4, 93.3, and 88.1%, respectively, for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, and yielded a slope of 0.87 and r^2 of 0.86 (Figure 2). In the current study, the mouse bioassay reported < detection limit, and 68.6 and 40.5% recovery for the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels. The AOAC collaborative study of the mouse bioassay (11) reported recoveries of 62.3% at spike levels similar to those in the current study (equivalent to 1000 μg STX diHCl equiv./kg) but higher recoveries of 81.5 and 96.0% were achieved at higher spike levels equivalent to 4000 and 8000 μg STX diHCl equiv./kg.

The observed poor recovery in the mouse bioassay at concentrations near and below the regulatory limit has been observed in other studies (2), and has been attributed to a salt or protective effect of the shellfish matrix, which, for concentrations at or below the regulatory limit of 800 μg/kg, is injected undiluted into the mouse. The spike recovery observed in the precolumn HPLC method in this study is also somewhat low, with 54.0, 62, and 51.5% recovery at the 200, 500, and 1200 μg STX diHCl equiv./kg spike levels, respectively. The AOAC collaborative study of the precolumn HPLC method reported 74.4–76.8% at similar spike levels following SPE C18 cleanup and 63.7–68.2% following SPE-COOH cleanup (3, 4). In comparison, the postcolumn HPLC method reported 88–104% recovery of STX spiked at levels somewhat lower than the current study. The higher recovery of the RBA than the HPLC method in the current study may reflect the use of the 0.1 M HCl extraction method in the RBA as compared to the acetic acid extraction used in the HPLC methods.

We previously established in the SLV study that the RBA performs well with shellfish extracted using either method (10). In that study, the RBA reported slightly higher toxicity values for shellfish extracts made using the 0.1 M HCl method than the acetic acid extraction, yielding a correlation of 0.99 with a slope of 1.23 (10). The higher toxicity reported by the RBA in 0.1 M HCl extracts may reflect the hydrolysis of less toxic congeners to more toxic congeners.

Assay Parameters and Quality Metrics

Table **2011.27D** summarizes the assay parameters and quality metrics for all laboratories. Eight of nine laboratories used microplate scintillation counters. Laboratory 4 used the manual counting method in which the microplate well filters are punched out, using an eight-place punch system, into traditional 4 mL scintillation vials and counted. Its performance using the manual counting method (RSD, 17.4%) was similar to or better than that of the laboratories using the microplate method, indicating that using the manual counting method does not affect the performance of the assay. Similarly, there was no apparent difference in assay parameters when the Packard Top Count (single detector) was used, compared to the Wallac Microbeta (coincidence detector), although the reference CPM values obtained on the Top Count generally were somewhat lower due to differences in counting efficiency inherent in the differences in detector geometry. Eight of nine laboratories used GraphPad Prism for curve-fitting, while only Laboratory 5 used Wallac MultiCalc software. Values reported by Laboratory 5 fell well within the range of values reported by laboratories using Prism.

All assays resulted in slopes between –0.8 and –1.2, as specified in the protocol. This specification reflects the fact that in a competitive binding assay for a ligand that interacts specifically at a single receptor site, the slope of the resulting standard curve should theoretically be 1.0. Although curve-fitting software packages often include a one-site binding curve that fixes the slope at 1.0, we specified in the protocol the use of the four-parameter logistic fit (also known as sigmoidal dose-response with variable slope), because it more readily identifies problems with the standard curve that may skew results. Laboratory 9 reported results using a one-site binding curve fit; in this case, the coordinating laboratory recalculated their raw data using the four-parameter logistic fit. The protocol also calls for RSD% < 30 on all standards. Most analysts did not experience variability problems in the standard wells. Infrequent high RSDs were most often associated with the well

Table 2. Congener profiles in shellfish homogenates included in the collaborative study[a]

Sample name	Species	STX	NEO	dcSTX	GTX1,4	GTX2,3	dcGTX2,3	B1	C1,2	C3,4	Total PSP	µg STX diHCl equiv./kg
MLV01	Surf clam	639.8		74.0		226.2	207.0				1146.9	894.3
MLV02	Almeja clam	298.3				1290.1		266.6			1855.0	802.1
MLV03	Chorito mussel	77.6				310.4					388.0	195.5
MLV04	Atlantic sea scallop	831.6				2785.6					3617.3	1890.2
MLV05	Atlantic sea scallop	193.8				576.2					770.0	412.8
MLV06	California mussel	912.8		10.9		0.0		233.8			1157.5	931.3
MLV07	Blue mussel, U.S. east coast	548.2				1097.3					1645.5	965.2
MLV08	Green mussel	164.2		63.5			272.3	454.8	3629.0		4419.6	340.8
MLV09	Blue mussel, U.S. west coast	432.3	124.9	8.7	353.7	727.8		506.4			2153.9	1070.9
MLV10	Butter clam	1763.5		40.6		533.2		203.5			2540.8	2000.9
MLV11	Almeja clam	159.1		12.2		185.5					356.8	236.9
MLV12	Blue mussel spike	108.4									108.4	108.4
MLV13	Blue mussel spike	310.2									310.2	310.2
MLV14	Blue mussel spike	618.5									618.5	618.5
MLV15	Blue mussel blank										0.0	0.0
MLV16	Chorito mussel	389.8		14.3		754.1					1158.1	684.9

[a] Values for individual congeners are in µg/kg. Values for composite toxicity are in µg STX diHCl equiv./kg. Abbreviations for congeners are as follows: STX – saxitoxin; NEO – neosaxitoxin; dcSTX – decarbamoyl saxitoxin; GTX1,4 – gonyautoxin 1 and gonyautoxin 4; GTX2,3 – gonyautoxin 2 and gonyautoxin 3; B1 – gonyautoxin 5 (also known as sulfocarbamoyl STX B1); C1,2 – sulfocarbamoyl STX C1 and sulfocarbamoyl STX C2; C3,4 – sulfocarbamoyl STX C3 and sulfocarbamoyl STX C4.

Table 3. Mouse bioassay results on collaborative study samples from three laboratories[a]

Sample No.	Sample ID	MBA Lab A	MBA Lab B	MBA Lab C	MBA Avg.	MBA s_R	MBA RSD_R, %
1	MLV05	400	415	340	385	39.7	10.3
2	MLV06	550	597	540	562	30.4	5.4
3	MLV08	440	<dl[b]	370	405	49.5	12.2
4	MLV09	670	612	760	681	74.6	11.0
5	MLV12	<dl	<dl	<dl	—	—	—
6	MLV14	489	489	480	486	5.2	1.1
7	MLV16	585	585	470	547	66.4	12.1
8	MLV01	750	716	600	689	78.6	11.4
9	MLV02	670	1115	590	792	282.9	35.7
10	MLV04	2040	<dl	1080	1560	678.8	43.5
11	MLV07	1480	748	670	966	446.8	46.3
12	MLV09	—	594	670	602	11.3	1.9
13	MLV11	380	379	<dl	380	—	—
14	MLV13	<dl	343	<dl	343	—	—
15	MLV03	400	364	<dl	382	—	—
16	MLV05	—	396	370	383	18.4	4.8
17	MLV06	—	702	630	666	50.9	7.6
18	MLV07	—	<dl	690	690	—	—
19	MLV10	1320	890	870	1027	254.2	24.8
20	MLV11	—	364	290	327	52.3	16.0
21	MLV15	<dl	<dl	<dl	—	—	—

[a] Values are in µg STX diHCl equiv./kg.

[b] dl = Detection limit.

in column 1 of the 96-well plate. Most analysts removed the suspect well from the curve-fitting process. When the RSD for a given standard was near the stated cutoff (e.g., 31–33%), and left in the curve-fitting process, there was no apparent effect on the curve parameters listed as criteria for assay acceptance.

The average IC_{50} among all 27 assays was 1.9 + 0.45 nM (RSD_R 23.5%). The other assay quality metric called for by the protocol is the analysis of the QC check sample, which should be 3 ± 0.9 nM STX (30% RSD, in-well concentration). Four of the 27 assays had QC values outside the stated limits, with no obvious error responsible for the variability. Among these, Laboratory 7 reported 6.5 nM for the QC check in assay 3 and an IC_{50} of 3.4 nM, which was outside the norm. Similarly, Laboratory 8 reported a QC of 1.5 nM in assay 2 and a low IC_{50} of 1.4 nM, which is at the lower edge of acceptability. In general practice, these values would trigger repeating the assay. However, because of the minimal number of laboratories participating in the study, both of these assays were retained in the study. In neither case were the reported sample values systematically higher or lower than those reported in the other assays.

LOD and LOQ

The LOD was calculated based on the measurement of the negative control shellfish matrix (MLV15) using the blank + 3×SD approach according to Eurachem guidelines (13), as recently applied to AOAC Method **2006.02**, an ELISA for domoic acid in shellfish using a similar four-parameter logistic curve (14). All laboratories reported <dl for this sample using the prescribed cutoff of B/B_0 <0.7 for quantification, with the exception of Laboratory 8, which was removed as an outlier as determined by Grubbs test ($P < 0.01$). If these samples are instead quantified using the B/B_0 values obtained, a mean of 5.5 ng/mL is obtained with an SD of 5.7 ng/mL, resulting in an LOD of 45 µg STX diHCl equiv./kg. Using the blank + 10 × SD definition, an LOQ of 126 µg STX di HCl equiv./kg is thus obtained. We previously established empirically that a 1/10 dilution of shellfish extracts is sufficient to remove matrix effects in the RBA (10), when a quantification cutoff of B/B_0 <0.7 is used. This is the basis for the ten-fold minimum sample dilution used in the current study. The IC_{70} values (B/B_0 0.7) for all standard curves run in the study are presented in Table **2011.27D**. An average of 0.80 ± 0.188 nM STX diHCl was obtained across all assays, following the removal of one outlier based on the Grubbs test ($P < 0.01$). Applying the blank + 3 × SD to this value, an LOD of 64 µg STX diHCl equiv./kg is obtained; applying the blank + 10 × SD to this value results in an LOQ of 131 µg STX diHCl equiv./kg for a sample diluted 1/10 and extracted as indicated in the study, in fair agreement with the value calculated above.

Correlation with HPLC and Mouse Bioassay

Comparison of the RBA results with the mouse bioassay

Nominal	Avg	S_R	RSD_R, %	Recovery, %
200	169	58	34.6	84.4
500	466	133	28.5	93.3
1200	1057	228	21.7	88.1

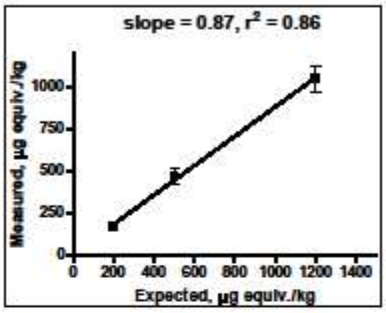


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

results yielded an r^2 of 0.84 and a slope of 1.64, indicating that the RBA reports somewhat higher STX equivalents in shellfish, relative to the mouse bioassay (Figure 3). This overestimate has been previously reported for both RBA and HPLC methods (2, 9) at the STX levels near or below the regulatory limit, which are the focus of the current study. Consistent with these findings, the HPLC method also reported higher values than the mouse bioassay in this study, with a slope of 1.33 and an r^2 of 0.84. RBA results correlated better with the precolumn oxidation HPLC method, with a slope of 1.20 and an r^2 of 0.92.

RBA Yielded No False Negatives Relative to the Regulatory Limit

When the data from the three methods were sorted by increasing µg STX diHCl equiv./kg as reported by the mouse bioassay, the RBA did not report any false negatives when compared to the regulatory limit of 800 µg STX equiv./kg (Table 2011.27E). When compared with the precolumn oxidation HPLC method, only Laboratory 9 reported values lower than the HPLC method. The fact that the RBA reports somewhat higher toxicity than the mouse bioassay or HPLC at levels near or below the regulatory limit is beneficial from a food safety standpoint. The higher values reported presumably arise from better recoveries, as demonstrated above. From a shellfish producer's perspective, the improved detection limits relative to the mouse bioassay and better recovery of low toxin levels compared to the HPLC can help to provide advance warning of developing toxicity, allowing producers to harvest early, delay harvest, or move cultures, as appropriate.

Participants' Comments

Laboratory 6 participated in the study without previous experience running receptor assays, and in doing so, identified several points needing clarification that have since been added to the proposed *Official Method* as enumerated in this report: (*1*) The vacuum required for filtration was not specified at 4–8" Hg, which is critical because insufficient vacuum pressure results in too slow a clearance of the wells, whereas too much pressure results in an airlock and no filtration at all. (*2*) Scintillation counting time for the microplates is 1 min/well. (*3*) Instructions have been added regarding how to calculate sample concentration if more than one dilution falls within B/B_0 0.2–0.7; specifically, an average value should be calculated from all sample dilutions falling within B/B_0 0.2–0.7. When corrected for dilution, serial sample dilutions should yield similar quantification. The absence of linearity between sample dilutions indicates either error in dilution or sample matrix interference; however, at the minimum sample dilutions recommended in the proposed *Official Method*, matrix effects from shellfish homogenates have not been encountered (10). In the current study, the nonlinearity of dilutions experienced in several samples by Laboratory 8 was not observed by the other laboratories, suggesting a systematic sample dilution issue rather than a sample matrix problem. Although experienced in RBAs in general, Laboratory 8 had not previously run the microplate filtration format of the assay for PST.

Laboratory 9, which reported generally lower values than the other laboratories, although familiar with the assay, had not performed it in more than a year. The lower values reported do not appear to be associated with conduct of the assay, or scintillation conduct of the assay, or scintillation counting, since the assay metrics are well within the averages reported by the other laboratories. Insufficient boiling or pH adjustment of sample extracts are a possible explanation. These points identified by the study participants should be added to the critical steps identified in the SLV study (10) that can affect precision and accuracy of the assay results, including: (*1*) ensure that the water is strongly boiling during extraction; (*2*) carefully adjust pH of extracts; (*3*) ensure even distribution of the membrane preparation across the microplate by frequent vortex-mixing or pipetting before and during its addition to the plate; (*4*) the wells must clear within 2–5 s during filtration; (*5*) the wash buffer should be ice-cold to minimize the rate of toxin release from the receptor; and (*6*) following addition of scintillant to the wells, incubate a minimum of 30 min to ensure that the scintillant fully penetrates the filters before counting.

Recommendations

The collaborative study of the RBA for PST was completed by nine laboratories representing six countries. Collaborators quantified PST as a composite toxicity value reported in µg STX di HCl equiv./kg in a variety of shellfish species from different regions of the world, containing varied toxin congener profiles. The study included laboratories with extensive experience as well as others with little or no previous experience. The study also included both microplate and scintillation counters as end points, because either instrument type could potentially be used by test laboratories. The study demonstrates that the RBA yields adequate repeatability, reproducibility, and recovery for routine determination and monitoring of PST in shellfish. The greater precision attained by laboratories that received prior training on the RBA and routinely implement this assay suggests that

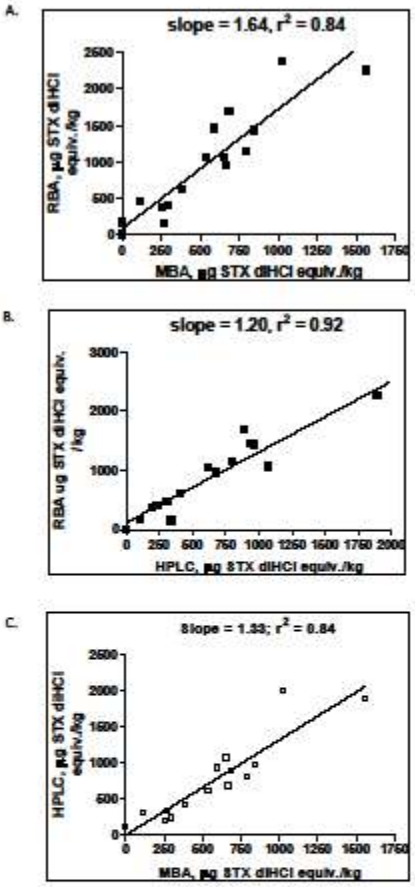


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

the overall interlaboratory reproducibility can be further improved. It is recommended that this method be accepted by AOAC INTERNATIONAL as Official First Action for the determination of PST in shellfish.

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Appendix: Rat Brain Membrane Preparation

The rat brain membrane preparation used in this assay can be produced in bulk, aliquotted, and stored at –80°C until use. Under this storage condition, the preparation is stable for a minimum of 6 months. The following protocol provides sufficient membrane preparation for a minimum of 125 plates and can be scaled up or down as needed.

A. Apparatus

(a) *Teflon/glass homogenizer.*—Motorized tapered Teflon pestle and glass tube, 15 mL.

(b) *Motorized tissue homogenizer.*—Polytron or small hand-held blender.

(c) *High-speed centrifuge and fixed angle rotor.*—Capable of 20000 × *g* (rcf).

(d) *Centrifuge tubes.*—12–15 mL rated for >20000 × *g* (rcf).

(e) *Plastic cryovials.*—2 mL.

(f) *Graduated beaker.*—300 or 500 mL.

(g) *Pipets.*—Disposable 5 and 10 mL.

(h) *Forceps.*

B. Reagents

(a) *20 Rat brains.*—Male, 6-week-old Sprague-Dawley (Hilltop Lab Animals, Inc., Scottdale, PA; http://hilltoplabs.com) or equivalent.

(b) *MOPS.*—pH 7.4 (Sigma, St. Louis, MO; Cat. No. M3183-500G).

(c) *Choline chloride.*—100 mM (Sigma; Cat. No. C7527-500G).

(d) *Phenyl methylsulfonyl fluoride (PMSF).*—Sigma; Cat. No. P7626.

(e) *Isopropanol.*

C. Procedure

(*1*) Prepare 1 L 100 mM MOPS buffer, pH 7.4, containing 100 mM choline chloride (detailed protocol in E, above) and 0.1 mM PMSF. PMSF must first be dissolved in isopropanol; dissolve 0.174 g PMSF in 10 mL isopropanol to make 100 mM stock. Aliquot and store at –20°C. Add PMSF (1/1000, 0.1 mM final concentration) to the MOPS/choline chloride buffer fresh on the day of use.

(*2*) Remove medulla and cerebellum from each brain using forceps and discard. Place the cerebral cortex (*see* Figure 1) in a small amount of ice-cold buffer and place on ice.

(*3*) Place one cerebral cortex in 12.5 mL MOPS/choline Cl/PMSF, pH 7.4, in glass/teflon homogenizer (two brains in 25 mL buffer will fit into 30 mL homogenizer tube). Homogenize at 70% full speed (385 rpm) with at least 10 up and down strokes (more if necessary to homogenize brain; there should be no visible chunks remaining in the homogenate). Keep tube in ice at all times. Pour homogenized tissue into 250 mL beaker on ice and repeat procedure with remaining cortices.

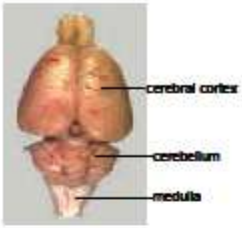


Figure 1. Rat brain.

(*4*) Transfer pooled homogenized tissue to centrifuge tubes, balance the tubes (pairwise; use ice-cold buffer to balance), and centrifuge at 20000 × g for 15 min at 4°C.

(*5*) Aspirate the supernatant and resuspend the pellets in ice-cold MOPS/choline Cl/PMSF buffer, using an adequate amount (~5 mL) to fully resuspend the pellet (can use clean glass stir rod to break up pellet), not exceeding 10 mL per brain.

(*6*) Pool resuspended membrane preparation in a small beaker. Rinse centrifuge tubes with a small amount of ice-cold buffer to recover all of the membrane preparation. Bring total volume to 200 mL total (keep on ice).

(*7*) Keeping the beaker on ice, Polytron (or use a small hand-held blender at low speed) at 70% full speed for 20 s to obtain a consistent homogenate.

(*8*) Aliquot 2 mL/tube into cryovials. It is critical to keep the preparation well mixed while dispensing, e.g., prior to each aliquot to ensure equal allocation of protein/receptors to each vial. Keep cryotubes on ice.

(*9*) Freeze and store at –80°C. This preparation is stable for at least 6 months. Use a permanent marker to label the preparation date on the storage container.

D. Protein Assay

(a) Determine protein concentration of membrane preparation using Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (microplate method) or No. 23225 (tube method) protein assay kit or equivalent protein assay (Thermo Fisher, Rockford, IL). The above protocol should yield 6–8 mg protein/mL of rat membrane preparation.

(b) Determine membrane dilution needed for the assay. The protein concentration in the daily working stock for the assay should be 1 mg/mL (this is diluted in the assay to yield 0.5 mg/mL in-assay concentration). Based on the protein concentration determined in the protein assay, determine the dilution needed to achieve 1 mg/mL. This is the dilution used in section E(e) above for all assays using this lot of membrane preparation. The protocol above typically yields a protein concentration that requires a dilution of 1/6–1/8. (Do not use less than 1/4 dilution or filtration wells may become clogged.) Protein concentration will need to be determined for each new batch of membrane preparation.

AOAC Official Method 2011.27
Paralytic Shellfish Toxins (PSTs) in Shellfish
Receptor Binding Assay
First Action 2011

[Applicable to the determination of paralytic shellfish toxins (PSTs), as µg STX diHCl equiv./kg, in shellfish (mussels, clams, scallops) at levels >149 µg STX diHCl equiv./kg, with a limit of detection (LOD) of 45 STX diHCl equiv./kg shellfish and a limit of quantification (LOQ) of 126 µg STX diHCl equiv./kg shellfish.]

Caution: Wear disposable gloves and protective laboratory coat while performing the assay. PSTs are neurotoxins that are harmful if ingested. The assay uses a tritium labeled tracer, [^{3}H] STX, at low concentration. All laboratories performing the assay must have approved radiation laboratory space and must follow procedures prescribed by their nuclear regulatory agency for receipt, use, and disposal of isotopes.

See Tables **2011.27A–E** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Test portions of shellfish homogenates are extracted using the AOAC mouse bioassay extraction protocol (**959.08**), modified by scale. The PST receptor assay is a competitive binding assay in which [^{3}H] STX competes with unlabeled STX in standards or mixtures of PST in samples for a finite number of available receptor sites (site 1 on the voltage gated sodium channel) in a rat brain membrane preparation. Following establishment of binding equilibrium at 4°C, unbound [^{3}H] STX is removed by filtration and bound [^{3}H] STX is quantified by liquid scintillation counting. A standard curve is generated using increasing concentrations of STX standard from 10^{-10} to 10^{-6} M STX, which results in a reduction in bound [^{3}H] STX that is directly proportional to the amount of unlabeled toxin present. The concentration of toxin in samples is determined in reference to the standard curve. Incubation is carried out in a microplate format to minimize sample handling and the amount of radioactivity used. Bound [^{3}H] STX (as counts per minute; CPM) can be determined either by conventional or by microplate scintillation counting. Both methods are included in this protocol.

B. Apparatus and Supplies

(a) *Traditional or microplate scintillation counter.*

(b) *Micropipettors.*—1–1000 µL variable volumes and disposable tips.

(c) *Eight channel pipettor.*—5–200 µL variable volume and disposable tips.

(d) *96-Well microtiter filter plate.*—With 1.0 µm pore size type GF/B glass fiber filter/0.65 µm pore size Durapore support membrane (Millipore, Bedford, MA, USA; Cat. No. MSFB N6B 50).

(e) *MultiScreen vacuum manifold.*—Millipore; Cat. No. NSVMHTS00.

(f) *Vacuum pump.*

(g) *Centrifuge tubes.*—15 and 50 mL, conical, plastic.

(h) *Mini dilution tubes in 96-tube array.*

(i) *Reagent reservoirs.*

(j) *Ice bucket and ice.*

(k) *Vortex mixer.*

(l) *Sealing tape.*—Millipore; Cat. No. MATA HCL00.

(m) *Volumetric flask.*—1 L.

(n) *–80°C freezer.*

(o) *Refrigerator.*

For traditional scintillation counter only:

(p) *MultiScreen punch device.*—Millipore; Cat No. MAMP 096 08.

(q) *MultiScreen disposable punch tips.*—Millipore; Cat. No. MADP 196 10.

(r) *MultiScreen punch kit B for 4 mL vials.*—Millipore; Cat. No. MAPK 896 0B.

(s) *Scintillation vials.*—4 mL.

For sample extraction:

(t) *Pipets.*

(u) *Centrifuge tubes.*—15 mL, conical, plastic.

(v) *Vacuum pump or house vacuum.*

(w) *pH meter or pH paper.*

(x) *Hot plate.*

(y) *Graduated centrifuge tubes.*—15 mL.

(z) *Centrifuge and rotor for 15 mL tubes.*

C. Reagents

(a) *[^{3}H] STX.*—0.1 mCi/mL, ≥10 Ci/mmol, ≥90% radiochemical purity (American Radiolabeled Chemicals, St. Louis, MO, USA, or International Isotopes Clearinghouse, Leawood, KS, USA).

(b) *STX diHCl.*—NIST RM 8642 (www.nist.gov).

(c) *3-Morpholinopropanesulfonic acid (MOPS).*—Sigma (St. Louis, MO, USA; Cat. No. M3183-500G), or equivalent.

(d) *Choline chloride.*—Sigma (Cat. No. C7527-500G), or equivalent.

(e) *Rat brain membrane preparation.—Appendix 1* [*J. AOAC Int.* (future issue)].

For traditional counter:

(f) *Scintiverse BD liquid scintillation cocktail.*—Fisher Scientific (Waltham, MA, USA; Cat. No. SX-18), or equivalent.

For microplate counter:

(g) *Optiphase liquid scintillation cocktail.*—PerkinElmer Life Sciences (Downers Grove, IL, USA; Cat. No. 1200-139), or equivalent.

For sample extraction:

(h) *Hydrochloric acid (HCl).*—1.0 and 0.1 M.

(i) *Sodium hydroxide.*—0.1 M.

(j) *Water.*—Distilled or deionized (18 µΩ).

D. Sample Extraction

Accurately weigh 5.0 g tissue homogenate into a tared 15 mL conical tube. Add 5.0 mL of 0.1 M HCl, vortex, and check pH. If necessary, adjust pH to 3.0–4.0 as determined by a pH meter or pH paper. To lower pH, add 1 M HCl dropwise with mixing; to raise pH, add 0.1 M NaOH dropwise with mixing to prevent local alkalinization and consequent destruction of toxin. Place the tube in a beaker of boiling water on hot plate for 5 min with the caps loosened. Remove and cool to room temperature. Check pH and adjust cooled mixture to pH 3.0–4.0 as described above. Transfer entire contents to graduated centrifuge tube and dilute volumetrically to 10 mL. Gently stir contents to homogeneity and allow to settle until portion of supernatant is translucent and can be decanted free of solid particles. Pour approximately 5 to 7 mL of the translucent supernatant into a centrifuge tube. Centrifuge at 3000 × *g* for 10 min. Retain clarified supernatant and transfer to a clean centrifuge tube. Store extracts at –20°C until tested in

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Table 2011.27A. Receptor binding assay results on individual samples (values are in µg STX diHCl equiv./kg shellfish tissue); summary statistics on all samples; summary statistics excluding Laboratory 9

	Sample		Lab									All labs				Labs 1–8			
Assay	No.	ID	1	2	3	4	5	6	7	8	9	Mean	S_R	RSD_R, %	HorRat	Mean	S_R	RSD_R, %	HorRat
Day 1	1	MLV05	370	610	620	410	690	1070	630	660	330	599	222	37.1	2.2	633	212	33.5	2.0
	2	MLV06	1100	1340	1320	1440	1260	1720	2080	2130	890	1476	422	28.6	1.9	1549	386	24.9	1.7
	3	MLV08	80	190	140	90	130	160	230	220	100	149	55	37.2	1.8	155	56	36.0	1.7
	4	MLV09	860	680	950	870	980	1120	1460	820	590	926	255	27.5	1.7	968	237	24.5	1.5
	5	MLV12	180[a]	200	200	150	150	100	150	290	100	168	62	37.2	1.8	177	60	34.1	1.7
	6	MLV14	950	940	1060	1130	1040	750	1460	1320	810	1051	228	21.7	1.4	1081	224	20.7	1.3
	7	MLV16	660	930	1080	870	840	1320	1490	2420[b]	490	960	329	34.3	2.1	1027	291	28.3	1.8
Day 2	8	MLV01	1360	1520	1580	1110	1700	3180	1400	2780	520	1683	818	48.6	3.3	1829	739	40.4	2.8
	9	MVL02	830	1180	1130	1150	1130	1780	1340	980	690	1134	311	27.4	1.8	1190	281	23.6	1.5
	10	MLV04	2440	2840	2910	1740	2150	1810	2690	2490	1210	2253	572	25.4	1.8	2384	446	18.7	1.3
	11	MLV07	1260	1540	1220	1980	1760	1530	1660	1210	840	1444	345	23.9	1.6	1520	279	18.3	1.2
	12	MLV09	810	1190	1130	810	1630	1390	1880	1120	870	1203	372	30.9	2.0	1245	375	30.1	2.0
	13	MLV11	270	370	480	340	640	490	240	600	110	393	174	44.3	2.4	429	148	34.4	1.9
	14	MLV13	400	1240[b]	560	450	650	530	500	440	200	466	133	28.5	1.6	504	85	16.8	1.0
Day 3	15	MLV03	330	270	410	180	590	680	370	1570[b]	90	365	197	54.0	2.9	404	176	43.5	2.4
	16	MLV05	580	670	250	430	910	700	860	940	300	627	257	41.1	2.4	668	242	36.2	2.1
	17	MLV06	1290	1520	1460	970	1800	2520	1470	870	1250	1461	488	33.4	2.2	1488	515	34.6	2.3
	18	MLV07	1010	1600	1390	1000	1720	1860	1520	2150	890	1460	429	29.4	2.0	1531	397	26.0	1.7
	19	MLV10	1640	2130	2800	2660	2330	1850	3390	2740	1830	2374	570	24.0	1.7	2443	569	23.3	1.7
	20	MLV11	430	350	460	280	550	620	1149[b]	410	250	419	127	30.2	1.7	443	115	26.0	1.4
	21	MLV15	ND[c]	ND	ND	ND	ND	ND	ND	180	ND	—	—	—		—	—	—	
Avg. RSD_R														33.2				28.7	
Avg. HorRat															2.0				1.8

[a] CV 41%; not used in calculations.

[b] Outlier; not used in calculations.

[c] ND = Not detected.

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Table 2011.27B. Summary statistics on blind duplicates, run in separate assays (values are in µg STX diHCl equiv./kg)

	MLV05		MLV06		MLV07		MLV09		MLV11		
Lab	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Avg.
1	370	580	1100	1290	1260	1010	860	810	270	430	
2	610	670	1340	1520	1540	1530	680	1190	370	350	
3	620	250	1320	1460	1220	1390	950	1130	480	401	
4	410	430	1440	970	1980	1000	870	810	340	280	
5	690	910	1260	1790	1760	1720	980	1630	640	550	
6	1070	700	1720	2520	1530	1860	1120	1390	490	620	
7	630	880	2090	1240	1750	1150	1460	1830	230*	1149*	
8	660	940	2130	870	1210	2150	820	1120	600	410	
9	330	300	890	1250	840	890	590	870	110	250	
Avg.		614		1453		1433		1062		416	
S_r		169		432		366		247		83	
S_R		239		444		387		338		152	
RSD_r, %		27.5		29.4		25.5		23.3		20.0	25.1
RSD_R, %		38.9		30.2		27.0		31.9		36.5	32.9
HorRat		2.3		2.0		1.8		2.0		2.0	2.0

* Outlier; not used in calculation.

receptor assay.

E. Preparation of Stock Solutions and Standards

(a) *Assay buffer.*—100 mM MOPS/100 mM choline chloride, pH 7.4. Weigh out 20.9 g MOPS and 13.96 g choline chloride and add to 900 mL dH_2O. Adjust pH to 7.4 with NaOH while stirring and bring to a final volume of 1 L with dH_2O. Store at 4°C.

(b) *Radioligand solution.*—Calculate the concentration of [^{3}H] STX stock provided by the supplier, which may vary between lots. Suppliers generally provide the specific activity in Ci/mmol (generally 10–30 Ci/mmol) and activity in mCi/mL (0.05–0.1 mCi/mL), from which the molar concentration can be calculated. Prepare 4 mL of a 15 nM working stock of [^{3}H] STX fresh daily in 100 mM MOPS/100 mM choline chloride buffer. This will provide sufficient volume for one 96-well plate at an in-well concentration of 2.5 nM. Measure total counts of each working stock prior to running an assay: add 35 µL of the working stock [^{3}H] STX in buffer to a liquid scintillation vial with 4 mL scintillant and count on a traditional liquid scintillation counter. This is done to confirm correct dilution prior to running the assay. Depending on the efficiency of the scintillation counter used, the corresponding CPM will vary, but should be consistent day-to-day and within 15% of the expected value.

(c) *Unlabeled STX standard working solution.*—The STX diHCl standard is provided at a concentration of 268.8 µM (100 µg/mL). A "bulk" standard curve can be made up in advance and stored at 4°C for up to 1 month. The use of a bulk standard curve minimizes the pipetting needed for setting up an assay routinely and improves day-to-day repeatability. Make up 3 mM HCl (e.g., from a 3 M stock, 50 µL in 50 mL), then perform the serial dilutions (*see* Table 2011.27F) of NIST RM 8642 STX diHCl (100 µg/mL = 268.8 µM) to make up the standard curve in 3 mM HCl. These standard stock solutions will be diluted 1/6 in the assay to yield the designated in-assay concentrations (*see* Table 2011.27F).

(d) *Interassay calibration standard (QC check).*—Prepare a reference standard containing 1.8×10^{-8} M STX standard (3.0×10^{-8} M STX in assay) in advance in 3 mM HCl and keep frozen (–80°C) in 1 mL aliquots for long-term storage. Aliquots should be thawed and stored at 4°C for routine use (stable up to 1 month) and analyzed in each assay. This serves as a QC check and confirms day-to-day performance of the assay.

(e) *Rat brain membrane preparation.*—Prepare rat brain membrane preparation in bulk [*Appendix 1; J. AOAC Int.* (future issue)] and store at –80°C until used in the assay. Thaw an aliquot of rat brain membrane preparation on ice. Dilute membrane preparation with cold (4°C) 100 mM MOPS/100 mM choline chloride, pH 7.4, to yield a working stock with a protein concentration of 1.0 mg/mL (this will be diluted in the assay plate to 0.5 mg/mL in-well concentration). Vortex vigorously to achieve a visibly homogeneous suspension. Keep the diluted membrane preparation on ice until ready to use.

F. Performing the Assay

(a) *Plate setup.*—When possible, use a multichannel pipet to minimize pipetting effort and increase consistency. Standard curve, QC check, and sample extracts are run in triplicate wells. Multiple dilutions of sample extracts should be analyzed in order to obtain a value that falls between 0.2–0.7 B/B_0 on the standard curve for quantification. For ease of analysis, it is convenient to use a standard plate layout that maximizes the number of samples and standards that can be analyzed on one plate. For shellfish extracts, a minimum dilution of 1:10 is used, which minimizes potential matrix effects, while still providing an LOQ of approximately 126 µg/kg shellfish (*see* Table 2011.27G).

(b) *Addition of samples and standards.*—Add in the following order to each of the 96 wells: 35 µL assay buffer; 35 µL STX standard, QC check, or sample extract; 35 µL [^{3}H] STX; 105 µL membrane preparation. The assay buffer is added first in order to wet the filter membrane. It is critical to continuously mix the membrane preparation by careful up-and-down pipetting immediately prior to

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Table 2011.27C. Performance of individual laboratories on blind duplicates (values are in µg STX diHCl equiv./kg)

Laboratory	ID	Day 1	Day 2	Mean	s,	RSD, %
1	MLV05	370	580	475	148	31.3
	MLV06	1100	1290	1195	134	11.2
	MLV07	1260	1010	1135	177	15.6
	MLV09	860	810	835	35	4.2
	MLV11	270	430	350	113	32.3
Avg.						18.9
2	MLV05	605	670	638	46	7.2
	MLV06	1340	1520	1430	127	8.9
	MLV07	1540	1530	1535	7	0.5
	MLV09	680	1190	935	361	38.6
	MLV11	370	350	360	14	3.9
Avg.						11.8
3	MLV05	620	250	435	262	60.1
	MLV06	1320	1460	1390	99	7.1
	MLV07	1220	1303	1262	59	4.7
	MLV09	950	1130	1040	127	12.2
	MLV11	480	460	470	14	3.0
Avg.						17.4
4	MLV05	410	430	420	14	3.4
	MLV06	1440	970	1205	332	27.6
	MLV07	1980	1000	1490	693	46.5
	MLV09	870	810	840	42	5.1
	MLV11	340	280	310	42	13.7
Avg.						19.2
5	MLV05	690	910	800	156	19.4
	MLV06	1260	1790	1525	375	24.6
	MLV07	1760	1720	1740	28	1.6
	MLV09	980	1630	1305	460	35.2
	MLV11	640	550	595	64	10.7
Avg.						18.3
6	MLV05	1070	700	885	262	29.6
	MLV06	1720	2520	2120	566	26.7
	MLV07	1530	1860	1695	233	13.8
	MLV09	1120	1390	1255	191	15.2
	MLV11	490	620	555	92	16.6
Avg.						20.4
7	MLV05	630	880	755	177	23.4
	MLV06	2090	1240	1665	601	36.1
	MLV07	1750	1150	1450	424	29.3
	MLV09	1460	1830	1645	262	15.9
	MLV11	230*	1150*			
Avg.						26.2
8	MLV05	660	940	800	198	24.7
	MLV06	2130	870	1500	891	59.4
	MLV07	1210	2150	1680	665	39.6
	MLV09	820	1120	970	212	21.9
	MLV11	600	410	505	134	26.6
Avg.						34.4
9	MLV05	330	300	315	21	6.7
	MLV06	890	1250	1070	255	23.8
	MLV07	840	890	865	35	4.1
	MLV09	590	870	730	198	27.1
	MLV11	110	250	180	99	55.0
Avg.						23.3
Overall avg.						22.2

* Outlier; not used in calculations.

dispensing into the 96-well plate to maintain an even suspension across the entire plate. Cover and incubate plate at 4°C for 1 h.

(c) *Assay filtration.*—Attach the vacuum manifold to the vacuum pump with an in-line side arm flask to catch filtrate from the plate filtration process. Set the vacuum pressure gauge on the pump or vacuum manifold to 4–8" Hg (135–270 millibar), as specified in the instructions provided with the filtration plates. Place the 96-well plate on the vacuum manifold. Fill empty wells with 200 µL MOPS/choline chloride buffer to ensure even vacuum pressure and filtration across the plate. Turn on vacuum. Optimum vacuum will pull the wells to dryness in 2–5 s. Pull contents of all wells through until all liquid is removed. (*Note*: Too low a vacuum will result in slow well clearance, but too high will result in an airlock and no well clearance.) With vacuum pump running, quickly rinse each well twice with 200 µL ice cold MOPS/choline chloride buffer using multichannel pipet. Maintain vacuum until liquid is removed.

(d) *Preparation of the assay for counting.*—Remove the plastic bottom from the plate. Blot the bottom once on absorbent toweling.

(*1*) *For counting in microplate scintillation counter.*—Place the microplate in a counting cassette. Seal the bottom of the 96-well plate with sealing tape. Add 50 µL Optiphase scintillation cocktail per well using multichannel pipet. Seal the top of the plate with sealing tape. Allow to incubate 30 min at room temperature. Place the plate in a counting cassette and count in a microplate scintillation counter for 1 min/well.

(*2*) *For counting in traditional scintillation counter.*—Place the microplate in the MultiScreen punch system apparatus. Place the disposable punch tips on top of the microplate. Punch the filters from the wells into scintillation vials and fill with 4 mL scintillation cocktail (Scintiverse or equivalent). Place caps on the vials and vortex. Allow vials to sit overnight in the dark, then count using a tritium window in a traditional scintillation counter.

G. Analysis of Data

For assays performed using the traditional counter, curve fitting is performed using a four-parameter logistic fit, also known as a sigmoidal dose response curve (variable slope; *see* Figure 2011.27), or Hill equation:

$$y = \min + \frac{\max - \min}{1 + 10^{(x - \log IC50)(HillSlope)}}$$

where max is the top plateau representing maximum binding in CPM in the absence of competing nonradiolabeled STX, also known as B_o; min is the bottom plateau, equal to nonspecific binding (in CPM) in the presence of saturating nonradiolabeled toxin; IC_{50} is the inhibitory concentration at which CPM are 50% of max-min (dashed lines; Figure 2011.27); Hill slope is the slope of the curve; x axis is the log concentration of STX; and y axis is total ligand binding in CPM (here represented as B/B_o, or bound/max bound). A curve fitting package such as Prism (Graph Pad Software, Inc.) is recommended. For the microplate counter users, receptor assay applications provided by the manufacturer may be used (e.g., MultiCalc; PerkinElmer Wallac, Gaithersburg, MD, USA).

(a) *Sample quantification.*—Sample quantification is carried out only on dilutions that fall within B/B_o of 0.2–0.7, where B represents the bound [^{3}H]STX (in CPM) in the sample and B_o represents the max bound [^{3}H]STX (in CPM). Where more than one dilution falls within B/B_o of 0.2–0.7 on the curve, all sample wells corresponding to these dilutions are used to calculate sample concentration. Sample concentration is calculated in µg STX diHCl

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Table 2011.27D. Calibration curve and QC check parameters in three receptor binding assays performed in nine participant laboratories

Lab	Assay day	Slope	IC_{50}, nM	QC, nM	Reference, CPM	IC_{50}, nM	Standards where RSD >30%; action	Curve fitting software	Scintillation counter	Manual/ microplate
1	1	–0.9	1.9	2.4	720	0.90	None	Prism v 3.02	Packard Top Count	Microplate
	2	–1.0	2.0	2.6	733	0.96	None			
	3	–1.1	2.1	3.2	1038	0.92	None			
2	1	–1.1	1.8	3.8	1160	0.66	3 nM; 1 well removed	Prism v 5.0	Packard Top Count	Microplate
	2	–1.2	2.2	3.9	1260	0.85	None			
	3	–1.0	1.6	3.2	1262	0.46	3 nM, 1 nM removed			
3	1	–1.0	2.0	2.3	2529	0.41	First column removed	Prism v 5.0	Wallac Microbeta	Microplate
	2	–0.9	2.0	2.5	1463	0.92	1000 nM; 1 well removed			
	3	1.0	1.6	2.8	2088	0.80	None			
4	1	-0.9	1.7	3.4	1125	0.61	None	Prism v 3.03	PerkinElmer Tricarb	Manual
	2	–1.2	1.7	3.2[a]	1611	0.77	None			
	3	–0.9	1.2	2.9	1324	0.45	30 nM 35%; 1 well removed			
5	1	–0.9	1.4	3.3	1566	0.64	1.0 nM; 1 well removed	MultiCalc	Wallac Microbeta	Microplate
	2	–1.2	1.8	3.6	1528	1.05	0.1 nM and 30 nM; 1 well removed			
	3	–1.2	1.8	2.9	1052	0.67	None			
6	1	–1.1	2.6	3.0	670	1.15	None	Prism v 4.0	Wallac Microbeta	Microplate
	2	–1.0	2.0	4.0[b]	1124	1.08	None			
	3	–1.1	3.4	6.5[b]	1030	2.04[c]	None			
7	1	–0.8	1.0	2.8[a]	919	0.33	None	Prism	Wallac Microbeta	Micropolate
	2	–1.0	1.6	2.7	619	0.70	None			
	3	–0.9	2.1	3.2[a]	693	0.82	None			
8	1	–1.2	1.7	3.7	1146	0.86	None	Prism	Wallac Microbeta	Microplate
	2	–1.1	1.4	1.5[b]	1095	0.78	None			
	3	–1.1	2.4	2.3	886	1.04	None			
9	1	–1.0	2.2	4.0[b]	1363	0.97	None	Prism	Wallac Microbeta	Microplate
	2	–1.0	2.0	3.2	1380	0.85	100 nM 33%; left in			
	3	–1.0	2.1	3.7	1532	0.92	None			

[a] One well removed.

[b] Outside of specifications.

[c] Outlier by Grubbs test.

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Table 2011.27E. Results of the receptor binding assay (RBA), mouse bioassay (MBA), and HPLC analyses of 21 shellfish extracts, sorted by mouse bioassay value (all values are in µg STX diHCl equiv./kg shellfish tissue; results in bold indicate toxicity above the 800 µg STX diHCl equiv./kg regulatory limit; all other results indicate toxicity below the regulatory limit)

Sample	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab 9	RBA, avg.	HPLC	MBA
21	ND[a]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
5	180	200	200	150	150	100	150	290	100	168	108	ND
15	330	270	410	180	590	680	370	**1570**[b]	90	365	196	182
13	270	370	480	340	640	290	240	600	110	371	236	299
20	430	350	460	280	550	490	**1150**[b]	410	250	403	236	299
14	400	**1240**[b]	560	450	650	530	500	440	200	466	625	343
1	370	610	620	410	690	**1070**[b]	**630**[b]	660	330	599	413	387
16	580	670	250	430	910	700	**860**[b]	**940**[b]	300	627	413	387
3	80	190	140	90	130	160	230	220	100	149	341	405
6	**950**	**940**	**1060**	**1130**	**1040**	750	**1460**	**1320**	**810**	**1051**	618	485
7	**660**	**930**	**1080**	**870**	**840**	**1320**	**1490**	**2420**	490	**960**	685	528
2	**1100**	**1340**	**1320**	**1440**	**1260**	**1720**	**2080**	**2130**	**890**	**1476**	**931**	595
17	**1290**	**1520**	**1460**	**970**	**1800**	**2520**	**1470**	**870**	**1250**	**1460**	**931**	595
4	**860**	**680**	**950**	**870**	**980**	**1120**	**1460**	**820**	590	**926**	**1070**	653
12	**810**	**1190**	**1130**	**810**	**1630**	**1390**	**1880**	**1120**	**870**	**1203**	**1070**	653
11	**1260**	**1540**	**1220**	**1980**	**1760**	**1530**	**1660**	**1210**	**840**	**1444**	**965**	714
18	**1010**	**1600**	**1390**	**1000**	**1720**	**1860**	**1520**	**2150**	**890**	**1452**	**965**	714
8	**1360**	**1520**	**1580**	**1110**	**1700**	**3180**	**1400**	**2780**	**520**	**1683**	**894**	752
9	**830**	**1180**	**1130**	**1150**	**1130**	**1780**	**1340**	**980**	690	**1134**	**802**	792
19	**1640**	**2130**	**2800**	**2660**	**2330**	**1850**	**3390**	**2740**	**1830**	**2374**	**2000**	**1027**
10	**2440**	**2840**	**2910**	**1740**	**2150**	**1800**	**2690**	**2490**	**1210**	**2252**	**1890**	**1080**

[a] ND = Not detected.

[b] Outlier; not used in average calculation.

equiv./kg shellfish, using the following formulas:

$$(\text{nM STX equiv.}) \times (\text{sample dilution}) \times \frac{(210\ \mu\text{L total volume})}{35\ \mu\text{L sample}} = \text{nM STX equiv. in extract}$$

$$(\text{nM STX diHCl equiv. in extract}) \times \frac{1\ \text{L}}{1000\ \text{mL}} \times \frac{372\ \text{ng}}{\text{nmol}} \times \frac{1\ \mu\text{g}}{1000\ \text{ng}} = \mu\text{g STX diHCl equiv./mL}$$

$$\mu\text{g STX diHCl equiv./mL} \times \frac{\text{mL extract}}{\text{g shellfish}} \times \frac{1000\ \text{g}}{\text{kg}} = \mu\text{g STX diHCl equiv./kg}$$

H. Assay Performance Standards

The following criteria must be met for assay acceptance:

(a) For a ligand that specifically binds at one receptor site, the slope of the resulting competition curve should theoretically be –1.0. If the slope of the curve for a given assay is outside of the acceptable range of –0.8 to –1.2, linearity of the assay will be compromised and quantification of the unknowns will be incorrect.

(b) RSDs of triplicate CPMs for standards should be below 30% as variability may affect the slope calculation and thereby quantification of samples.

(c) If the IC_{50} is out of the acceptable range (2.0 nM ± 30%) then the assay should be considered suspect and rerun, as a shift in the curve will result in over- or underestimation of sample concentrations.

(d) QC check should be 3 nM STX ± 30% (in-well concentration).

Table 2011.27F. Dilution series to prepare bulk solutions for standard curve

	Stock, M	In-assay, M
100 µL 268.8 µM STX + 4.38 mL 0.003 M HCl	6×10^{-6}	1×10^{-6}
500 µL 6×10^{-6} M + 4.5 mL 0.003 M HCl	6×10^{-7}	1×10^{-7}
1.5 mL 6×10^{-7} M + 3.5 mL 0.003 M HCl	1.8×10^{-7}	3×10^{-8}
500 µL 6×10^{-7} M + 4.5 mL 0.003 M HCl	6×10^{-8}	1×10^{-8}
500 µL 1.8×10^{-7} M + 4.5 mL 0.003 M HCl	1.8×10^{-8}	3×10^{-9}
500 µL 6×10^{-8} M + 4.5 mL 0.003 M HCl	6×10^{-9}	1×10^{-9}
500 µL 6×10^{-9} M + 4.5 mL 0.003 M HCl	6×10^{-10}	1×10^{-10}
5 mL 0.003 M HCl	0	Reference

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Table 2011.27G. Recommended microplate layout for ease of handling triplicate wells of standard curve, QC check sample, and unknown samples; each sample is run at three dilutions (1:10, 1:50, 1:200); standard curve is run in columns 1–3 (values are in M STX)[a]

Microplate row	Microplate column											
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-6}	10^{-6}	10^{-6}	QC	QC	QC	U3 1:50	U3 1:50	U3 1:50	U6 1:10	U6 1:10	U6 1:10
B	10^{-7}	10^{-7}	10^{-7}	U1 1:10	U1 1:10	U1 1:10	U3 1:200	U3 1:200	U3 1:200	U6 1:50	U6 1:50	U6 1:50
C	3×10^{-8}	3×10^{-8}	3×10^{-8}	U1 1:50	U1 1:50	U1 1:50	U4 1:10	U4 1:10	U4 1:10	U6 1:200	U6 1:200	U6 1:200
D	10^{-8}	10^{-8}	10^{-8}	U1 1:200	U1 1:200	U1 1:200	U4 1:50	U4 1:50	U4 1:50	U7 1:10	U7 1:10	U7 1:10
E	3×10^{-9}	3×10^{-9}	3×10^{-9}	U2 1:10	U2 1:10	U2 1:10	U4 1:200	U4 1:200	U 1:200	U7 1:50	U7 1:50	U7 1:50
F	10^{-9}	10^{-9}	10^{-9}	U2 1:50	U2 1:50	U2 1:50	U5 1:10	U5 1:10	U5 1:10	U7 1:200	U7 1:200	U7 1:200
G	10^{-10}	10^{-10}	10^{-10}	U2 1:200	U2 1:200	U2 1:200	U5 1:50	U5 1:50	U5 1:50			
H	REF	REF	REF	U3 1:10	U3 1:10	U3 1:10	U5 1:200	U5 1:200	U5 1:200			

[a] REF = Reference; QC = quality control check; U = unknown sample. [Note: The same standard curve may be used for multiple plates (i.e., 11 samples can be run on subsequent plates in a series if the standard curve is not included).]

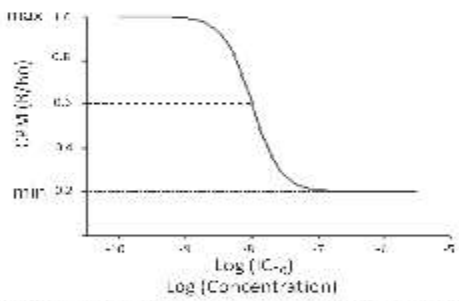


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}

Assays with a QC check sample out of specifications should trigger a check of the IC_{50} value.

The following criteria must be met for acceptability of a sample measurement:

(a) Sample quantification should be done only on dilutions that fall within B/B_0 of 0.2–0.7. In the event that all sample dilutions fall below B/B_0 0.2 (i.e., concentration is too high), further dilutions must be made and the sample reanalyzed. In the event that the sample concentration is too low to be quantified (i.e., $B/B_0 > 0.7$), the sample is reported as below LOD. If more than one dilution falls on the linear part of the curve, an average value calculated from all dilutions should be used. If there is disagreement between different dilutions in final concentration reported, check for error in the sample dilution process.

(b) RSD of the sample CPMs should be ≤30%.

Reference: *J. AOAC Int.* **95**, 795(2012)

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Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	ISSC Laboratory Committee
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6. City, State, Zip	Richmond, VA 23227
7. Phone	(803) 330-6380
8. Email	Issc@issc.org
9. Proposal Subject	Provide Guidance on Performing Laboratory Method Platform Extensions
10. Specific NSSP Guide Reference	Section IV: Guidance Documents, Chapter III. Laboratory, @.02 Methods and Single Lab Validation For Laboratory Methods page in the Laboratory Section of the ISSC Website
11. Text of Proposal/ Requested Action	New guidance document attached.
12. Public Health Significance	Modifications to validated laboratory methods are sometimes necessary in order to improve accessibility, adapt to new equipment or technology, or adapt to changes in availability or suitability of materials utilized in the original method. Additional studies must be done to demonstrate that these modified methods perform equivalently to the approved method before they can be used within the NSSP. In some situations, the modifications to the method are significant enough that a full Single Lab Validation is necessary. In other situations, a smaller extension study may be appropriate. These documents provide guidance on how and when to conduct a platform extension study in order to validate certain modifications to laboratory methods.
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Section IV: Guidance Documents, Chapter III. Laboratory, @.02 Methods, .04 Laboratory Method Platform Extensions

Laboratory methods approved for use within the National Shellfish Sanitation Program (NSSP) must be validated for their intended use before being adopted into the program. Each method approval is specific to the equipment, materials, and laboratory procedures used as part of the original validation study.

In some circumstances, modifications to validated methods are necessary to improve accessibility, adapt to new equipment or technology, or adapt to changes in availability or suitability of materials utilized in the original method. Additional studies must be done to demonstrate that these modified methods perform equivalently to the approved method before they can be used within the NSSP. In some situations, the modifications are significant enough that a full Single Lab Validation (SLV) is necessary. In other situations, a smaller extension study may be appropriate.

In the case of Real-Time PCR Methods, extension studies can be used to demonstrate equivalent method performance when the method modification involves any of the following:

1) New Platform/Instrument
    a. New company, same chemistry
    b. Same company, new model
2) Analysis changes (i.e. use of auto-thresholds instead of set thresholds)
3) Changes in detection hardware (i.e. optical system updated, closed vs. automated system)
4) Changes in cycle conditions (i.e. temperature or time)
5) Changes in extraction protocols
6) Changes in enzyme
7) Changes in reagents
8) Changes in primer/probe (i.e. new manufacturer, new structure, new type, new fluorophore)

If a method modification falls into any of these categories, then a platform extension study should be conducted and the data submitted to the ISSC for review following the process outlined in the ISSC Constitution, Bylaws, and Procedures, Procedure XV, starting with the required pre-proposal, and moving on to the validation study.

For guidance on performing a platform extension study for a Real-Time PCR Method, please refer to the platform extension guidelines available on the ISSC website.

Guidance has not yet been developed on what types of biotoxin method modifications can be validated through a platform extension. If you believe that your proposed biotoxin method modification can be validated through a platform extension, please contact the ISSC Laboratory Committee for consultation on how to proceed before undertaking the validation.

****Note: Pages 2-7 of this document are intended to be hosted on the Laboratory section of the ISSC website along with the other method validation guidelines and SOPs****

ISSC Platform Extension Guidelines

Suggested Procedures for Validating a Real-Time PCR Method Platform Extension

This document provides a detailed outline of the types of information and data the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Committee (LC) requests from submitters for extension of current NSSP methods to additional instrument platforms. These recommendations are intended for methods which have already undergone single laboratory validation (SLV) and are being considered for use with a different instrument or platform, which requires further validation.

Platform Extension Pre-Study Requirement

Prior to initiation of the platform extension study, an initial study should be conducted to determine the likelihood of successful completion of the platform extension. This initial study should be conducted in the absence of matrix (i.e. a pure culture isolate).

Pure culture of the target organism should be grown and diluted serially 10-fold to beyond the limit of detection. Dilutions should be spread plated on a non-selective media in triplicate, and the results should be averaged to determine the concentrations. Equivalent dilutions of three independently grown cultures of the same organism, each analyzed in triplicate, should be used to generate standard curves.

From these standard curves, the following performance criteria should be evaluated:

1) Method Sensitivity – Based on the lowest detectable copy number.
2) Reaction Efficiency – Based on the formula $E = 100 \times (-1+10^{(-1/slope)})$.
3) Repeatability – Based on the standard deviation of generated C_t values.

To be acceptable, method sensitivity should be <20 CFU/rxn, reaction efficiency should be 90-110%, and repeatability should average ≤ 0.5 C_t. If these criteria are met, then the full Platform Extension Study should be conducted in the appropriate matrix(ces) as described below. If these criteria are not met, the method should be further modified to produce acceptable results on the new platform, and a full SLV may be required.

Platform Extension Study

Note: These guidelines are designed for use by a laboratory that has access to the original method platform as well as the intended new platform. If the original platform is not available, it is recommended that the laboratory attempt to collaborate with a laboratory that has the original platform available, or that retains samples analyzed using the original platform, to generate the necessary data.

The purpose of the platform extension study is to generate data that can be analyzed to determine if a modified platform performs in an equivalent manner to the original method platform. Unlike with other ISSC validations, however, this suggested procedure does not directly assess performance characteristics for the modified method. Instead, characteristics like Accuracy, Sensitivity, Specificity, Precision, and overall Comparability are indirectly assessed by comparing the results generated by the original platform with results generated by the modified platform to ensure equivalent performance. This assessment evaluates the following:

- The modified method should correctly detect the presence or absence of a target pathogen or DNA sequence at an equivalent level to the original method.
- The modified method should have false negative and false positive rates equivalent to or better than the original method.
- The modified method should reliably detect the presence of the analyte at the Limit of Detection (LOD) that was determined through the original SLV.
- The modified method should demonstrate a comparable cycle threshold (Ct) to the original method.

To generate this data, the following experiments should be conducted, with all samples analyzed on both the validated platform and the proposed platform. The data from both experiments will be compiled into one complete dataset for analysis.

Experiment 1: Use shellfish free of the target organism(s). For each shellfish matrix of interest, use a minimum of 12 animals and prepare as a homogenate. Take four aliquots of the sample homogenate appropriately sized for the work and spike the first aliquot at or near the LOD for the validated method. Spike the second aliquot at a concentration 0.5 to 1 log higher and spike the third aliquot at the highest level of interest. Do not spike the fourth aliquot, as this will be used as the sample blank. To ensure the accuracy of the spikes, determine the concentrations of the organisms in each spike by plating (in triplicate and calculating the average) on a non-selective media.

For each aliquot, excluding the sample blank sub-aliquot for three replicate analyses. Process each replicate, as well as the blank (a total of 10 MPNs) according to the NSSP validated protocol. Run each extract on both the validated platform and the proposed platform. Repeat this process for a minimum of three shellfish samples collected from different growing areas, the same growing area harvested on different days, or from different process lots. Use the same target spike levels on each of the three shellfish samples. (Figure 1)

Experiment 2: Use naturally incurred samples. For each shellfish matrix of interest, use a minimum of 12 animals per sample and prepare as a homogenate. Process each sample using the established NSSP method and run each extract on both the validated platform and the proposed platform. Repeat this process for a minimum of five shellfish samples collected from different growing areas, the same growing area harvested on different days, or from different process lots. (Figure 2)

Data Analysis

Note: Because this analysis is assessing the relative performance of the two PCR platforms, each MPN tube subsequently tested via PCR counts as an individual "sample" for all the assessments below.

Data analysis for a platform extension should answer the following questions:

1) Does the modified method correctly detect the presence or absence of a target pathogen or DNA sequence at an equivalent level to the original method?
2) Does the modified method have false positive and false negative rates equivalent to or better than the original method?
3) Does the modified method reliably detect the presence of the analyte at the Limit of Detection that was determined through the original SLV?
4) Does the modified method demonstrate comparable cycle thresholds (Cts) to the original method?
5) Is this modified method comparable to the original method?

Several of the analyses below make use of this contingency table to determine the values to input into the equations. Complete this table utilizing the compiled results from experiments 1 and 2.

Contingency Table

	Validated PCR (+)	Validated PCR (-)	Total
Modified Assay (+)	A (True Positive)	B (False Positive)	A+B
Modified Assay (-)	C (False Negative)	D (True Negative)	C+D
Total	A+C	B+D	N (Total Samples)

Cohen's Kappa Coefficient (K) quantifies the level of agreement between the methods above what is expected by chance alone. Calculate K using this online calculator: https://www.graphpad.com/quickcalcs/kappa1/

Percent Positive Agreement (Estimated Sensitivity) = 100 x (A/(A+C))
Percent Negative Agreement (Estimated Specificity) = 100 x (D/(B+D))
Overall Percent Agreement (Total Accuracy) = 100 x ((A+D)/N)

McNemar's Test determines if the differences between discordant pairs (i.e. original method vs. modified method) are significant. Run McNemar's Test using the contingency table to complete this online calculator: https://www.graphpad.com/quickcalcs/mcnemar1/

The **Bland-Altman Plot** is a visual method used to analyze the agreement between two different measurement methods/instruments. Utilizing the C_t values from Experiments 1 and 2, create a data table as follows:

a. X = mean of each pair of measurements (validated platform and proposed platform)
b. Y = difference between each pair of measurements

Example:

Sample	X	Y
1	19.9	1.2
2	19.8	1.1
3	23.4	-1.2
4	36.6	-0.3
5	21.1	0.8

Create a scatter plot using these X,Y coordinate pairs. Calculate the overall average of all the differences and draw a horizontal line on the plot at this value (Bias Line). Calculate the standard deviation (SD) of all the differences and draw horizontal lines at the Bias Line ± 1.96(SD) (Limits of Agreement).

Use the following information to determine the success of the platform extension study:

Cohen's Kappa (K)	**Strength of Agreement**
0.81 to 1.00	Almost Perfect
0.61 – 0.80	Substantial
0.41 – 0.60	Moderate
<0.40	Fair to Poor (Method Re-optimization Required)

The Cohen's Kappa (K) must be between 0.81 to 1.00, indicating a near perfect-to-perfect agreement between the platforms.

Percent positive agreement, percent negative agreement, and overall percent agreement should all exceed 95%.

The results of the McNemar's Test should have a p-value >0.05, indicating no significant difference in results between the two platforms.

The data points on the Bland-Altman Plot should be tightly clustered around the zero line, the bias line should be close to the zero line, and the separation between the limits of agreement should be small.

Reporting

The report included in the final proposal's submission should include all the following:

1) A completed "ISSC Method Application and Single Lab Validation Summary of Required Elements for Acceptance of a Method for Use in the NSSP" form, available through the ISSC website. Section C of that form does not need to be completed for a platform extension. Information to complete Sections A, B, and D should focus only on the method modifications being assessed, not on the original method.
2) Raw data and summary results from the pre-study.
3) All the raw data associated with the platform extension study
4) A summary of the data analysis, highlighting specific results.
5) A revised laboratory evaluation checklist accounting for the changes made.

Figure 1: Flow diagram of Experiment 1. (Repeat two (2) additional times for a total of three (3) independent experiments.

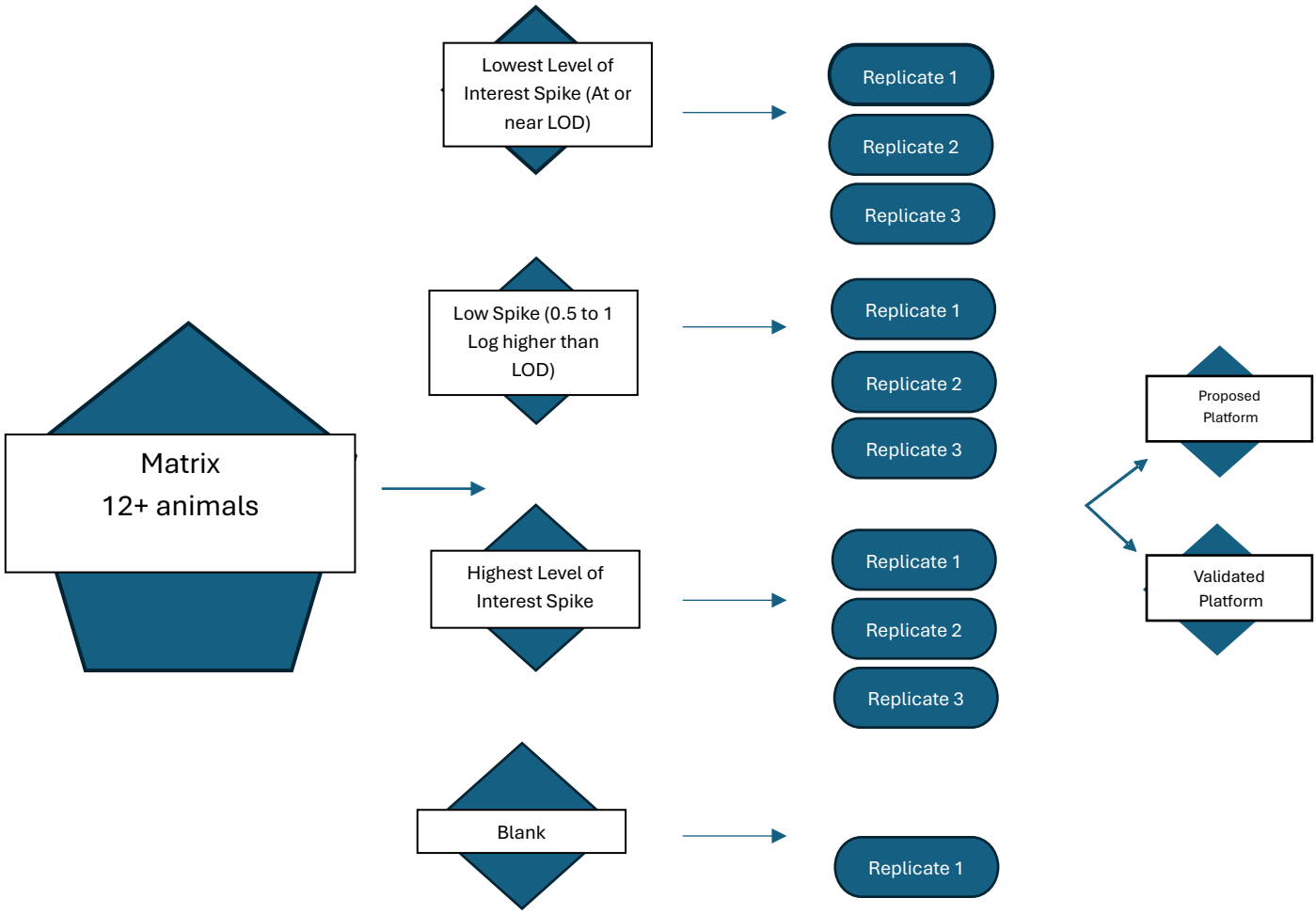
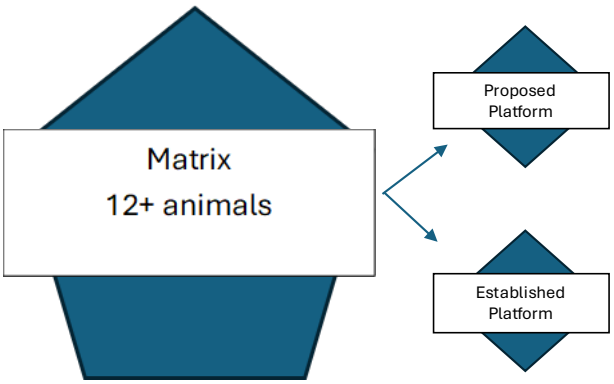


Figure 2: Flow diagram of Experiment 2. (Repeat four (4) additional times for a total of 5 independent experiments)



References:

1. Liu, K.C., Pires, G.S., Furseth, H.A., and Jinneman, K.C., **Report of a Single Laboratory Validation Study: Platform Extension of the Simplex Real-time PCR Method for Detection of Prohibited Materials in Animal Feed**., US Food and Drug Administration, LIB #4656
2. Saah, A.J., Hoover, D.R., **"Sensitivity" and "Specificity" Reconsidered: The Meaning of These Terms in Analytical and Diagnostic Settings**., *Annals of Internal Medicine*. 1997;126:91-94.
3. Association of Public Health Laboratories. **Laboratory test Verification and Validation Toolkit**. APHL website. Accessed August 14, 2026.
4. U.S. Food and Drug Administration Foods Program. (2023) **Guidelines for the validation of analytical methods for nucleic acid sequence-based analysis of food, feed, cosmetics, and veterinary products**. (Edition 1.2).
5. U.S. Food and Drug Administration Foods Program. (2019) **Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds**. (Edition 3.0).

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Laboratory Committee
3. Affiliation	ISSC
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7. Phone	(803) 330-6380
8. Email	issc@issc.org
9. Proposal Subject	Update Guidance on Analytical Method Verification
10. Specific NSSP Guide Reference	Section IV: Guidance Documents, Chapter III. Laboratory, @.02 Methods, .02
11. Text of Proposal/ Requested Action	New guidance attached.
12. Public Health Significance	Verification of method performance within a lab is a necessary step before a laboratory may begin to implement a method to support a state shellfish sanitation program. This proposal is intended to update the existing guidance documents section on method verification to provide clearer instruction on how to carry out a method verification, and to add in new information on how to verify a laboratory method that does not include original SLV data.
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Section IV – Guidance Documents, Chapter III – Laboratory, @.02 Methods

.02 Analytical Method Verification

This guidance is provided to aid laboratories in verifying the performance of an NSSP Approved or Approved Limited Use Method of Analysis, as adopted, within their laboratory before implementation. Method performance verification is an NSSP Laboratory Quality Systems Checklist requirement. Specifically, Item 3.2.1 reflects, "*The laboratory will internally verify new methods to confirm with objective evidence that the intended protocols are demonstrated, and outcomes are fulfilled*." Further, NSSP Checklist Item 3.2.2 states, "*Methodologies do not deviate from the validated/verified method and the laboratory's internal verification remains on file in the laboratory.*" If a laboratory has reason to significantly modify/alter/deviate from a validated approved method, a full method validation is required.

When a laboratory intends to implement an NSSP method for the first time, the lab should contact the FDA Laboratory Evaluation Officer (LEO) Team to discuss the verification process, review their verification plan before implementation, and schedule the applicable Technical Laboratory Evaluation. The verification plan should include:

1. Type of verification and purpose of study.
2. Purpose of test and method description, including Standard Operating Procedure (SOP).
3. Details of study design, including:
    a. Number and type(s) of samples.
    b. Type of quality assurance (QA) and quality controls (QC) that will be used.
    c. Number of replicates, including how many days and how many analysts.
4. Performance characteristics that will be evaluated and the acceptance criteria.
5. Materials, equipment, and any other resources needed.
6. Safety considerations.
7. Expected timeline for completion.

After that, a series of experiments, based on the guidance provided here, should be conducted to verify proper method performance within the laboratory. Upon completion of those experiments, the full data package should be submitted to the FDA LEO Team for review. Once the verification data package has been approved by the LEO Team and the laboratory is conforming or provisionally conforming to the applicable NSSP Laboratory Evaluation Checklist requirements, the laboratory may begin to implement that method to support a State shellfish sanitation program or other interested parties intending to introduce bivalve mollusks into interstate commerce.

Each method verification is matrix specific. If the new laboratory method is utilized to test shellfish, these verification procedures must be repeated for each shellfish matrix category of interest. Shellfish matrix categories can be found in the table included in NSSP Guide for the Control of Molluscan Shellfish, Section IV – Guidance Documents, Chapter III: Laboratory, .03 – Laboratory Method Matrix Extensions. Verification experiments can be completed using a single species from each category of interest or can make use of a mix of species from within each category, as long as the total number of samples meets the criteria described in the guidelines below.

If a planned verification study deviates from these guidelines, the submitter is encouraged to consult with the FDA LEO Team to determine if the alternative procedures are acceptable prior to initiation of the study.

Any deviations from these suggested procedures should be highlighted in the final submission, along with a rationale for why an alternative procedure was used.

Method Verification Performance Characteristics

When verifying a method, the laboratory is required to demonstrate that they can achieve specific performance characteristics. These performance characteristics can vary by method, but may include some or all of the following:.

Accuracy/Trueness - The closeness of agreement between a test result and an accepted reference value. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work.

Recovery – The fraction or percentage of an analyte recovered following sample analysis. The recovery of the target analyte must be consistently good over the range of concentrations of importance to the application to be of benefit in the intended work.

Measurement Uncertainty – The possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. A narrow range of values indicates that the method as implemented by the laboratory produces reliable results.

Linear Range – The range within the working range where the results are proportional to the concentration of the analyte present in the sample.

Limit of Detection – The minimum concentration at which the analyte can be identified.

Limit of Quantitation – The minimum concentration of the analyte that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Precision (Repeatability) –The measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.

Microbiological Method Verification

Recommended Procedure: For shellfish meat samples, use samples free of the target organism(s). For each shellfish matrix of interest, use a minimum of 12 animals per sample (the quantity of meat and liquor should be sufficient to cover the blender blades, or additional shellfish should be used to ensure homogeneity) and prepare as a homogenate. For seawater or wastewater, locally collected samples autoclave- or filter-sterilized should be used for all aliquots.

1. For Vibrio methods, take a minimum of six (6) aliquots of the water or shellfish homogenate appropriately sized for the work, and spike five (5) of the aliquots with five (5) different concentrations of the target organism(s) spanning the desired working range. For MPN, Membrane Filtration, or ETCP Methods, take a minimum of four (4) aliquots of the water or shellfish homogenate appropriately sized for the work, and spike three (3) of the aliquots with three (3) different concentrations of the target organism spanning the desired working range. Vortex each aliquot after spiking.
2. Do not spike one aliquot, which will serve as the sample blank.

3. Determine the concentration of the target organism(s) used to spike each aliquot with an appropriate enumeration method (e.g., spread plating on the appropriate media in triplicate).
4. Process and analyze each spiked sample aliquot in triplicate, with only a single analysis for the sample blank.
5. Analyze each replicate according to the NSSP method, including the sample blank.
6. Repeat this process with a minimum of three samples collected from different growing areas, the same growing area harvested/collected on different days, or from different process lots (applicable to shellfish only). Target the same spike levels for each sample analyzed.

Microbiological Method Verification Data Handling and Analysis:

- ***For methods with existing SLV,*** utilizing the procedures described in the data handling sections of the appropriate SOPs here: https://www.issc.org/single-lab-validation-laboratory-methods, determine the values and ranges for each of the performance characteristics listed above.
    - Compare the method verification results for each applicable performance characteristic with the values obtained in the original SLV. It should be noted that acceptance criteria are method-specific, and because analytical methods are developed to be fit for purpose, there is no single universal tolerance that applies across all NSSP methods. Verification results do not need to perfectly replicate original SLV values to pass. However, any significant deviations must be statistically evaluated against protocol limits before the method is approved for routine use. The laboratory must demonstrate performance that satisfies established fit-for-purpose limits.
    - Within the Method Verification Report, the laboratory should determine if the method verification results are satisfactory and if the method has been successfully transferred. The FDA LEO Team will review the report and determine if they concur or do not concur with the laboratory's determination.
    - Data associated with those original SLVs can be accessed under the "Reference" column of the Laboratory Methods table located on the ISSC website at: https://www.issc.org/laboratory-method-references.
- ***For quantitative methods without documented SLV data***, utilize the results generated using the NSSP method as well as the results generated in step 3 of the "Recommended Procedure" above ("Confirmatory Test") to create a Bland-Altman Plot as described below.

  1) Create a data table as follows:

    a. X = mean of each pair of measurements (NSSP Method and Confirmatory Test Method)
    b. Y = difference between each pair of measurements

Example:

Sample	X	Y
1	19.9	1.2
2	19.8	1.1
3	23.4	-1.2
4	36.6	-0.3
5	21.1	0.8

2) Create a scatter plot using these X,Y coordinate pairs.
3) Calculate the overall average of all the differences and draw a horizontal line on the plot at this value (Bias Line).
4) Calculate the standard deviation (SD) of all the differences and draw horizontal lines at the Bias Line ± 1.96(SD) (Limits of Agreement).

5) The data points on the Bland-Altman Plot should be tightly clustered around the zero line, the bias line should be close to the zero line, and the separation between the limits of agreement should be small.

Marine Biotoxin Verification

Recommended Procedure: Use samples free of the target analyte(s). For each shellfish matrix of interest, use a minimum of 12 animals per sample (the quantity of meat and liquor should be sufficient to cover the blender blades, or additional shellfish should be used to ensure homogeneity) and prepare as a homogenate.

1. Take a minimum of six (6) aliquots of the homogenate appropriately sized for the work, and spike five (5) of those aliquots with five (5) different concentrations of the target analyte(s) spanning an appropriate range of values, based on total toxicity of all the target analytes in shellfish. Spiking levels should be appropriate for what is described in the associated Biotoxin Management Plan, and generally should include levels at half, at, and above the action level. In other situations, spikes focused on the lower part of the range may be necessary to determine linear range, limit of detection, and limit of quantitation. Vortex each aliquot after spiking.
2. Do not spike the sixth aliquot, as this will be used as the sample blank.
3. For marine biotoxin experiments, determine the concentration of the analytes(s) used to spike each aliquot.
4. Process and analyze each spiked sample aliquot in triplicate, with only a single analysis for the sample blank.
5. Analyze each replicate according to the NSSP method, including the sample blank.
6. Repeat this process with a minimum of three samples collected from different growing areas, the same growing area harvested/collected on different days, or from different process lots. Use the same spike levels for each sample analyzed.
7. For methods that need to be evaluated for matrix effects, matrix-matched standards (MMS) should be prepared by taking six (6) sub-aliquots of the extract of the sample blank and spiking the target analyte(s) into five (5) of the six sub-aliquots with concentrations matching those used in the experiments described above.
    a. Generate a calibration curve using the five (5) MMS for each sample and compare with that made from solvent standards. Calculate the matrix effects: Matrix Effects (%) = (Slope of MMS/Slope of Solvent Standards – 1) × 100. Adjust method parameters to have the three tested samples exhibit the same range of Matrix Effects if necessary. Determine MMS or solvent standard calibration curve is used for determination of toxins in samples based on the results of matrix effects. Test each aliquot including the replicates, along with the remaining sample blank, to determine the concentration of the target analyte(s) of interest. Repeat this process for each sample analyzed as part of this verification.

Marine Biotoxin Method Verification Data Handling and Analysis:

- Utilizing the procedures described in the data handling sections of the appropriate SOPs here: https://www.issc.org/single-lab-validation-laboratory-methods, determine the values and ranges for each of the performance characteristics listed above.
- For methods with existing SLVs, compare the method verification results for each performance characteristic with the values obtained in the original SLV. The acceptance criteria are determined in the SLV and are specific to the analytical method. When the method verification results are within the values determined during the SLV it can be concluded that the method has been successfully transferred.
    - Differences between the performance characteristics of the method verification data and SLV data may be observed. When direct comparison with SLV data is not possible, alternative comparisons may be acceptable instead. As an example, the values included in Reference 4 at the end of this document may be used to determine the acceptability of a method verification.
- Data associated with those original SLVs can be accessed under the "Reference" column of the Laboratory Methods table located on the ISSC website at: https://www.issc.org/laboratory-method-references.
- For methods without documented SLV data, determine the values and ranges for each performance characteristic using the same procedures as above. To evaluate for successful implementation, utilize established norms and refer to published guidelines such as those included in References 4, 5, and 6 at the end of this document. Discuss the acceptance criteria and comparison to reference standards in the laboratory's method verification report.

Method Verification Documentation

Method verification documentation should be submitted to the FDA LEO Team in a report that includes:

1. A written summary of the work performed, including how sample was prepared and analyzed, equipment used, etc.
2. Analytical testing data generated during the verification study, including raw data generated in appendices.
3. Statistical evaluation of verification data, noting any statistical programs used.
4. Determination of if the method verification results are satisfactory, and assessment of successful method transfer.
5. Plan for proficiency testing or other means of demonstrating continued laboratory analyst effectiveness
    a. This plan should describe a proficiency testing program, interlaboratory study, or other means of comparative analysis that the laboratory plans on participating in each year in order to demonstrate continued successful analyst performance of the method.

Visual Aides

Visual Aide 1: Examples of method verification bench sheet.

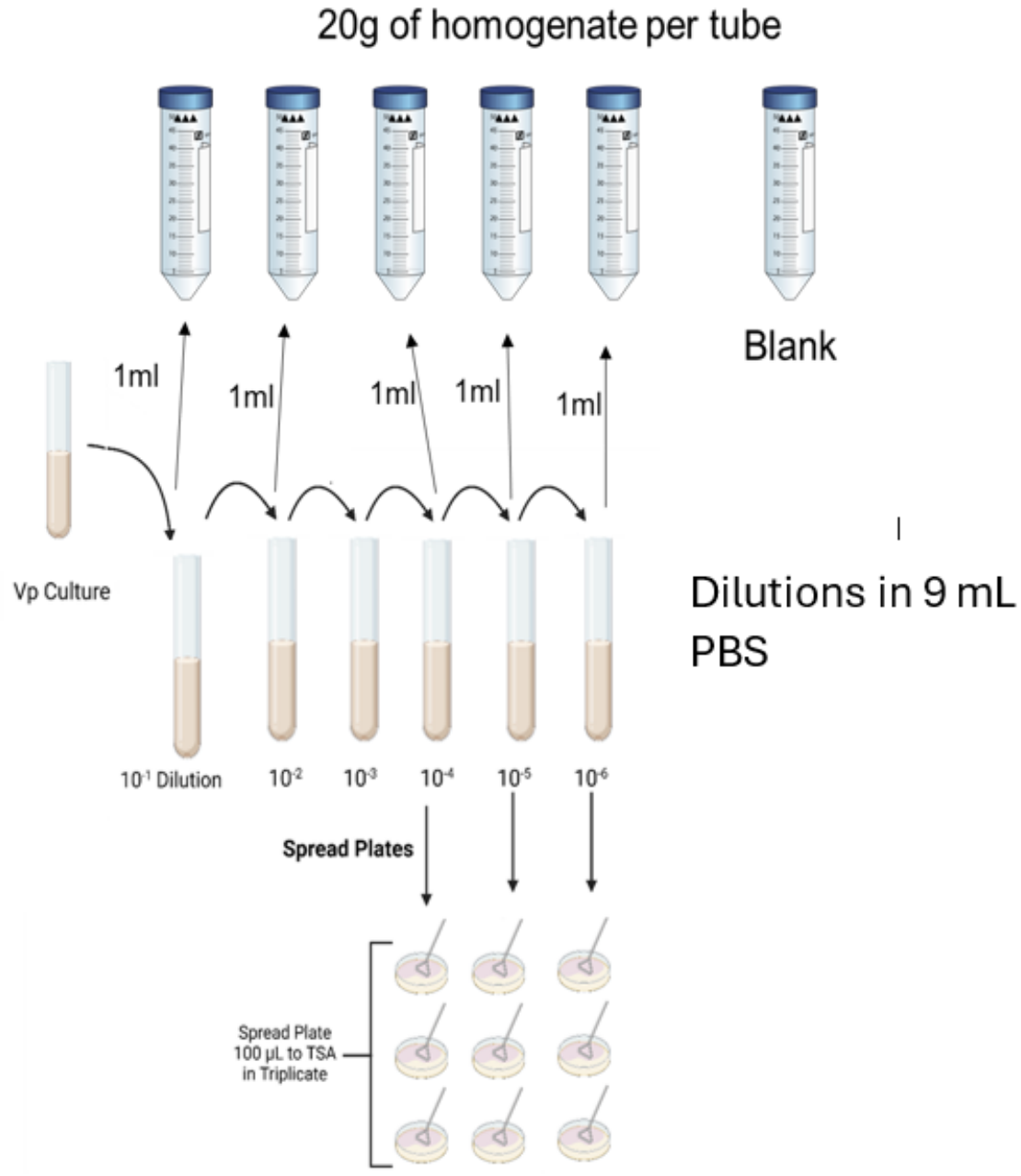
	Spike #1)			Spike #2			Spike #3			Spike #4			Spike #5			
	1A	1B	1C	2A	2B	2C	3A	3B	3C	4A	4B	4C	5A	5B	5C	Blank
Sample 1																
Sample 2																
Sample 3																

		Sample 1	Sample 2	Sample 3	Analyst Initials	Comments
Spike 1	1A					
	1B					
	1C					
Spike 2	2A					
	2B					
	2C					
Spike 3	3A					
	3B					
	3C					
Spike 4	4A					
	4B					
	4C					
Spike 5	5A					
	5B					
	5C					
Blank						

Standard series & matrix	Calibration curve slope	Matrix effects %
Sample 1 MMS		
Solvent standards		
Sample 2 MMS		
Sample 3 MMS		

Visual Aide 2: Suggested method verification set up for MPN real-time PCR for detection of Vibrio.

Suggested Method Verification Set Up for MPN real-time PCR for detection of Vibrio



References

1. The Analytical Laboratory Accreditation Criteria Committee, How to Meet ISO 17025 Requirements for Method Verification, AOAC International, 2007 - https://www.aoac.org/wp-content/uploads/2019/09/ALACC-method-verification.pdf

2. A2LA, G108 – Guidelines for Estimating Uncertainty for Microbiological Counting Methods - https://a2la.qualtraxcloud.com/ShowDocument.aspx?ID=10938

3. United States Food and Drug Administration (FDA), ORA Lab Manual Vol. II – Methods, Method Verification and Validation - https://www.fda.gov/media/73920/download

4. United States Food and Drug Administration, Guidelines for the Validation of Chemical Methods in Food, Feed, Cosmetics, and Veterinary Products, 3rd Edition – Table on Page 23: https://www.fda.gov/media/81810/download

5. European Union Reference Laboratory for Marine Biotoxins, EU-Harmonized Standard Operating Procedure for Determination of Domoic Acid in Shellfish and Finfish by RP-HPLC Using UV Detection, Annex A - https://www.aesan.gob.es/AECOSAN/docs/documentos/laboratorios/LNRBM/archivo4_EU-Harmonised-SOP-ASP-HPLC-UV_Version1.pdf

6. Codex Alimentarius – Recommended Methods of Analysis and Sampling, CXS 234-1999, pages 23-24.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	ISSC Laboratory Committee
3. Affiliation	ISSC
4. Address Line 1	4801 Hermitage Rd
5. Address Line 2	
6. City, State, Zip	Richmond, VA 23227
7. Phone	(804) 330-6380
8. Email	issc@issc.org
9. Proposal Subject	Update SOP for Quantitative Biotoxin Method Validation
10. Specific NSSP Guide Reference	ISSC Website – Laboratory – Single Laboratory Validation Protocol for Submission to the ISSC
11. Text of Proposal/ Requested Action	Procedure Attached
12. Public Health Significance	Laboratory method validation is a foundational component of the NSSP Laboratory Program, ensuring that laboratory methods are fit for their intended use within the program before they are approved for use. The existing SOPs providing guidance on how to conduct a single laboratory validation study were originally added to the program in the 1990s, and since that time, a lot has been learned as they have been applied to the many laboratory methods that have been submitted for adoption into the program. Using feedback from both method developers and method reviewers, an improved SOP for Quantitative Biotoxin Method Validation has been developed.
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

ISSC Single Laboratory Method Validation Guidelines

Suggested.Procedures.for.Validating.Quantitative.Methods.for.Biotoxins

Introduction: The purpose of laboratory method validation in the National Shellfish Sanitation Program (NSSP) is to ensure that methods under consideration for adoption by the NSSP are fit for their intended use within the Program. This document provides a summary of the types of information and data that the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Committee (LC) requests from submitters for the validation of quantitative biotoxin methods. Included are the method performance criteria that should be examined as part of a validation package, suggested testing procedures, suggested data handling procedures, and an outline of how it is recommended that the data be presented in a final submission. In some circumstances, data being generated as part of this study can be used to evaluate multiple performance parameters. These areas of overlap will be highlighted throughout these guidelines.

Method validations are matrix specific, so each set of experiments described below should be repeated for each shellfish matrix category of interest. Alternatively, once a method has been validated and adopted into the program for a single matrix, method developers can then utilize matrix extensions to validate the method on additional shellfish matrices. Shellfish matrix categories and information on matrix extensions can be found in the NSSP Guide for the Control of Molluscan Shellfish, Section IV – Guidance Documents, Chapter III: Laboratory, .03 – Laboratory Method Matrix Extensions. Validation experiments can be completed using a single species from each category of interest, or can make use of a mix of species from within each category, as long as the total number of samples meets the criteria described in the guidelines below.

It should be noted that these guidelines represent one way to design and implement a validation study. If a planned validation study deviates from these guidelines, the method submitter is encouraged to consult with the LC through their assigned method liaison to determine if the alternative procedures are acceptable prior to initiation of the study. Any deviations from these suggested procedures should be highlighted in the final submission, along with a rationale for why an alternative procedure was used.

Validation Criteria

Matrix.Effects – An alteration of the analyte response due to interfering and often unidentified components in the sample matrix.

Accuracy—Trueness - The closeness of agreement between a test result and an accepted reference value. Trueness is the closeness of the mean of the measured values to the accepted reference value (i.e., bias). Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work.

Recovery – The fraction or percentage of an analyte recovered following sample analysis. The recovery of the target analyte must be consistently fit for purpose over the range of concentrations of importance to the application to be of benefit in the intended work.

Linear.Range – The range where the results are proportional to the concentration of the analyte present in the sample.

Limit.of.Detection – The minimum concentration at which the analyte can be identified.

Limit.of.Quantitation – The minimum concentration of the analyte that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Measurement.Uncertainty – The possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. A narrow range of values indicates that the method as implemented by the laboratory produces reliable results.

Repeatability – The measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.

Precision – The closeness of agreement between independent test results obtained under stipulated conditions.

Intermediate.Precision – Within laboratory precision obtained under variable conditions such as different days, different analysts, or different instrumentation.

Specificity–Selectivity – The ability of a method to measure what it is intended to measure.

Ruggedness–Robustness – The ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.

Cross_Reactivity – In immunoassays, the degree to which an antibody binds to compounds that are structurally similar to the target analyte, essentially measuring how well the antibody can detect related compounds. This criterion may also apply to activity-based assays (e.g. receptor-binding assays) in which the response of related toxin congeners may vary.

Comparability – The acceptability of a new or modified analytical method as an alternative or substitute for an established method in the NSSP.

Validation Criteria – Experiment #1

Matrix.Effects.Evaluation

Suggested Procedure:

If you have determined that matrix effects are not a concern for your method, please include data or text explanation supporting that assertion within the final submission package.

For methods that need to be evaluated for matrix effects, matrix-matched standards (MMS) should be prepared by taking five sub-aliquots from a sample blank extract, and spiking each extract sub-aliquot with a different concentration of the target analyte(s) spanning the desired working range and including levels at half, at, and above the action level if these three levels are within the method linear working range. Generate a calibration curve using these five MMS from a sample and compare with neat standards (in solvent) to assess matrix effects. A minimum of ten samples collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots, should be analyzed following these same procedures, using the same spike levels to generate MMS for each sample.

For LC-MS methods, analyte sensitivity can drift over time, and this drift must be assessed during the validation. One potential method of assessment is to alternate injections between neat standards and the five MMS from a sample (yielding a neat standard calibration curve and an MMS calibration curve for each sample) in one LC-MS sequence to determine matrix effects for all ten samples. Implementation of matrix effects evaluation can also be done on different days along with routine samples as long as solvent standard and MMS calibration curves are run side by side. For non-LC-MS methods, only one calibration curve from solvent standards per day is necessary to complete the table. For the purposes of completing Table 1, Matrix Effects (%) = (Slope of MMS/Slope of Solvent Standards – 1) × 100. Adjust method parameters to ensure the ten tested samples exhibit the same range of Matrix Effects if necessary. Determine whether the MMS or solvent standard calibration curve should be used for determination of toxins in samples based on the results of matrix effects.

Table 1:

Sample #	1	2	3	4	5	6	7	8	9	10
Matrix effects										

Validation Criteria – Experiment #2

Criteria.To.Be.Assessed¿Accuracy–Trueness·.Recovery·.Linear.Range·.Limit.of.Detection·.Limit.of.Quantitation.

(Full-method repeatability, intermediate precision, recovery precision, and measurement uncertainty are assessed in Experiment #3 using independent spiking and extraction replicates.)

Suggested Procedure:

Use shellfish free of the target analyte(s). For each shellfish matrix of interest, use a minimum of 12 animals per sample and prepare as a homogenate. Select a minimum of 10 samples collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots.

For each of the 10 samples, following the appropriate process for the method being validated, prepare one spiked aliquot at each of five target analyte concentrations spanning the intended working range, including levels at half, at, and above the action level (at least 1.5x, as appropriate for the method and toxin being assessed) (Table 2). In cases where a toxin class has multiple congeners with varying toxicity, a cocktail of congeners with total toxin equivalents at the appropriate concentrations can also be used. Additional spikes in the lower part of the range may be required to obtain linear range, limit of detection, and limit of quantitation. Spike the analytes(s) into shellfish homogenate and vortex well before toxin extraction. Additionally, create a single unspiked replicate of each sample to serve as the sample blank.

*For three selected representative samples prepare three independent spiking and extraction replicates at three decision-relevant levels. The first result from each of these replicate subsets will be used in experiment #2, and the additional replicates will be used in later experiments.

Analyze each spiked aliquot once to determine the concentration of the target analyte(s). Additional spikes in the lower part of the range may be required to obtain linear range, limit of detection, and limit of quantitation.

Table 2:

Sample	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5	Blank
Sample 1	1 extract; 1 analysis	1 extract; 1 analysis	3 independent extracts	3 independent extracts	3 independent extracts	Blank
Sample 2	1 extract; 1 analysis	1 extract; 1 analysis	3 independent extracts	3 independent extracts	3 independent extracts	Blank
Sample 3	1 extract; 1 analysis	1 extract; 1 analysis	3 independent extracts	3 independent extracts	3 independent extracts	Blank
Sample 4	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	Blank
Sample 5	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	Blank
Sample 6	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	Blank
Sample 7	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	Blank

Sample	Spike 1	Spike 2	Spike 3	Spike 4	Spike 5	Blank
Sample 8	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	Blank
Sample 9	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	1 extract; 1 analysis	Blank
Sample 10	1 extract; 1 analyses	1 extract; 1 analyses	1 extract; 1 analyses	1 extract; 1 analyses	1 extract; 1 analyses	Blank

Data Handling and Analysis:

a. Accuracy/Trueness

i. Use Experiment #2 data from all 10 samples and five spike levels. For each sample-level combination, use the measured result from the independently spiked and extracted aliquot.
   a. For the replicate subset, utilize the first result from each spike concentration and sample for the Experiment #2 matrix summary. (Retain the three independent extraction results for Experiment #3 precision analyses.)

ii. For each spike level, calculate Relative Error using the sample-level measured values from all 10 samples. As appropriate for the method being assessed, calculate relative bias as well. Summarize results by spike level across the 10-sample matrix set, and provide an overall summary across all spike levels to characterize performance throughout the intended working range.

Relative Error (%) = [(Measured value - Reference value) / Reference value] x 100

Relative Bias (%) = [(Mean measured value - Reference value) / Reference value] x 100

b. Recovery

Use Experiment #2 data from all 10 samples and five spike levels to calculate Recovery across representative matrices.

i. For each sample-level combination, calculate Recovery (%) using the sample-level measured value.
Recovery (%) = (Measured value / Reference value) x 100
   a. For the replicate subset, utilize the first result from each spike concentration and sample for the Experiment #2 matrix summary. (Retain the three independent extraction results for Experiment #3 precision analyses.)

ii. For each spike level, summarize Recovery across the 10-sample matrix set by reporting mean Recovery (%), standard deviation, relative standard deviation, and range.

Variance-based estimates of repeatability, intermediate precision, recovery precision, and measurement uncertainty are evaluated in Experiment #3 using independent replicate extractions.

c. Linear Range

i. Perform the linear regression analysis for the standard calibration curves. Perform the linear regression analysis for each spiked sample at all spike levels with Y-axis using the mean response and X-axis using the mean measured concentration at each spike level.
   a. For the three selected samples at the three decision-relevant levels, use the first result from each spike concentration and sample.

ii. Compare the linear regression analysis obtained from the standards and the spiked samples, determine the linear working range for the samples. If the linear working range covers a wide range of the target analyte concentration, evaluate whether a weighting factor is appropriate

d. Limit of Detection

There are several approaches for determining LOD, depending on the method being assessed (ICH harmonized Tripartite guideline, Validation of analytical procedures: text and methodology Q2(R1), 1994, 1996, 2005). As one option, LOD and LOQ may be estimated from Experiment #2 if the low-level spikes and blanks provide adequate data near the expected detection and quantitation limits. If not, perform an additional low-level study using concentrations near the expected LOD/LOQ.

i. Based on Signal-to-Noise Ratio. This approach can only be applied to analytical procedures that exhibit baseline noise. A signal-to-noise ratio of 3 is generally considered acceptable for estimating the LOD.

ii. Based on the Standard Deviation of the Response and the Slope. LOD = 3.3σ/S. S is the slope of the calibration curve. σ is the standard deviation of the response, which can be estimated based on the standard deviation of the blank or the calibration curve. When calibration curve is used for LOD estimate, a special calibration curve should be performed using samples containing the target analyte in the range of LOD. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

e. Limit of Quantitation

There are several approaches for determining LOQ, similar to LOD.

i. Based on Signal-to-Noise Ratio. This approach can only be applied to analytical procedures which exhibit baseline noise. A signal-to-noise ratio of 10 is typically considered acceptable for estimating the LOQ.

ii. Based on the Standard Deviation of the Response and the Slope. LOD = 10σ/S. S is the slope of the calibration curve. σ is the standard deviation of the response, which can be estimated based on the standard deviation of the blank or the calibration curve. When calibration curve is used for LOQ estimate, a special calibration curve should be performed using samples containing the target analyte in the range of LOQ. The residual standard deviation of a regression line or the standard deviation of y-intercepts of regression lines may be used as the standard deviation.

Validation Criteria – Experiment #3

Criteria.To.Be.Assessed¿Measurement.Uncertainty·.Repeatability.(Within_Run.Precision)?and. Intermediate.Precision

Note: Experiment #2 and Experiment #3 are intentionally linked to avoid duplicate analyses. The three selected samples and three decision-relevant spike levels prepared as independent replicate extractions in Experiment #2 serve as Run 1 / Day 1 / Analyst 1 of Experiment #3.

Suggested Procedure:

Select three representative samples from Experiment #2. At each of three decision-relevant concentrations, approximately 0.5x the action level / limit, 1x the action level / limit, and above the action level / limit (at least 1.5x, as appropriate for the method and toxin being assessed), prepare three independently spiked and extracted replicates. This is Run 1 and is generated during Experiment #2. Repeat this complete procedure (sample preparation, spiking, extraction, and analysis) a second time, processing by different analysts or by the same analysts on a different day, for a total of three times, producing three independent validation runs.

Table 3:

Sample	Level	Run 1 from Exp. 2	Run 2	Run 3
Sample 1	0.5x limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 1	1x limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 1	Above limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 2	0.5x limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 2	1x limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 2	Above limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 3	0.5x limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 3	1x limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3
Sample 3	Above limit	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3	Rep 1, Rep 2, Rep 3

Data Handling and Analysis

A. Repeatability (Within-Run Precision)

Repeatability is estimated from the three independently prepared replicates within each analytical run.
For each sample and spike level:

Calculate the within-run standard deviation (Sr).

Calculate the Relative Standard Deviation (RSDr).

RSDr (%) = (Sr / Mean Measured Value) × 100

Report RSDr for each sample and spike level.

B. Intermediate Precision

Intermediate Precision evaluates the variability introduced by different analytical runs performed on different days, by different analysts, or both. For each sample and spike level, perform a one-way ANOVA using analytical run as the grouping factor.

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F	p-value	Fcrit
Between Runs	Sb	2	$M_b=S_b/2$	Mb/Mw		
Within Runs	Sw	6	$M_w=S_0/6$			
Total	Sb + Sw	8				

Calculate:

Repeatability for Sample 1

Standard deviation: Sr = SQRT(M_w)

Relative standard deviation RSD_r = (S_r/average of all 9 replicates with sample 1)×100

Intermediate Precision standard deviation for Sample 1:

Standard deviation: S_{IP} = SQRT((M_b-M_w)/n+M_W)

Relative standard deviation RSD_{IP} = (S_{IP}/average of all 9 replicates with sample 1)×100

Since variability including analysts or days cannot be less than within analysts or days, if S_R is less than S_r, S_{IP} is set equal to S_r.

Repeat calculation of S_r and S_{IP} for sample 2 & 3.

C. Measurement Uncertainty

Calculate the standard deviation for each sample and spike level. Estimate measurement uncertainty as approximately ±2 times the standard deviation (S_{IP}). Summarize the resulting uncertainty estimates across samples at each spike level by reporting the range of at each spike level.

D. Summary of Method Precision

Summarize the following for each of the three samples and spike levels:

Mean Recovery (%)
RSDr (%)
RSD_{IP} (%)
Measurement Uncertainty (± 2SD)

These values represent the performance of the complete analytical method over the concentration range of greatest regulatory importance.

Validation Criteria – Experiment #4

Criteria To Be Assessed¿Specificity–Selectivity (not required for LC MS_MS methods? as they combine LC and MS–MS selective techniques for toxin identification and quantification)

Suggested Procedure:

Use shellfish free of the target analyte(s). For each shellfish matrix of interest, use a minimum of 10-12 animals per sample and prepare as a homogenate. For each sample, take a minimum of three aliquots of the homogenate appropriately sized for the work. For one aliquot, create five replicates spiked at a low but determinate level of the target analyte. For the second aliquot, create five replicates at the same low level of the target analyte and also spike with a moderate to high level of a suspected interfering toxin or compound. Use the third aliquot to create a single unspiked replicate of the sample to serve as the sample blank. Spike the analyte(s) into shellfish homogenate and vortex well before extraction. Process replicates of each sub-aliquot and the sample blank to determine the concentration of the target analyte(s). Repeat this process for all suspected interfering toxins or compounds identified.

Table 4. For Each Potential Interfering Toxin or Compound:

	Sample Spiked with Low but Determinate Level of Target Analyte	Sample Spiked with Target Analyte and Suspected Interfering Compound	Sample Blank
Replicate 1			
Replicate 2			
Replicate 3			
Replicate 4			
Replicate 5			

Data Handling and Analysis:

a. Calculate the Specificity Index (SI) as follows:

$$SI = \frac{Sample\ Spiked\ With\ Target\ of\ Interest\ Only}{Sample\ Spiked\ With\ Both\ Target\ and\ Suspected\ Interference}$$

b. SI should equal one in the absence of interferences. To test the significance of an SI other than one, a two-sided t-test at the 0.05 significance level is performed to determine if the arithmetic mean of the SI differs from one. Repeat for all interfering compounds/toxins tested.

Validation Criteria – Experiment #5

Criteria.To.Be.Assessed¿Ruggedness–Robustness

Note: Data from Experiment #2 at the action level can be used to fulfill the "Following Method" data requirement described below in the section about assessing the effects of modifications to analysis parameters. Additional replicates of those samples will be needed in order to address the "Method with Modification" component of this test.

Suggested Procedure:

This experiment is to determine whether the method, as implemented, is sufficiently rugged to withstand the types of changes anticipated to occur in routine use. Identify critical performance parameters (i.e., lot of reagents, temperature, incubation time, pH, chemical stability of target analyte during storage and analysis, etc.) and compare results generated with and without modification to these parameters. Only one modification at a time should be examined.

To assess the effect of modifications to extraction parameters, the following procedure should be followed: Use shellfish free of the target analyte(s). For each shellfish matrix of interest, use a minimum of 10-12 animals per sample and prepare as a homogenate. Take two aliquots of the homogenate appropriately sized for the work. Following the appropriate process for the method being validated, spike one aliquot with the target analyte(s) at the action level. Aliquot 1 should be processed following the method procedure. Aliquot 2 should be processed following the method procedure, with modification to a single critical performance parameter. Spike the analyte(s) into the shellfish homogenate and vortex well before extraction. Repeat this process for a minimum of 10 samples collected from a variety of growing areas, the same growing area harvested on different days, or from different process lots.

Table 5:

	Following Method	Method with Modification
Sample 1		
Sample 2		
Sample 3		
Sample 4		
Sample 5		
Sample 6		
Sample 7		
Sample 8		
Sample 9		
Sample 10		

Data Handling and Analysis:

For each sample, calculate the paired difference between the result obtained following the method as written and the result obtained following the modified method.

Paired Difference = (Modified Method Result – Method Result)

Examine the distribution of the paired differences. If the paired differences are approximately symmetric and free of substantial outliers, analyze the data using a paired t-test. If the paired differences exhibit marked skewness or contain substantial outliers, analyze the data using the Wilcoxon signed-rank test.

Data Summary:

a. mean result under method conditions
b. mean result under modified conditions
c. mean paired difference
d. p-value
e. Test used
f. Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples (Y/N)

Validation Criteria – Experiment #6

Criteria.To.Be.Assessed¿Cross.Reactivity.and.Extraction.Efficiency.of.Multiple.Target.Analyte.Congeners.(If.Necessary)

For antibody- or activity-based assays that may detect congeners within a toxin class to varying degrees, cross-reactivity and extraction efficiency should be assessed for each congener, if possible.

Suggested Procedure:

a. Cross reactivity

Use shellfish free of the target analyte(s). For each shellfish matrix of interest, use a minimum of 10-12 animals per sample and prepare as a homogenate. Follow the appropriate process for the method being validated to create sufficient blank (unspiked) replicate extracts to complete the steps below.

Prepare matrix-matched standards (MMS) by taking five sub-aliquots from a sample blank extract and spiking each extract sub-aliquot with a different concentration of the target analyte spanning the desired working range as described in Experiment 1. The target analyte is the toxin congener specified in the method for calibration and sample quantitation.

Spike three replicate sub-aliquots from a sample blank extract with a (single) different congener (Congener 1) at the action level for the toxin class. Determine the concentration of Congener 1 in each replicate using the matrix-matched target analyte standards as the calibration solution. Repeat the above process for each available analyte congener.

Table 6a. Cross reactivity in sample matrix

	Replicate 1	Replicate 2	Replicate 3	Sample blank
Congener 1 MMS				
Congener 2 MMS				
Congener 3 MMS				
Congener 4 MMS				
Congener 5 MMS				
Congener 6 MMS				
Congener 7 MMS				

b. Extraction efficiency

Extraction efficiency should be evaluated for those congeners demonstrating cross-reactivity in the assay. Use shellfish free of the target analyte(s). For each shellfish matrix of interest, use a minimum of 10-12 animals per sample and prepare as a homogenate. Following the appropriate process for the method being validated, create three replicates of an aliquot spiked with Congener 1 at the action level for the toxin class. Spike the congener into the shellfish homogenate and vortex well before extraction. Additionally, create a single unspiked replicate of the sample to serve as the sample blank.

Prepare matrix-matched standards (MMS) of Congener 1 by taking five sub-aliquots from the sample blank extract and spiking each extract sub-aliquot with a different concentration of Congener 1, spanning the desired working range as described in Experiment 1.

Determine the concentration of Congener 1 using the corresponding Congener 1 MMS as the calibration solution to determine method extraction efficiency. Repeat the above process for each analyte congener available.

Table 6b. Extraction Efficiency and Matrix Effects

	Replicate 1	Replicate 2	Replicate 3	Sample blank
Congener 1				
Congener 2				
Congener 3				
Congener 4				
Congener 5				
Congener 6				
Congener 7				

Data Handling and Analysis:

Calculate the average and standard deviation of the three replicates of each congener examined. Calculate cross-reactivity (from 6a) and extraction efficiency (from 6b) as a percentage of the spike concentration added.

Table 6c:

	Congener cross reactivity (%) ± S.D. (6a)	Extraction Efficiency (%) ± S.D. (6b)
Congener 1		
Congener 2		
Congener 3		
Congener 4		
Congener 5		
Congener 6		
Congener 7		

Validation Criteria – Experiment #7

Criteria.To.Be.Assessed¿Comparability.(If.Necessary)

Note: Comparability only needs to be assessed if the proposed method is intended as an alternative or substitute for an established method that has already been adopted by the NSSP.

Suggested Procedure:

Naturally incurred samples are preferred for this analysis, although equivalent spiked samples can be used if naturally incurred samples are unavailable. For each shellfish matrix of interest, use a minimum of 10-12 animals per sample and prepare as a homogenate. For each sample, take two aliquots, analyze one using the officially recognized NSSP method, and analyze the second using the proposed method. A minimum of thirty paired samples should be analyzed, including a variety of toxin concentrations that span the range of the intended application of the method, including primarily determinate results. Samples should come from a variety of different geographic areas or different seasons.

Table 7:

	NSSP Method	Proposed Method
Sample 1		
Sample 2		
Sample 3		
Sample 4		
Sample 5		
Sample 6		
Sample 7		
Sample 8		
Sample 9		
Sample 10		
Sample 11		
Sample 12		
Sample 13		
Sample 14		
Sample 15		
Sample 16		
Sample 17		
Sample 18		
Sample 19		
Sample 20		

	NSSP Method	Proposed Method
Sample 21		
Sample 22		
Sample 23		
Sample 24		
Sample 25		
Sample 26		
Sample 27		
Sample 28		
Sample 29		
Sample 30		

Data Handling and Analysis:

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. Since samples may cover a wide concentration range of the target analyte, regression line analysis is used for comparing the new method with the established method. Perform regression analysis with y-axis for the new method and x-axis for the established method, obtain the results of product-moment correlation coefficient (r), the slope (b) and the intercept (a) along with their lower and upper 95% confidence limit of the linear regression line from Regression analysis table (e.g., Excel-data analysis-regression). The ideal values for b = 1 and a = 0 should be included in the 95% confidence limits.

Data Reporting

Upon completion of the necessary experiments and data analysis, a written report summarizing the findings should be completed for submission with the SLV. The report should include the following:

1) Method title
2) Method submitter(s) and contact information
3) Intended use
4) Rationale for this method in the NSSP – Does this method meet an immediate or continued need, improve analytical capability, reduce cost, ease implementation, etc.?
5) Method principle/basis – Receptor binding assay, immunoassay, LC-MS, etc.
6) Target matrix/matrices – Describe the shellfish matrix or matrices that will be validated using the matrix category table available at NSSP Guide for the Control of Molluscan Shellfish, Section IV – Guidance Documents, Chapter III: Laboratory, .03 – Laboratory Method Matrix Extensions. Include details on the specific species to be used to represent each matrix category.
7) Target toxin(s)
8) Existing certifications of the method
9) Equipment required – Provide a list of specialized equipment needed to perform the method.
10) Reagents required – Provide a list of specialized chemicals, reagents, etc. needed to perform the method.
11) Proprietary aspects
12) Laboratory safety requirements – Describe the safety measures, beyond those of routine laboratory practices, required to perform the method.
13) Method cost – Provide an estimate of start-up costs, cost per analysis, etc.
14) Sample throughput – Provide a description of how many samples can be analyzed by this method in a given time frame.
15) References – Provide references that are pertinent and supplemental to the validation data submitted. This may include peer-reviewed publications in which the method was validated and/or applied, validation packages submitted to other entities, etc.
16) Standard Operating Procedure – Provide a detailed method procedure that is adequate to allow for replication of the method in additional laboratories.
17) Draft Method Evaluation Checklist – Following the format of other laboratory evaluation checklists (available at https://www.issc.org/nssp/2023-nssp-guide-control-molluscan-shellfish), and using the Standard Operating Procedure as a starting point, develop a draft checklist that laboratory evaluation officers could utilize to evaluate laboratory implementation of the method.
18) Overview of Quality Systems – Provide an overview of the quality assurance/quality control systems utilized in the developer(s)/submitter(s) laboratory. This should include any laboratory accreditation or conformance status, if available, or the laboratory Quality Assurance Manual.
19) Analysis of each parameter described in the "Validation Criteria" section below, including data tables/figures, summary of calculations, and final results highlighted, and an explanation of why each result demonstrates that the method fit for purpose.

20) All raw data used in the analyses should be presented in table form as appendices to the written report using the spreadsheets included in Appendix 1 of this document.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☑ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Leanne Flewelling
3. Affiliation	Florida Fish and Wildlife Conservation Commission
4. Address Line 1	100 8th Avenue SE
5. Address Line 2	
6. City, State, Zip	Saint Petersburg, FL 33701
7. Phone	727-386-3437
8. Email	leanne.flewelling@myfwc.com
9. Proposal Subject	AOAC OMA 2005.06, Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection
10. Specific NSSP Guide Reference	Section IV Guidance Documents – Chapter III. Laboratory @.02 Methods 0.1 Approved NSSP Laboratory Tests 2. Approved Methods for Marine Biotoxin Testing
11. Text of Proposal/ Requested Action	Adoption of AOAC Official Method 2005.06 (OMA 2005.06) as an Approved Method of PSP testing in mussels, clams, oysters, and scallops. In the ISSC Constitution Bylaws and Procedures, updated July 22, 2024, Procedure XV. Procedure for the approval of analytical methods for the NSSP, section vii(a) states that: *AOAC approved methods that have undergone the AOAC Official Methods of Analysis (OMA) or FDA Office of Foods Level 3 or 4 validations may be accepted as an NSSP method without Single Lab Validation providing the AOAC or FDA multi-laboratory validation was performed in the raw molluscan shellfish matrix for which the Conference intends it to be used and is deemed by ISSC as fit for purpose.* The European Union (EU) integrated OMA 2005.06 into its regulatory monitoring in 2006, and in 2019, officially adopted OMA 2005.06 as the EU Reference Method for the detection of PSP toxins in shellfish. This method has been rigorously vetted and validated and has been deemed to be fit for purpose across the EU. This proposal is for adoption of OMA 2005.06 as published. Subsequent refinements and improvements (including additional toxins), as adopted by the EU, will be submitted as method extensions in future submissions.
12. Public Health Significance	Paralytic Shellfish Poisoning (PSP) occurs worldwide and poses a serious threat to shellfish consumers. Currently approved methods in the NSSP include the APHA Mouse Bioassay (for all matrices covered by the NSSP), the Receptor Binding Assay (for mussels only), and the PCOX HPLC method (for mussels, clams, oysters, and scallops). Each method has its advantages and drawbacks, and NSSP laboratories benefit from choices that may be driven by access to equipment, the matrices of interest, or local PSP profiles.

13. Cost Information	$94,000 – $240,000 for equipment. $14 - $25 per sample for consumables, depending on the toxins present.
14. Changes Needed to Guidance (if proposed change is adopted)	Section IV Guidance Documents – Chapter III. Laboratory @.02 Methods 0.1 Approved NSSP Laboratory Tests Add to table: 2. Approved Methods for Marine Biotoxin Testing AOAC Official Method 2005.06[6] Indicate applicability to columns: Biotoxin Type Paralytic Shellfish Poisoning (PSP) Application: Growing Area Survey & Classification Sample Type: Shellfish Application: Dockside Testing Program Sample Type: Shellfish Application: Controlled Relaying Sample Type: Shellfish Add to Footnotes: 6. AOAC International. (2005). AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish: Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection. In Official Methods of Analysis of AOAC International. Gaithersburg, MD: AOAC International. doi: 10.1093/9780197610145.003.4019. Applicable to the determination of STX; NEO; GTX2,3; GTX1,4; dcSTX; B-1/GTX5; C1,2; and C3,4 in mussels, clams, oysters, and scallops.

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-2151/2055/4960 FAX 301-436-2601
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Paralytic Shellfish Poisoning-PSP (OMA 2005.06, HPLC-FLD)

LABORATORY:		
ADDRESS:		
TELEPHONE:	FAX:	EMAIL:
DATE OF EVALUATION:	DATE OF REPORT:	LAST EVALUATION:

LABORATORY REPRESENTED BY:	TITLE:
LABORATORY EVALUATION OFFICER:	SHELLFISH SPECIALIST:
	REGION:
OTHER OFFICIALS PRESENT:	TITLE:

Items which do not conform are noted by:

C – Critical K - Key O - Other NA - Not Applicable Conformity is noted by a "1"

PART I – QUALITY ASSURANCE			
Code	REF		Item Description
			1.1 Quality Assurance (QA) Plan
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check '1 those that apply) a. Organization of the laboratory. b. Staff training requirements. c. Standard operating procedures. d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established. e. Laboratory safety. f. Internal performance assessment.
C	5		**1.1.2 QA Plan is implemented.**
			1.2 Educational/Experience Requirements
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	**State's Human Resources Department**		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	**USDA Microbiology & EELAP**		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	5		**1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.**
			1.3 Work Area
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
			1.4 Laboratory Equipment
O	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit. [Only applicable if using the sample cleanup procedure]
K	5		1.4.2 The pH meter is calibrated daily when in use. Results are recorded and records are maintained. [Only applicable if using the sample cleanup procedure]
K	8		1.4.3 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.

K	8		1.4.4 The pH meter manufacturer instructions are followed for calibration, or a minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e., pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.
K	5, 11		1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	6, 2		1.4.6 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8, 9		1.4.7 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.8 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	1		1.4.10 Freezer temperature is maintained at -20 ºC or below.
K	8		1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	13		**1.4.12 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.13 All glassware is clean.
K	3,4		1.4.14 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 2.0 mL/min, b. injection loop suitable for 25-100 µL injections, c. fluorescence detector with excitation set at 340 nm and emission at 395 nm d. a data collection system (e.g., computer, integrator) Not required by OMA2005.06, but recommended: e. solvent degasser f. refrigerated autosampler that can maintain 6 °C.
K	2		1.4.15 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.16 A solid phase extraction (SPE) vacuum manifold or automated SPE system capable of holding 3 mL cartridges is used.
O	3		1.4.17 A centrifuge capable of holding 50 mL polypropylene tubes is used.
			1.5 Reagents and Reference Solution Preparation and Storage
C	3		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
C	3, 8		**1.5.2 Water is glass distilled or deionized and exceeds 0.5 megaohm resistance or is less than 2 µSiemens/cm conductivity at 25 ºC to be tested and recorded monthly for resistance or conductivity and the results are recorded. (Not required for bottled HPLC grade water when used immediately upon opening.)**
K	8		1.5.3 Water is analyzed for residual chlorine monthly and is at a nondetectable level (≤0.1 ppm) Results are recorded and records are maintained.

K	8		1.5.4 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	3		**1.5.5 The binary mobile phase system used to analyze PSP toxins consists of: A: 0.1 M ammonium formate in water and B: 0.1 M ammonium formate in 5% acetonitrile/95% water, both adjusted to pH 6.0.**
C	3		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: ___**
C	7		**1.5.7 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.8 All standards used are within expiration date.**
C	**7**		**1.5.9 All standards are prepared either gravimetrically or using appropriate positive displacement pipettes or syringes.**
C	3		**1.5.10 Analytical standard working stocks are made up from primary standard by dilution with deionized water.**
C	3		**1.5.11 Working standard mixes, if used, are made up from working stocks by dilution with water and are adjusted to approximately pH 4 with 0.1 M acetic acid.**
C	3		**1.5.12 Periodate oxidant is prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate, and 0.3 M Na_2HPO_4 and adjusting the pH to 8.2 with 0.2 M NaOH using a pH meter.**
C	3		**1.5.13 Hydrogen peroxide (H_2O_2), 10% aqueous solution is prepared daily by mixing 1 volume of 30% H_2O_2 with 2 volumes of deionized water (1:2, v/v)**
			1.6 Collection and Transportation of Samples
O	6		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	6		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**
K	14		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	6		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**

PART II – EXAMINATION OF SHELLFISH FOR PSP TOXINS			
			2.1 Preparation of Sample
C	6, 1		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., 3 geoduck gut balls).**
O	6		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	6		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8 Pieces of shell and drainage are discarded.
C	6		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
			2.2 Sample Extraction
K	6		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 °C or below.
C	3		**2.2.2 Five (5.0 ± 0.1 g) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and 3ml of 1% acetic acid is added.**
C	3, 4		**2.2.3 Sample is mixed on a vortex for 2 minutes and heated in a boiling water bath at 100 °C for 5 minutes.**
K	3		2.2.4 After boiling, sample is allowed to cool in a refrigerator or ice bath for 5 minutes, remixed on a vortex, and then centrifuged for 10 minutes at 3600 xg. Supernatant is transferred to a graduated 15 ml conical tube.
K	3		2.2.5 3 mL of 1% acetic acid solution is added to the centrifuge tube containing the solid residue. The sample is mixed with a Vortex mixer for 2 minutes and centrifuged again for 10 minutes at 3600 xg.
K	3		2.2.6 The supernatant is transferred into the same graduated conical test tube that contains the first portion of extract, and the volume is made up to 10 mL with water.
			2.3 Sample Cleanup
K	3		2.3.1 SPE C18 cartridges: Supelclean LC-18 cartridges (500 mg/3 mL volume) (Supelco) or equivalent are used.
K	3		2.3.2 The SPE cartridge is conditioned with 6 mL methanol, followed by 6 mL water.
K	3		2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.

K	3		2.3.4 The flow rate is kept between 2-3 mL/min for all elutions.
K	3		2.3.5 The supernatant is transferred into the same tube that contains the first portion of extract and the volume is made up to 10.0 mL with water.
K	3		2.3.6 SPE C18 cartridges: Supelclean LC-18 cartridges (500 mg/3 mL volume) (Supelco) or equivalent are used.
C	**3**		**2.3.7 The SPE cartridge is conditioned with 6 mL methanol, followed by 6 mL water.**
K	3		2.3.8 The cartridge is not allowed to run dry during conditioning through sample loading.
K	3		2.3.9 The flow rate is kept between 2-3 mL/min for all elutions.
C	3		2.3.10 One (1) mL of crude extract (0.5g shellfish equivalent) is loaded onto the cartridge, and effluent is collected in a 15 ml conical test tube.
C	3		2.3.11 Cartridge is washed with 2 mLs water and combined with the effluent.
K	3		2.3.12 Flow rate is kept between 2 and 3 mL/min for all elutions.
C	3		2.3.13 pH of extract is adjusted to 6.5 with 1.0 M NaOH and diluted to exactly 4.0 ml with deionized water and thoroughly mixed before withdrawing an aliquot for analysis.
K	3		2.3.14 Extracts found to contain N-1 Hydroxylated PSP analogues after C18 cleanup are fractionated using SPE-COOH ion exchange.
K	3		2.3.15 Bakerbond Carboxylic Acid silane (COOH) bonded to silica gel (500 mg/3 mL volume) (J.T. Baker) or equivalent are used.
C	3		2.3.16 The 3 mL SPE-COOH cartridge is conditioned by passing 10 mL of 0.01 M ammonium acetate through it. Keep the flow rate between 2-3 mL/min for all elutions. Do not let the cartridge run dry.
K	3		2.3.17 The cartridge is not allowed to run dry during conditioning through sample loading.
K	3		2.3.18 The flow rate is kept between 2-3 mL/min for all elutions.
C	3		**2.3.19 Fraction #1 (contains C toxins): 2 (two) ml of SPE C18 cleaned extract is passed through the COOH cartridge and collected in graduated tube marked Fraction #1. Then 4.0 mL of water is passed through the cartridge and collected into the same tube. The volume is brought to exactly 6.0 ml with water.**
C	3		**2.3.20 Fraction #2 (contains GTX 1,4; GTX2,3; B1; B2; and dcGTX 2,3): 4.0 mL of 0.05 M NaCl is passed through the same cartridge used for Fraction #1 and collected in a graduated tube marked as Fraction #2. The final volume is adjusted to 4.0 mL if necessary.**
C	3		**2.3.21 Fraction #3 (contains STX, NEO and dcSTX): 5.0ml of 0.3 M NaCl is passed through the same cartridge and collected in a graduated tube marked Fraction #3. The final volume is adjusted to 5.0 mL if necessary.**
K	3		2.3.22 If fractions need to be concentrated to achieve required sensitivity, fractions are evaporated in a 50 mL round bottom flask to approximately 1 mL on a rotary evaporator with a water bath set at 45 °C.
O	3		2.3.23 If concentrating fractions, the reduced solution is transferred to a 2 or 5 mL volumetric flask or graduated tube using a Pasteur pipet. The 50 mL round bottom flask is rinsed 3 times with 0.2-0.3 mL of water each time,

			transferring the rinse into the graduated tube. The final volume of extract is adjusted to 2.0 mL.
			2.4 Analysis
C	2		2.4.1 **A standard calibration curve (of at least six concentrations) of each toxin or mix of toxins is performed upon initial instrument set up, following any major hardware maintenance activity, changes to the analytical system, or with new standard lots. Results are recorded and records are maintained.**
K	3		2.4.2 50-100 µL of extract is injected for periodate oxidation.
K	3		2.4.3 25-50 µL of extract is injected for peroxide oxidation.
O	4		2.4.4 If a column heater is used, the temperature is maintained at 35°C during the analysis.
C	3		2.4.5 **The appropriate analytical column is used: reversed phase C18 column, 15 cm x 4.6 mm id, 5 µm particle size (Supelcosil LC-18 or equivalent).**
K	2		2.4.6 The column is stored following the manufacturer's instructions when not in use.
O	2		2.4.7 If a precolumn in-line filter and/or a compatible guard column are/is used, rejection criteria are established to determine when to change the filter/guard column.
K	2		2.4.8 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.
			2.5 System Suitability
C	2		**2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.98 for each individual toxin.**
C	3		**2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of PSP toxins.**
K	2		2.5.3 Peak asymmetry is routinely monitored to evaluate the performance of the column. Results are recorded and records maintained.
C	2, 14		**2.5.4 The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3.**
C	2, 14		**2.5.5 Daily injection schedules must include the adequate frequency of injection standards based on an assessment of individual standard toxin variability. Variability in response of peaks bracketing samples must be less than 10% for calculation of toxicity in samples.**
			2.6 Calculation of Sample Toxicity
C	3		2.6.1 The toxicity of the individual toxins is calculated as follows: $$\frac{A_x \times C_s \times V_x \times D \times 100}{A_s \times M}$$ where: A_x = peak area of the toxin in the extract analyzed A_s = peak area of the nearest standard

			C_s = concentration of standard (µg/mL) **V_x = final volume of extract analyzed (mL): 4 mL for C18 SPE; 6 mL for SPE-COOH Fraction 1; 4 mL for Fraction 2; and 5 mL for Fraction 3.** **D = dilution factor (for those extracts where PSP toxins are above the highest standard and require dilution and reanalysis)** **M = amount of test portion (g) carried through cleanup procedure: 0.5 g for SPE C18 and 0.25 g for SPE-COOH cleanup** **100 = factor to convert results from µg/g to µg/100 g**
C	3		**2.6.2 If both NEO and dcSTX are present, the concentration of NEO is calculated by difference in accordance with AOAC OMA 2005.06.**
C	3, 10		**2.6.3 The total toxicity in saxitoxin equivalents (STX eq) is calculated by multiplying each toxin analog concentration by its toxicity equivalent factor (TEF) as determined by Oshima (1995), corrected for its molecular weight, and summing them.** **Total toxicity in shellfish (µg STX eq/100 g) =** $$\sum TEF_i \times C_{xi}$$ **where:** **TEF_i = toxicity equivalent factor for each analog detected:** ***STX = 1; NEO = 0.88; dcSTX = 0.6; GTX1,4 = 0.63; GTX2,3 = 0.38; B1 = 0.051; C1,2 = 0.032; C3,4 = 0.022*** **C_{xi} = concentration of each toxin analog measured in the shellfish sample (µg/100g)**
C	12		**2.6.4 Any value at or above 80 µg STX equivalents/100 g of meat is actionable.**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. AOAC Official Methods of Analysis (2005). AOAC Official Method 2005.06 Paralytic Shellfish Poisoning Toxins in Shellfish. Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection.
4. European Union Reference Laboratory for Marine Biotoxins. (2020). Standard operating procedure for the analysis of paralytic shellfish toxins (PST) by precolumn HPLC-FLD according to OMA AOAC 2005.06.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 192. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Oshima, Y. (1995). Postcolumn derivation liquid chromatographic method for paralytic shellfish toxins. Journal of AOAC International, 78(2), 528–532.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C.and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. *Compendium of Methods for the Microbiological Examination of Foods*, 3rd Edition, pg. 901.

LABORATORY:			DATE OF EVALUATION:
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	

AMNESIC SHELLFISH POISON (ASP or domoic acid) COMPONENT: PARTS I AND II

A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the ASP (domoic acid) Component

1. **Conforms Status: The ASP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The ASP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The ASP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥4**.
   - b. or the total # of Key nonconformities is **≥6.**
   - c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform – Provisionally Conforms – Conforms

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____ Date:_____

LEO Signature: Date:

AOAC Official Method 2005.06
Paralytic Shellfish Poisoning Toxins in Shellfish

phic Oxidation and Liquid Chromatography vith Fluorescence Detection
First Action 2005

[Applicable to the determination of saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins 2 and 3 (GTX 2, 3; together), gonyautoxins 1 and 4 (GTX 1, 4; together), decarbamoyl saxitoxin (dcSTX), B-1, C-1 and C-2 together, and C-3 and C-4 (together) in shellfish (mussels, clams, oysters and scallops). In this application, statistics were acceptable with HorRat values <2.0 observed for total paralytic shellfish poisoning (PSP). The method is applicable to quantifying saxitoxin, >22 μg/kg; GTX 2,3 together, >125 μg/kg (lowest concentration tested); B-1, >27 μg/kg; dcSTX, >8 μg/kg; NEO, >40 μg/kg; GTX 1,4 together, >50 μg/kg; C1,2 together, >93 μg/kg; and C3,4 together, >725 μg/kg.]

See Tables **2005.06A–R** for the results of the interlaboratory orting acceptance of the method.

A. Principle

Test portions are extracted by heating with acetic acid solution. Extracts are cleaned up using solid-phase exraction (SPE) C18 cartridges. After periodate and peroxide oxidation, they are analyzed by high-performance liquid chromatography (HPLC) with fluorescence detection. Most toxins (STX, C1,2, B-1, dcSTX, and GTX2,3) can be quantified after simple SPE-C18 cleanup. Extracts containing the toxins NEO, GTX1,4, C3,4, and B-2 must be further purified by using SPE-COOH cleanup/separation. PSP toxin concentrations are calculated by comparison of peak areas or heights in the test samples with those of the standards.

B. Apparatus and Materials

(a) *LC system.*—Producing a binary gradient; equipped with an injection port capable of injecting up to 100 μL solution. Use a reversed-phase C18 column with dimensions of 15 × 4.6 mm id, 5 μm particle size (for example, Supelcosil LC-18) for separation of the toxin oxidation products. If a column of different dimensions is used, the gradient and flow rate conditions must be altered to obtain the necessary separation of the PSP oxidation products within an approximately 15 min chromatographic run. The suggested mobile phase gradient used to elute the PSP oxidation products consists of 2 mobile phases [*see Reagents* (o)] under the following conditions: 0–5% mobile phase B in the first 5 min, 5–70% B for the next 4 min and back to 0% B over the next 2 min; then at 0% B for another 3 min before the next injection.

The flow rate is 2 mL/min. For monitoring the LC effluent, a dual monochromator fluorescence detector, with excitation set to 340 nm and emission to 395 nm. The detector must be sensitive enough to produce a peak height response of at least 3 cm with a peak-to-peak baseline noise of 3 mm (10:1, signal-to-noise ratio) for an injection of 400 pg STX standard carried through the peroxide oxidation procedure under the chromatographic conditions described above.

Table 2005.06A. Interlaboratory study results for the determination of STX in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, μg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	11(1)	471.00		41.16	8.74	115.25	0.49
1	Scallop	15(0)	1048.07		229.23	21.87	641.84	1.38
2	Clams	15(0)	338.27		90.88	26.87	254.46	1.43
3	Oyster	15	ND					
4	Mussel	15	ND					
5	Clams	15(0)	522.20		69.39	13.29	194.29	0.75
5D	Clams	15(0)	517.87		73.89	14.27	206.89	0.81
6	Clams	15	ND[c]					
7	Mussel	14(0)	23.36		8.48	36.30	23.74	1.29
8	Mussel	15(0)	309.60		78.07	25.22	218.60	1.32
8D	Mussel	15(0)	315.53		70.28	22.27	196.78	1.17
9	Mussel	15(0)	132.47		33.38	25.20	93.46	1.16
10	Oyster	15(0)	136.13		46.14	33.89	129.19	1.57
10D	Oyster	15(0)	144.47		41.64	28.82	116.59	1.35
11	Oyster	15(0)	257.67		59.49	23.09	166.57	1.18
12	Mussel	15(0)	783.87		215.24	27.46	602.67	1.65
Spike 1	Mussel	15(0)	223.07	74.4	52.46	23.52	146.89	1.17
Spike 2	Mussel	15(0)	447.00	74.5	112.49	25.17	314.97	1.39
Spike 3	Mussel	15(0)	51.27	93.2	16.96	33.08	47.49	1.32
Spike 4	Mussel	15(0)	169.00	76.8	43.00	25.44	120.40	1.22
Spike 5	Mussel	11(0)	13.18		8.65	65.63	24.22	2.14

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06B. Interlaboratory study results for the determination of STX in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	S_R	RSD_R, %	R	HorRat
Practice sample	Mussel	15(0)	451.80		121.68	26.93	340.70	1.49
1	Scallop	15(0)	955.93		187.77	19.64	525.76	1.22
2	Clams	14(0)	294.57		82.80	28.11	231.84	1.46
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	15(0)	509.53		85.78	16.84	240.18	0.95
5D	Clams	15(0)	474.40		82.56	17.40	231.17	0.97
6	Clams	14	ND					
7	Mussel	12(0)	21.33		6.80	31.88	19.04	1.12
8	Mussel	15(0)	270.13		68.83	25.48	192.72	1.31
8D	Mussel	13(0)	269.08		49.81	18.51	139.47	0.95
9	Mussel	14(0)	119.64		38.16	31.90	106.85	1.45
10	Oyster	14(0)	140.00		45.24	32.31	126.67	1.50
10D	Oyster	15(0)	123.67		26.92	21.77	75.38	0.99
11	Oyster	15(0)	232.87		37.89	16.27	106.09	0.82
12	Mussel	14(0)	644.00		78.89	12.25	220.89	0.72
Spike 1	Mussel	13(0)	191.08	63.7	39.19	20.51	109.73	1.00
Spike 2	Mussel	15(0)	409.27	68.2	122.65	29.97	343.42	1.64
Spike 3	Mussel	11(0)	43.55	79.2	11.36	26.08	31.81	1.02
Spike 4	Mussel	15(0)	147.93	67.2	31.42	21.24	87.98	1.00
Spike 5	Mussel	9(0)	10.44		4.48	42.91	12.54	1.35

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

Figure **2005.06A** shows expected chromatographic responses for the toxins after periodate and peroxide oxidation. The chromatographic system must be able to separate with baseline resolution, the main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B-1 and STX, within a 15 min chromatographic run as shown in Figure **2005.06B**. A chromatography data processing workstation is recommended to analyze chromatograms and generate reports.

(**b**) *Hot plate.*

(**c**) *Vortex mixer.*

(**d**) *pH meter.*

(**e**) *Benchtop centrifuge.*—3600 × *g*.

(**f**) *Centrifuge tubes.*—Polypropylene, 50 mL.

(**g**) *Test tubes.*–Glass, graduated 5 and 15 mL.

(**h**) *SPE cartridges.*—Ensure that SPE cartridges used give minimum 80% recovery with standards and the correct elution patterns according to the directions in **F** and **G**, below.

(**i**) *C18 cartridges.*—Supelclean LC-18 cartridges (500 mg/3 mL volume; Supelco, Bellefonte, PA, USA; www.supelco.com).

(**j**) *SPE-COOH ion exchange cartridges.*—Bakerbond Carboxylic Acidsilane (COOH) bonded to silica gel (500 mg/3 mL volume; J.T. Baker, Phillipsburg, NJ, USA). Phenomenex (Torrance, CA, USA) or Isolute SPE-COOH cartridges are also suitable with the procedure.

C. Reagents

(**a**) *Analytical standards of STX, NEO, GTX1,4, GTX2,3, C1,2, B-1, and dcSTX.*—Separate solutions in 0.1 M acetic acid with concentrations ranging from 100 to 2000 µg/mL; available from National Research Council of Canada (Halifax, Canada). Dilute with water to obtain working stock solutions with concentrations ranging from 8 to 100 µg/mL. These are stable for at least a month.

Note: For convenience, standards can be combined into 3 mixtures by appropriate dilution of stock solutions in water. Those solutions must be adjusted to about pH 4 with 0.1 M acetic acid. All further dilutions should be made using 0.1 mM acetic acid, so that the solutions are kept at about pH 4. The mixtures are as follows: *Mix I.—For periodate oxidation.*—GTX1,4 and NEO; *Mix II.*—For peroxide oxidation—GTX2,3, STX, B-1, and dcSTX; *Mix III.—For periodate and peroxide oxidation.*—C1,2 and C3,4.

Figure **2005.06B** shows typical chromatograms of such mixtures.

Make dilutions with 0.1 mM acetic acid as described above. Store dilute standard solutions in plastic vials in glass containers deactivated by soaking the vials overnight in sodium hydroxide rinsed with water followed by methanol, and dried.

The oxidation products are not affected by the type of the vials; therefore, the oxidation reactions can be carried out in glass autosampler vials without deactivation.

(**b**) *Matrix modifier for periodate oxidation.*—Prepare by extracting from PSP-free oysters as in **E** and **F**. Adjust extract to pH 6.5 with 1 M NaOH and let stand 2–3 days to precipate coextracted material. Decant the supernate or filter it through a 0.45 µm Acrodisk filter and store in refrigerator when not in use. The initial PSP-free oyster extract can be stored frozen for 2 months at –20°C, but the matrix modifier must be prepared from it every

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Table 2005.06C. Interlaboraatory study results for the determination of NEO in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	10(2)	565.50		113.31	20.04	317.27	1.15
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
5	Clams	7(1)	33.14		12.75	38.47	35.70	1.44
5D	Clams	9(1)	40.00		17.41	43.53	48.75	1.68
6	Clams	15	ND					
8	Mussel	14(0)	283.86		91.76	32.33	256.93	1.67
8D	Mussel	14(0)	278.29		70.58	25.36	197.62	1.31
9	Mussel	13(0)	140.08		45.88	32.75	128.46	1.52
12	Mussel	15(0)	871.07		227.63	26.13	637.36	1.60
Spike 1	Mussel	14(0)	222.64	55.7	88.03	39.54	246.48	1.97
Spike 2	Mussel	15(0)	455.40	56.9	116.94	25.68	327.43	1.43
Spike 3	Mussel	14(0)	158.29[d]		39.36	24.87	110.21	1.18
Spike 4	Mussel	12(0)	213.33[e]		85.77	40.21	240.16	1.99
Spike 5	Mussel	12(0)	344.08[e]		163.71	47.58	458.39	2.53

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] NEO together with dcSTX but calculated as NEO.
[e] No NEO present in these samples. Peaks due to dcSTX but calculated as NEO.

2 weeks. Analyze the matrix modifier for PSP toxins by the periodate and peroxide oxidation to ensure absence of toxins before use.

(c) *Doubly deionized water.*

(d) *Acetic acid.*—Glacial.

(e) *Methanol.*—Analytical grade.

(f) *Acetonitrile.*—Analytical grade.

(g) *Ammonium formate.*—0.3 and 0.1 M aqueous solutions.

(h) *Ammonium acetate.*—0.01 M aqueous solution.

(i) *Sodium chloride.*—3 and 0.05 M aqueous solutions.

(j) *Sodium hydroxide.*—1 M aqueous solution.

(k) *Hydrogen peroxide solution.*—10% aqueous solution (store in refrigerator).

(l) *Disodium hydrogen phosphate (Na_2HPO_4).*—0.3 M aqueous solution.

(m) *Periodic acid solution.*—0.03 M aqueous solution (store solution in refrigerator).

(n) *Periodate oxidant.*—Prepare daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate, and 0.3 M Na_2HPO_4, and adjust pH to 8.2 with 0.2 M NaOH using pH meter.

(o) *Mobile phase.*—A: 0.1 M ammonium formate; B: 0.1 M ammonium formate in 5% acetonitrile, both adjusted to pH 6 by adding 6 mL 0.1 M acetic acid.

Table 2005.06D. Interlaboratory study results for the determination of NEO in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	15(0)	642.80		147.95	23.02	414.26	1.35
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	10(0)	38.80		13.02	33.56	36.46	1.29
5D	Clams	10(0)	42.20		18.27	43.29	51.16	1.68
6	Clams	14	ND					
8	Mussel	13(1)	254.62		89.85	35.29	251.58	1.80
8D	Mussel	15(0)	266.20		92.93	34.91	260.20	1.79
9	Mussel	14(0)	97.64		46.37	47.49	129.84	2.09
12	Mussel	14(1)	842.93		199.36	23.65	558.21	1.44
Spike 1	Mussel	14(0)	212.29	53.1	68.05	32.06	190.54	1.59
Spike 2	Mussel	15(0)	492.43	61.6	159.58	32.41	446.82	1.82

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06E. Interlaboratory study results for the determination of NEO in the presence of dcSTX in spiked shellfish after SPE-COOH cleanup—Spike 3

Lab No.	NEO, µg/kg	Rec., %
2	107	53.5
6[a]	137	68.5
12[a]	154	77.2
15	86	43.0
18	75	37.5
19[a]	92	46.0
22	112	56.0
23	153	76.5
Mean	114.48	57.28
s_R		30.49
RSD_R	26.63	
R		85.37
HorRat	1.20	

[a] Calculation carried out in the coordinator's laboratory using results provided by the collaborators.

D. Preparation of Shellfish Tissue

As with the mouse bioassay [*see* 959.08 (*see* 49.10.01)], thoroughly clean outside of shellfish with fresh water, open shellfish, remove tissue, and transfer it to a homogenizer. Blend until homogeneous.

E. Extraction

In a 50 mL polypropylene centrifuge tube, mix 5.00 ± 0.10 g homogenized shellfish with 3.0 mL 1% acetic acid solution on a Vortex mixer, cap loosely (to avoid pressure buildup during heating), and place in a boiling water bath (100°C) so that contents of tube are below water line. Heat for 5 min. Do not place too many tubes in bath at once that the water bath stops boiling or temperature drops for more than 30 s. Remove from water bath, remix on a Vortex mixer, and cool by placing in a refrigerator or a beaker of cold water for 5 min. Centrifuge for 10 min at 4500 rpm (3600 × *g*) and decant the supernatant solution into a 15 mL graduated conical test tube. Add 3 mL 1% acetic acid solution to the centrifuge tube containing the solid residue, mix well on a Vortex mixer, and centrifuge again for 10 min at 4500 rpm (3600 × *g*). Collect the supernatant solution into the same graduated conical test tube that contains the first portion of extract and dilute to 10.0 mL with water.

F. SPE C18 Cleanup

Condition a 3 mL (500 mg sorbent) SPE C18 cartridge with 6 mL methanol followed by 6 mL water. Add 1 mL (0.5 g shellfish equivalent) of the crude extract to the cartridge. Keep flow rate between 2 and 3 mL/min for all elutions. Collect the effluent into a 5 mL graduated conical test tube. Do not let cartridge run dry. Wash cartridge with 2 mL water and combine washings with the effluent.

Adjust the extract to pH 6.5 with 1M NaOH using pH indicator paper (calibrated against a pH meter) and then dilute to exactly 4.0 mL with water. Use aliquots of this extract for oxidation with periodate and peroxide as described below. Also, analyze an aliquot of the extract without oxidation to verify that the PSP toxin peaks found in the chromatograms are indeed due to PSP, and not to naturally fluorescent coextractives, by mixing a volume of extract and matrix modifier with water instead of periodate oxidant before injecting an aliquot into the LC system. The resulting chromatogram will permit identification of peaks arising from naturally fluorescent coextractives. PSP toxins do not produce peaks under these conditions.

G. SPE-COOH Cleanup

SPE-COOH ion exchange cleanup is used only for extracts that are found to contain *N*-1-hydroxylated PSP toxins after C18 cleanup. Condition a 3 mL SPE-COOH cartridge by passing 10 mL 0.01 M ammonium acetate solution through it. Keep flow rate

Table 2005.06F. Interlaboratory study results for the determination of dcSTX in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	9(0)	18.78		21.61	115.07	60.51	3.95
1	Scallop	13(0)	12.31		4.33	35.17	12.12	1.13
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
5	Clams	14(0)	8.14		2.71	33.29	7.59	1.01
5D	Clams	12(1)	7.42		1.83	24.66	5.12	0.74
6	Clams	15	ND					
7	Mussel	11(1)	10.55		2.02	19.15	5.66	0.60
11	Oyster	11(0)	3.45		1.63	47.25	4.56	1.26
12[d]	Mussel							
Spike 3	Mussel	15(0)	83.73	83.7	41.17	49.17	115.28	2.12
Spike 4	Mussel	15(0)	293.87	73.5	63.35	21.56	177.38	1.12
Spike 5	Mussel	14(1)	385.64	64.27	40.60	10.53	113.68	0.57

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] Not calculated. Only 7 laboratories reported numerical data; the remainder reported as not detected.

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Table 2005.06G. Interlaboratory study results for the determination of dcSTX in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	10(0)	12.60		12.38	98.25	34.66	3.18
1	Scallop	12(1)	9.88		3.89	39.37	10.89	1.23
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	11(0)	8.36		4.86	58.13	13.61	1.77
5D	Clams	10(0)	6.80		3.33	48.97	9.32	1.44
6	Clams	14	ND					
7	Mussel	13(0)	11.62		3.04	26.16	8.51	0.84
11[d]	Oyster							
12	Mussel	10(0)	7.80		3.22	41.28	9.02	1.24
Spike 3	Mussel	13(0)	69.08	69.1	17.09	24.74	47.85	1.03
Spike 4	Mussel	15(0)	276.00	69.0	52.83	19.14	147.92	0.99
Spike 5	Mussel	15(0)	396.27	66.04	93.72	23.65	262.42	1.29

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] Not calculated. Only 6 laboratories reported numerical data; the remainder reported as not detected.

between 2 and 3 mL/min for all elutions. Do not let cartridge run dry. Discard the effluent. Pass a 2 mL aliquot (0.250 g shellfish tissue equivalent) of shellfish extract from the SPE C18 cleanup through the cartridge and collect the effluent in a 15 mL graduated conical test tube marked as Fraction No. 1. Then pass 4.0 mL water through the cartridge and collect into the same tube. Adjust final volume to 6.0 mL in total. This fraction contains the C toxins. Then pass 4.0 mL 0.05 M NaCl solution through the same cartridge, and collect in a 5 mL graduated conical test tube marked as Fraction No. 2. Ensure that the final volume is 4.0 mL. This fraction contains the toxins GTX1,4, GTX2,3, B-1, B-2, and dcGTX2,3. Next, pass 5.0 mL 0.3 M NaCl solution through the cartridge and collect in a 5 mL

Table 2005.06H. Interlaboratory study results for the determination of GTX1,4 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	11(0)	1437.55		649.76	45.20	1819.33	2.98
1	Scallop	10(0)	199.30		170.10	85.35	476.28	4.19
2	Clams	11(1)	285.27		60.08	21.06	168.22	1.09
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	11(0)	61.36		28.46	46.38	79.69	1.91
5D	Clams	11(0)	62.09		17.73	28.56	49.64	1.17
6	Clams	16	ND					
7	Mussel	15(0)	1489.13[d]		417.08	28.01	1167.82	1.86
8	Mussel	12(0)	572.67		156.87	27.39	439.24	1.57
8D	Mussel	13(0)	672.23		164.05	24.40	459.34	1.44
9	Mussel	13(0)	254.46		69.57	27.34	194.80	1.39
11	Oyster	12(0)	81.00		54.86	67.73	153.61	2.90
12	Mussel	10(0)	1815.70[d]		427.69	23.56	1197.53	1.61
Spike 1	Mussel	10(0)	240.10	60.0	115.87	48.26	324.44	2.43
Spike 2	Mussel	11(0)	1440.45[d]		274.83	19.08	769.52	1.26
Spike 5	Mussel	12(1)	2236.25[d]		571.98	25.58	1601.54	1.80

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] GTX1,4 together with C3,4 but calculated as GTX1,4.

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Table 2005.06I. Interlaboratory study results for the determination of GTX1,4 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	16(0)	1279.38		316.92	24.77	887.38	1.61
1[c]	Scallop							
2	Clams	13(1)	364.23		141.21	38.77	395.39	2.08
3	Oyster	15	ND[d]					
4	Mussel	15	ND					
5	Clams	13(0)	73.54		17.18	23.36	48.10	0.99
5D	Clams	13(0)	71.46		22.60	31.63	63.28	1.33
6	Clams	16	ND					
7	Mussel	14(1)	240.07		64.50	26.87	180.60	1.35
8	Mussel	15(1)	645.00		147.94	22.94	414.23	1.34
8D	Mussel	15(1)	674.73		120.99	17.93	338.77	1.06
9	Mussel	14(0)	235.57		44.94	19.08	125.83	0.96
11	Oyster	8(1)	48.88		13.03	26.66	36.48	1.06
12	Mussel	16(0)	2079.31		394.95	18.99	1105.86	1.33
Spike 1	Mussel	14(0)	267.50	66.9	86.80	32.45	243.04	1.66
Spike 2	Mussel	15(0)	628.07	78.5	167.26	26.63	468.33	1.55
Spike 5	Mussel	14(0)	265.21[e]		68.76	25.93	192.53	1.33

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.

[c] Not calculated. Half of the laboratories reported not detected.

[d] ND = Not determined.

[e] No GTX1,4 present in this sample. Peaks due to dcGTX2,3 (confirmed by peroxide oxidation) but calculated as GTX1,4.

Table 2005.06J. Interlaboratory study results for the determination of GTX2,3 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	12(1)	3291.17		523.82	15.92	1466.70	1.19
1	Scallop	16(0)	2526.00		652.30	25.82	1826.44	1.86
2	Clams	15(1)	2537.73		479.37	18.89	1342.24	1.36
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	16(0)	119.75		21.78	18.19	60.98	0.83
5D	Clams	16(0)	113.75		24.44	21.49	68.43	0.97
6	Clams	16	ND					
7	Mussel	14(0)	348.64		92.95	26.66	260.26	1.42
8	Mussel	16(0)	813.13		252.98	31.11	708.34	1.89
8D	Mussel	16(0)	757.31		180.55	23.84	505.54	1.43
9	Mussel	16(0)	316.44		65.95	20.84	184.66	1.10
10	Oyster	16(0)	367.25		88.99	24.23	249.17	1.30
10D	Oyster	15(1)	336.20		78.60	23.38	220.08	1.24
11	Oyster	15(1)	673.00		182.03	27.05	509.68	1.59
12	Mussel	15(1)	1855.93		505.27	27.22	1414.76	1.87
Spike 1	Mussel	14(1)	227.36	75.8	42.71	18.79	119.59	0.94
Spike 2	Mussel	16(0)	529.31	88.2	167.47	31.64	468.92	1.80
Spike 5	Mussel	16(0)	954.31	79.5	293.49	30.75	821.77	1.91

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.

[c] ND = Not determined.

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Table 2005.06K. Interlaboratory study results for the determination of GTX2,3 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	15(1)	3314.33		594.49	17.94	1664.57	1.34
1	Scallop	16(0)	2347.94		522.01	22.23	1461.63	1.58
2	Clams	16(0)	2697.50		506.20	18.77	1417.36	1.36
3	Oyster	15	ND[c]					
4	Mussel	14	ND					
5	Clams	15(1)	132.00		25.33	19.19	70.92	0.88
5D	Clams	16(0)	115.94		28.43	24.52	79.60	1.11
6	Clams	15	ND					
7	Mussel	15(0)	342.80		80.23	23.40	224.64	1.25
8	Mussel	16(0)	804.63		164.42	20.43	460.38	1.24
8D	Mussel	16(0)	745.94		164.78	22.09	461.38	1.32
9	Mussel	16(0)	320.88		58.22	18.14	163.02	0.96
10	Oyster	15(1)	369.47		71.38	19.32	199.86	1.04
10D	Oyster	15(0)	341.60		86.11	25.21	241.11	1.34
11	Oyster	16(0)	701.31		203.93	29.08	571.00	1.72
12	Mussel	16(0)	1873.94		345.02	18.41	966.06	1.27
Spike 1	Mussel	15(0)	261.27	87.1	42.72	16.35	119.62	0.84
Spike 2	Mussel	16(0)	563.56	93.9	116.81	20.73	327.07	1.19
Spike 5	Mussel	16(0)	1024.88	85.4	174.72	17.05	489.22	1.07

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

Table 2005.06L. Interlaboratory study results for the determination of B-1 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	11(0)	435.36		47.84	10.99	133.95	0.61
1	Scallop	13(0)	91.08		25.12	27.58	70.34	1.20
2	Clams	11(0)	31.00		7.36	23.74	20.61	0.88
3	Oyster	16	ND[c]					
4	Mussel	16	ND					
5	Clams	13(1)	42.77		8.36	19.55	23.41	0.76
5D	Clams	13(1)	40.54		7.22	17.81	20.22	0.69
6	Clams	16	ND					
7	Mussel	15(0)	145.07		27.11	18.69	75.91	0.87
8	Mussel	15(0)	328.00		47.09	14.36	131.85	0.76
8D	Mussel	15(0)	334.13		51.52	15.42	144.26	0.82
9	Mussel	15(0)	119.80		25.34	21.15	70.95	0.96
10	Oyster	12(0)	37.67		9.26	24.58	25.93	0.94
10D	Oyster	14(0)	39.57		12.97	32.78	36.32	1.26
11	Oyster	14(0)	73.14		16.47	22.52	46.12	0.95
12	Mussel	15(0)	946.87		263.88	27.87	738.86	1.73
Spike 2	Mussel	14(0)	53.36		16.01	30.00	44.83	1.21
Spike 3	Mussel	15(0)	311.13	77.8	69.98	22.49	195.94	1.18
Spike 4	Mussel	15(0)	1212.53	75.8	239.77	19.77	671.36	1.27
Spike 5	Mussel	14(1)	691.00	86.4	223.40	32.33	625.52	1.91

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06M. Interlaboratory study results for the determination of B-1 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	14(0)	349.71		58.75	16.80	164.50	0.90
1	Scallop	12(1)	71.75		13.02	18.15	36.46	0.76
2	Clams	9(0)	27.11		4.43	16.34	12.40	0.59
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	12(0)	42.00		11.07	26.36	31.00	1.02
5D	Clams	12(0)	40.75		9.73	23.88	27.24	0.92
6	Clams	14	ND					
7	Mussel	13(0)	120.85		17.71	14.65	49.59	0.67
8	Mussel	14(0)	285.79		43.73	15.30	122.44	0.79
8D	Mussel	14(0)	274.14		52.47	19.14	146.92	0.98
9	Mussel	14(0)	123.71		29.11	23.53	81.51	1.07
10	Oyster	10(0)	30.60		5.99	19.58	16.77	0.72
10D	Oyster	12(0)	37.58		10.93	29.08	30.60	1.11
11	Oyster	12(0)	63.00		11.09	17.60	31.05	0.73
12	Mussel	14(0)	784.86		73.41	9.35	205.55	0.56
Spike 2	Mussel	13(0)	46.00		8.78	19.09	24.58	0.75
Spike 3	Mussel	13(0)	292.54	73.1	43.03	14.71	120.48	0.76
Spike 4	Mussel	14(0)	1064.71	66.5	147.33	13.84	412.52	0.87
Spike 5	Mussel	15(0)	619.67	77.5	85.39	13.78	239.09	0.80

[a,b] Number of laboratories where a=number of laboratories retained after Dixon statistical outliers removed and b = number of outlier laboratories.
[c] ND = Not determined.

Table 2005.06N. Interlaboratory study results for the determination of C1,2 in shellfish after SPE C18 cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	12(0)	299.00		50.81	16.99	142.27	0.89
2	Clams	13(1)	253.62		56.83	22.41	159.12	1.14
3	Oyster	16	ND[c]					
4	Mussel	15	ND					
5	Clams	16(0)	256.50		74.66	29.11	209.05	1.48
5D	Clams	15(1)	239.13		50.81	21.25	142.27	1.07
6	Clams	16	ND					
7	Mussel	15(0)	912.40		301.90	33.09	845.32	2.04
8	Mussel	12(0)	118.08		63.81	54.04	178.67	2.45
8D	Mussel	10(1)	96.30		24.10	25.03	67.48	1.10
9	Mussel	9(0)	76.56		80.34	104.9	224.95	4.46
10	Oyster	11(0)	161.91		43.73	27.01	122.44	1.28
10D	Oyster	15(0)	189.73		58.51	30.84	163.83	1.50
11	Oyster	16(0)	357.69		58.20	16.27	162.96	0.87
12	Mussel	16(0)	256.50		67.38	26.27	188.66	1.34
Spike 2	Mussel	13(1)	734.85	73.5	108.97	14.83	305.12	0.88
Spike 5	Mussel	14(1)	1566.50	78.3	227.74	14.54	637.67	0.97

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06O. Interlaboratory study results for the determination of C1,2 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
Practice sample	Mussel	14(0)	300.93		77.77	25.84	217.76	1.35
2	Clams	14(1)	261.07		66.24	25.37	185.47	1.30
3	Oyster	14	ND[c]					
4	Mussel	14	ND					
5	Clams	14(1)	237.07		64.30	27.12	180.04	1.37
5D	Clams	15(0)	238.07		80.34	33.75	224.95	1.70
6	Clams	15	ND					
7	Mussel	15(0)	983.40		255.06	25.94	714.17	1.62
8	Mussel	9(0)	108.56		40.27	37.09	112.76	1.66
8D	Mussel	10(0)	93.40		35.75	38.28	100.10	1.67
9[d]	Mussel							
10	Oyster	14(0)	204.36		57.38	28.08	160.66	1.38
10D	Oyster	14(0)	200.71		48.51	24.17	135.83	1.19
11	Oyster	15(0)	349.07		81.27	23.28	227.56	1.24
12	Mussel	12(1)	257.75		39.23	15.22	109.84	0.78
Spike 2	Mussel	14(0)	859.64	86.0	204.53	23.79	572.68	1.45
Spike 5	Mussel	15(0)	1687.13	84.4	342.42	20.30	958.78	1.37

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.
[d] Not calculated. Only 3 laboratories reported numerical data; the remainder reported as not detected.

graduated conical test tube marked as Fraction No. 3. Ensure that the final volume is 5.0 mL. This fraction contains STX, NEO, and dcSTX. Proceed with oxidation and HPLC analyses.

If problems with detector sensitivity are encountered, concentrate each fraction from the ion exchange column in 50 mL round-bottom flasks instead of graduated conical test tubes and evaporate to ca 1 mL on a rotary evaporator with a water bath set at 45°C. Transfer the solution into a 5 mL graduated conical test tube using a Pasteur pipet. Rinse the 50 mL round-bottom flask 3 times with about 0.2–0.3 mL water each time, transferring the rinse into the graduated tube so that the final volume of extract is 2.0 mL. *Note*: A graduated 2 mL volumetric flask can also be used. Analyze Fraction Nos. 1–3 by LC after periodate and peroxide oxidations as described below.

H. Oxidation Reactions

(a) *Periodate oxidation.*—Dispense all reagents and solutions used in the oxidation reactions with autopipets (Eppendorf or equivalent) with disposable plastic tips. Add a 100 µL standard solution or test extract after SPE C18 or ion exchange cleanup to 100 µL of matrix modifier solution in a 1.5 mL microcentrifuge tube (or autosampler vial, if automated chromatographic analysis will be performed). Then add 500 µL periodate oxidant and mix well on a Vortex mixer. Let solution react at room temperature for 1 min; then add 5 µL concentrated acetic acid, and mix. Let mixture stand for at least 10 min at room temperature before injecting 50–100 µL into the LC system. This solution is stable for about 1 day for the nonhydroxylated toxins. However, for NEO, B-2, and GTX1,4, a slow degradation of the oxidation products occurs (about 30% over 8 h). Quantification can still be carried out after this if the standards

Table 2005.06P. Interlaboratory study results for the determination of C3,4 in shellfish after SPE-COOH cleanup

Sample No.	Matrix	Labs[a,b]	Mean, µg/kg	Rec., %	s_R	RSD_R, %	R	HorRat
3	Oyster	15	ND[c]					
4	Mussel	15	ND					
6	Clams	15	ND					
7	Mussel	13(0)	837.15		227.24	27.14	636.27	1.65
12	Mussel	10(1)	237.50		106.35	44.78	297.78	2.25
Spike 2	Mussel	9(0)	724.78	80.5	180.88	24.96	506.46	1.49
Spike 5	Mussel	15(0)	1425.07	79.2	301.97	21.19	845.52	1.40

[a,b] Number of laboratories: a = number of laboratories retained after Dixon statistical outliers removed; b = number of outlier laboratories.
[c] ND = Not determined.

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Table 2005.06Q. Interlaboratory study results for the determination of PSP in shellfish after SPE C18 cleanup—blind duplicates statistical treatment

PSP	Youden pairs	Matrix	Labs[a,b]	Mean, µg/kg	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HorRat
STX	5 and 5D	Clams	15(0)	520.03	31.38	6.03	71.46	13.74	87.85	200.10	0.78
	8 and 8D	Mussel	15 (0)	312.57	67.80	21.69	73.23	23.43	189.84	205.05	1.23
	10 and 10D	Oyster	15(0)	140.30	24.96	17.79	43.91	31.29	69.87	122.94	1.46
NEO	8 and 8D	Mussel	13(0)	280.00	43.14	15.41	83.82	29.94	120.79	234.70	1.55
dcSTX	5 and 5D	Clams	12(0)	7.46	0.68	9.08	2.02	27.04	1.90	5.65	0.81
GTX1,4	5 and 5D	Clams	9(0)	64.61	12.85	19.89	24.13	37.35	35.98	67.57	1.55
	8 and 8D	Mussel	11(0)	601.45	119.96	19.95	155.53	25.86	335.89	435.48	1.50
GTX2,3	5 and 5D	Clams	16(0)	116.75	15.77	13.51	23.15	19.83	44.15	64.82	0.90
	8 and 8D	Mussel	16(0)	785.22	135.94	17.31	220.26	28.05	380.64	616.72	1.69
	10 and 10D	Oyster	15(0)	347.13	66.64	19.20	81.10	23.36	186.59	227.09	1.25
B-1	5 and 5D	Clams	13(0)	41.65	5.26	12.62	7.82	18.78	14.72	21.90	0.73
	8 and 8D	Mussel	15(0)	331.07	27.86	8.42	49.17	14.87	78.01	137.68	0.79
	10 and 10D	Oyster	12(0)	38.58	8.35	21.63	11.69	30.30	23.37	32.73	1.16
C1,2	5 and 5D	Clams	15(0)	241.47	37.53	15.54	53.39	22.11	105.09	149.49	1.12
	8 and 8D	Mussel	8(0)	101.00	28.62	28.34	38.38	38.00	80.14	107.46	1.68
	10 and 10D	Oyster	11(0)	168.77	53.48	31.69	53.48	31.69	149.75	149.75	1.52

[a,b] Number of laboratories: a = number of laboratories retained after Cochran statistical outliers removed; b = number of outlier laboratories.

Table 2005.06R. Interlaboratory study results for the determination of PSP in shellfish after SPE-COOH cleanup—blind duplicates statistical treatment

PSP	Youden pairs	Matrix	Labs[a,b]	Mean, µg/kg	s_r	RSD_r, %	s_R	RSD_R, %	r	R	HorRat
STX	5 and 5D	Clams	15 (0)	491.97	43.99	8.94	85.73	17.43	123.16	240.04	0.98
	8 and 8D	Mussel	13 (0)	273.31	36.19	13.24	60.05	21.97	101.32	168.15	1.13
	10 and 10D	Oyster	14(0)	132.14	32.87	24.88	37.88	28.67	92.05	106.08	1.32
NEO	8 and 8D	Mussel	13(0)	262.65	67.73	25.79	89.95	34.24	189.65	251.79	1.75
	5 and 5D	Clams	10 (0)	40.50	7.82	19.30	15.86	39.15	21.89	44.40	1.51
dcSTX	5 and 5D	Clams	9(0)	6.89	0.88	12.80	3.15	45.76	2.47	8.83	1.35
GTX1,4	5 and 5D	Clams	12 (0)	73.92	11.10	15.02	19.36	26.19	31.09	54.20	1.11
	8 and 8D	Mussel	15 (0)	659.87	85.84	13.01	135.04	20.47	240.36	378.12	1.20
GTX2,3	5 and 5D	Clams	15(0)	124.47	19.88	15.97	28.14	22.61	55.65	78.80	1.03
	8 and 8D	Mussel	16(0)	775.28	91.07	11.75	166.54	21.48	380.64	616.72	1.69
	10 and 10D	Oyster	14(0)	357.21	84.30	23.60	84.30	23.60	236.03	236.03	1.26
B-1	5 and 5D	Clams	11(0)	40.68	5.04	12.39	10.37	25.48	14.11	29.03	0.98
	8 and 8D	Mussel	14(0)	279.96	40.58	14.49	48.02	17.15	113.62	134.44	0.89
	10 and 10D	Oyster	10(0)	35.25	8.49	24.10	9.56	27.11	23.78	26.76	1.02
C1,2	5 and 5D	Clams	14(0)	230.43	46.06	19.99	62.14	26.97	128.97	174.01	1.35
	8 and 8D	Mussel	8(0)	100.94	19.99	19.80	40.52	40.14	55.96	113.45	1.78
	10 and 10D	Oyster	14(0)	202.54	37.12	18.33	52.66	26.00	103.93	147.46	1.28

[a,b] Number of laboratories: a = number of laboratories retained after Cochran statistical outliers removed; b = number of outlier laboratories.

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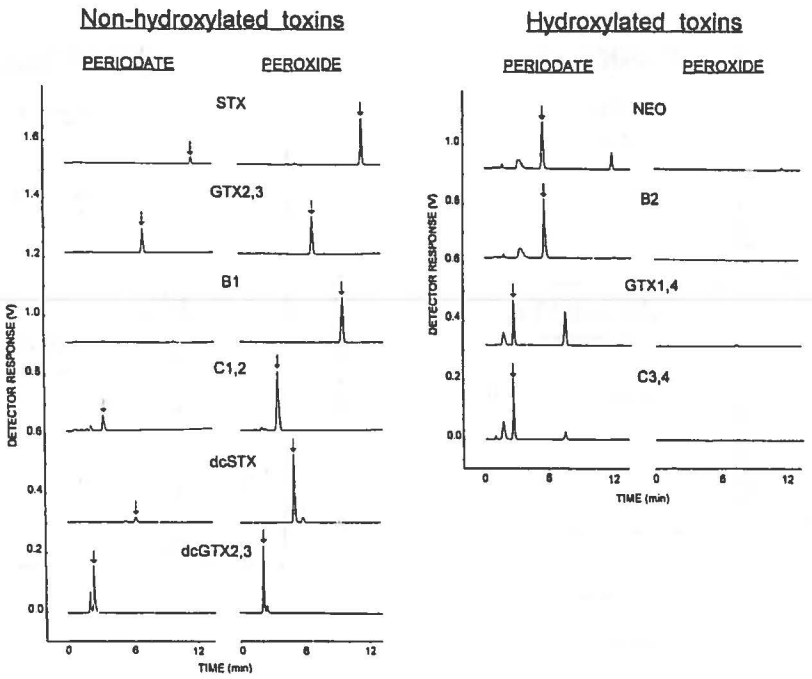


Figure 2005.06A. Chromatographic patterns showing oxidation products formed after periodate and peroxide oxidations of toxins included in this study. The same quantity of each toxin was used for each oxidation reaction. Arrows indicate peaks used for quantitation.

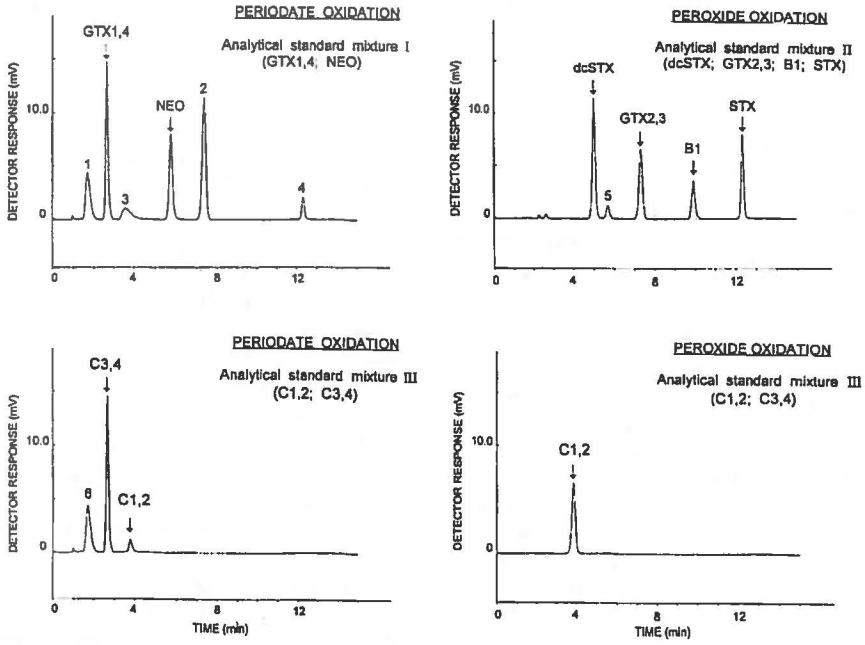


Figure 2005.06B. Typical chromatographic patterns obtained with 3 mixtures of analytical standards of PSP toxins. The hydroxylated toxins are oxidized with periodate and the nonhydroxylated toxins with peroxide. Arrows indicate peaks used for quantitation. Peaks 1 and 2 are secondary oxidation products of GTX1,4; peaks 3 and 4 are secondary oxidation products of NEO; peak 5 is a secondary oxidation product of dcSTX; and peak 6 is a secondary oxidation product of C3,4.

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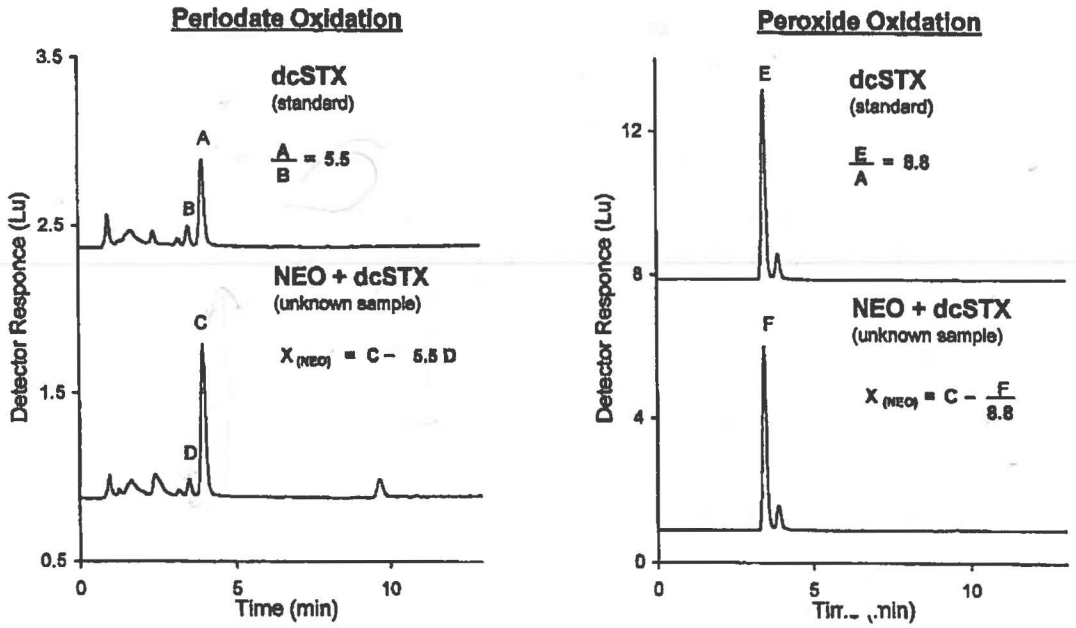


Figure 2005.06C. Chromatograms explaining the calculation of NEO in the presence of dcSTX. A, B, and E are the peak areas of dcSTX standard after periodate and peroxide oxidation; C, D, and F are the peak areas of an unknown sample contaminated with dcSTX and NEO after periodate and peroxide oxidation.

and test solutions are stored under the same conditions and analyzed together.

(**b**) *Peroxide oxidation.*—Add 25 µL 10% (w/v) aqueous H_2O_2 to 250 µL of 1M NaOH in a 1.5 mL plastic microcentrifuge tube (or autosampler vial, if automated chromatographic analysis will be performed), and mix. Then add 100 µL standard solution or test extract after SPE C18 or ion exchange cleanup. Mix and allow to react for 2 min at room temperature. Add 20 µL concentrated acetic acid and mix the solution. Inject 25–50 µL of this solution into the LC system. (*Note*: Injecting more than 50 µL may cause peak broadening.) The solution is stable for at least 8 h.

I. Calculations

Each toxin is quantitatively determined in shellfish tissue by direct comparison to analytical standards at similar concentrations as anticipated in the test portion [*see* below, section (**a**)]. For convenience, 3 analytical standard mixtures can be used for quantitating the toxins as described above.

STX, GTX2,3, B-1, and C1,2 produce single oxidation products with both oxidation reactions, while dcSTX produces 2 oxidation products with both reactions. However, NEO, B-2, GTX1,4, and C3,4 each produce 3 peaks after periodate oxidation but only the second eluting peaks are used for quantitation (Figure **2005.06B**). Because some PSP toxins (NEO and B-2; GTX1,4, and C3,4) give the same oxidation products, their quantitative determination can be made only after separation by COOH ion exchange chromatography as described above. If present in a high enough concentration, dcSTX will interfere with NEO quantification after periodate oxidation. NEO can be quantified by using mathematical ratios of peaks after periodate and peroxide oxidation as described below.

(**a**) *Calculate the concentration of PSP (in µg/kg).*—

$$\text{Toxin concentration, } \mu g/kg = \frac{Ax\ Cs\ Vx\ D\ 1000}{As\ M}$$

where Ax = peak area of the toxin in the extract analyzed; As = peak area of the nearest standard; Cs = concentration of standard (µg/mL); Vx = final volume of extract analyzed (mL); D = dilution factor [for those extracts where PSP toxins are above the most concentrated standard mixture, dilute with water after SPE cleanup, so that the toxin concentrations fall in the range of the standards, and reanalyze beginning at step **H** above (note the dilution factor)]; M = amount of test portion (g) carried through cleanup procedure, for example, 0.5 g for SPE C18 and 0.25 g for SPE-COOH cleanup; 1000 = factor to convert results from µg/g to µg/kg.

(**b**) *Calculating NEO in the presence of dcSTX.*—The peak area of NEO in a test portion containing both NEO and dcSTX can be calculated in 2 ways. Figure **2005.06C** shows chromatograms after periodate and peroxide oxidation of a dcSTX standard and an unknown test portion containing a mixture of NEO and dcSTX.

Mark the peak areas with letters as follows: *dcSTX standard.*—A = second (larger) peak, periodate oxidation; B = first (smaller) peak, periodate oxidation; E = first (larger) peak, peroxide oxidation. *Unknown sample.*—C = second (larger) peak, periodate oxidation; D = first (smaller) peak, periodate oxidation; F = first (larger) peak, peroxide oxidation.

Method 1.—If the concentration of dcSTX in the test portion containing a mixture of NEO and dcSTX is significant to produce a well-integrated first peak (peak D, Figure **2005.06C**) with periodate

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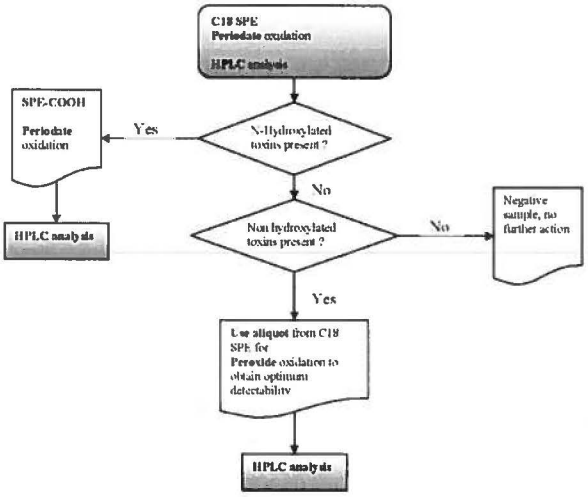


Figure 2005.06D. Schematic diagram of procedure.

oxidation, then calculate the peak area corresponding to NEO (peak $X_{(NEO)}$) from the ratio of the 2 dcSTX peak areas produced with periodate oxidation of the standard.

For the dcSTX standard analyzed with periodate oxidation, the ratio of the 2 peak areas is calculated as

$$\frac{A}{B} = \frac{X_{(dcSTX)}}{D} \Rightarrow X_{(dcSTX)} = \frac{D\,A}{B}$$

where $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - \frac{D\,A}{B}$$

Method 2.—If the concentration of dcSTX in the test portion containing NEO and dcSTX is not significant enough to produce a well-integrated first peak (peak D, Figure **2005.06C**) with periodate oxidation, then calculate the peak area corresponding to NEO (peak $X_{(NEO)}$) from the ratio of the dcSTX peak produced with periodate oxidation (peak A, Figure **2005.06C**) and the dcSTX peak area produced with peroxide oxidation (peak E, Figure **2005.06C**) in the standard.

For a dcSTX standard analyzed with periodate and peroxide oxidations, the ratio of the 2 peak areas is calculated as

$$\frac{E}{A} = \frac{F}{X_{(dcSTX)}} \Rightarrow X_{(dcSTX)} = \frac{F\,A}{E}$$

where $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - \frac{F\,A}{E}$$

J. Application of the Method for Routine Analysis

To efficiently apply this method to routine analysis where large numbers of test samples need to be analyzed, the following procedure is recommended (*see* Figure **2005.06D**). (*1*) Analyze test extracts after C18 SPE cleanup and periodate oxidation. If no peaks correspond to any of the PSP standards, the test sample is negative and no further analyses are required. (*2*) If any of the nonhydroxylated toxins, STX, dcSTX, GTX2,3, B-1, or C1,2, are present but the *N*-hydroxylated toxins, NEO, GTX1,4, and C3,4, are absent, then the nonhydroxylated toxins can be quantified by direct comparison to known standards carried through the periodate oxidation. However, the peroxide oxidation reaction is much more sensitive for B-1, C1,2, and dcSTX and would be preferred if optimum detectability is required. The peroxide oxidation reaction may be used to confirm findings of all toxins obtained by the periodate oxidation. (*3*) If both *N*-hydroxylated and nonhydroxylated toxins are present, the nonhydroxylated toxins are quantified after another aliquot of the test extract is oxidized using hydrogen peroxide, and the peaks are compared to standards similarly oxidized. The reasons for this are that with periodate oxidation some of the hydroxylated toxins produce peaks that elute with their nonhydroxylated analogues and that for some toxins (especially B-1, C1,2, and dcSTX) peroxide oxidation yields much better detectability. (*4*) For quantification of any *N*-hydroxylated toxins, another portion of the test extract is cleaned up using the SPE-COOH cartridge step, and the appropriate fraction is oxidized after periodate oxidation. This step separates C3,4 from GTX1,4 and B-2 from NEO, enabling all toxins to be quantified by direct comparison to standards carried through the same periodate oxidation procedure.

Reference: J. AOAC Int. 88, 1714(2005).

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ISSC Method Application and Single Lab Validation Summary of Required Elements for Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A summary of required elements has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria in this document have been defined and accompany the summary of required elements as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish; Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection
Name of the Method Developer/Submitter	Developed by James Lawrence, Barbara Niedwiadek, and Cathie Menard, Health Canada. Submitted by Leanne J. Flewelling, Florida Fish and Wildlife Conservation Commission
Developer Contact Information	100 8th Avenue SE, St. Petersburg, FL 33701 727-386-3437 leanne.flewelling@myfwc.com
Required Elements	**Brief Description**
A. Need for the New Method	
1. Clearly define the need for which the method has been developed.	Paralytic Shellfish Poisoning (PSP) occurs worldwide and poses a serious threat to shellfish consumers. AOAC Official Method 2005.06 (OMA 2005.06) was developed to provide a quantitative chemical method for determination of PSP toxins in shellfish. Currently approved methods in the NSSP include the APHA Mouse Bioassay (for all matrices covered by the NSSP), the Receptor Binding Assay (for mussels only), and the PCOX HPLC method (for mussels, clams, oysters, and scallops). Each method has its advantages and drawbacks, and NSSP laboratories benefit from choices that may be driven by access to equipment, the matrices of interest, or local PSP profiles.
2. What is the intended purpose of the method?	The intended purpose of the method is as an Approved Method of PSP testing in mussels, clams, oysters, and scallops.
3. Is there an acknowledged need for this method in the NSSP?	Yes. PSP toxin testing is required to support shellfish safety decisions under the NSSP. AOAC Official Method 2005.06 provides a validated chemical method that may be used to support regulatory monitoring and offers advantages over the MBA and RBA, including toxin-specific quantitation and reduced reliance on animal testing.
4. What type of method? i.e. chemical, molecular, culture, etc.	Chemical analytical method: High performance liquid chromatography with fluorescence detection (HPLC-FLD) following prechromatographic oxidation of PSP toxins.
B. Method Documentation	
1. Method documentation includes the following information:	

Method Title	AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish; Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection (OMA 2005.06)
Method Scope	The published AOAC method is applicable to determination of STX; NEO; GTX2,3; GTX1,4; dcSTX; B-1/GTX5; C1,2; and C3,4 in mussels, clams, oysters, and scallops.
References	AOAC International (2005). Official Method 2005.06, Paralytic Shellfish Poisoning Toxins in Shellfish; Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection. Official Methods of Analysis of AOAC International. Appendix D. Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005). Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: Collaborative study. Journal of AOAC International, 88, 1714-1732. Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., & Lees, D.N. (2009). Refinement and extension of AOAC Method 2005.06 to include additional toxins in mussels: Single-laboratory validation. Journal of AOAC International, 92(1), 190-207. Turner, A.D., Hatfield, R.G., Rapkova-Dhanji, M., Norton, D.M., Algoet, M., & Lees, D.N. (2010). Single-laboratory validation of a refined AOAC HPLC Method 2005.06 for oysters, cockles, and clams in UK shellfish. Journal of AOAC International, 93(5), 1482-1493.
Principle	Shellfish tissues are extracted with acetic acid. Extracts are cleaned up using SPE-C18 cartridges, with SPE-COOH fractionation applied when required for N-hydroxylated toxins. Toxins are oxidized with periodate and/or peroxide to fluorescent derivatives and analyzed by reversed-phase HPLC with fluorescence detection. Concentrations are calculated by comparison with toxin standards and summed as saxitoxin equivalents.
Any Proprietary Aspects	No proprietary method components are required.
Equipment Required	Required equipment: HPLC system with fluorescence detection, centrifuge, vortex mixer, pH meter, boiling water bath or hot plate, SPE manifold, and standard laboratory equipment. Consumables: Reversed-phase C18 column, SPE cartridges
Reagents Required	Reagents: acetic acid, ammonium formate, acetonitrile, methanol, sodium hydroxide, hydrogen peroxide, periodic acid/periodate oxidant components, sodium chloride solutions, matrix modifier, and certified PSP toxin standards.
Sample Collection, Preservation and Storage Requirements	At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection,

	shellfish should be placed in dry storage between 0- and 10-degrees C. Shellfish not shucked on the day of collection should be refrigerated. Refrigeration must not exceed 48 hours. If shellfish are refrigerated, only live animals are used in the analysis. The outside of shellfish are cleaned with fresh water. Adductor muscles are cut and the shell is opened. The inside of the shellfish is rinsed with fresh water to remove sand and other foreign material. Meats are shucked from the shells being careful not to cut or damage the body of the mollusk. Approximately 100-120 grams of meat are collected, in a single layer, on a number 10 sieve, and the sample is drained for 5 minutes. Any pieces of shell are discarded. Drained meats are blended at high speed until homogenous (60-120 seconds) and extracted for PSP toxins (see protocol in Appendix B). Samples must be processed within 24 hours of shucking.
Safety Requirements	General chemical safety practices (e.g. personal protective equipment including gloves, safety glasses and laboratory coat) must be followed.
Clear and Easy to Follow Step-by-Step Procedure	See protocol in Appendix B.
Quality Control Steps Specific for this Method	A multipoint calibration (at least 6 points) of each of the PSP analogs or standard mix solutions is performed during initial method verification and at least annually thereafter to confirm the established analytical working range and detector response for each analog. Calibration linearity R^2 must be ≥ 0.98. More frequent calibrations are performed as warranted by instrument performance, following significant service or changes to the analytical system or procedure that may affect detector response or quantitation, and with a new standard lot. Routine sample quantitation is performed using appropriate analytical standards in accordance with AOAC OMA 2005.06. The standards are analyzed at the beginning of the batch and periodically thereafter at a frequency established by the laboratory to verify stability of detector response and retention time. Additional standards are analyzed at the end of the batch. Peaks must have S/N > 10 and be above the lowest calibration curve point for quantitation. The main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B1, and STX must be separated with baseline resolution. Peak asymmetry is routinely monitored to evaluate the performance of the column. The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3. Identification retention time of sample vs standard must be within ±0.2 min.
Laboratory Evaluation Checklist for Use During Evaluations of Proper Method Implementation	See Appendix C.

C. Validation Criteria	
1. Accuracy / Trueness	Accuracy/trueness is supported by data in the AOAC collaborative study on fortified mussels (summarized in Appendix A Table A1). Published data were handled as directed in ISSC SOP VII.1 and calculated accuracies were STX 75.8%; NEO 58.5%; dcSTX 69.4%; GTX1,4 74.6%; GTX2,3 81.5%; B-1/GTX5 79.1%; C1,2 76.7%; C3,4 79.6%.
2. Measurement Uncertainty	Measurement uncertainty was not reported as a primary endpoint of the AOAC collaborative study. However, the study provides strong uncertainty-related evidence through the blind duplicates, including repeatability, reproducibility, repeatability limits, reproducibility limits, and HorRat values (results reported in Lawrence et al. 2005 Tables 2005.06Q and 2005.06R). These data are directly relevant to the precision component of measurement uncertainty. The spike recovery tables provide additional information relevant to recovery bias and interlaboratory variability in recovery (Appendix A Table A1). Additional supportive data for measurement uncertainty is provided in Turner et al. 2009 (Table 16 and Appendix A Table A3). Here method uncertainty for PSP toxins in mussel tissue was calculated using standardized uncertainty components derived from precision, recovery, and reproducibility/long-term performance. Combined expanded uncertainty (k = 2) for individual toxins ranged from 0.34 to 1.03. Turner et al. 2010 applied the same approach to oysters and hard clams. Combined expanded uncertainty (k = 2) for individual toxins ranged from 0.22 to 0.70 (Table 13 and Appendix A Table A5).
3. Precision Characteristics (repeatability and reproducibility)	The AOAC collaborative study provides interlaboratory reproducibility statistics, including RSDR and HorRat values, across multiple PSP toxins and matrices. Repeatability and reproducibility of three blind duplicate treatments are reported in Tables 2005.06Q and 2005.06R, with all HorRat values <2.0. Repeatability is further supported by Turner et al. 2009 (Tables 11 and 12 and Appendix A Table A3) for mussels and Turner et al. 2010 selected OMA-relevant data for oysters and hard clams (Appendix A Table A5).
4. Recovery	The AOAC collaborative study included fortified mussel samples and published recoveries for individual toxin analogs in the collaborative tables. Recoveries for non-hydroxylated PSP toxins using SPE C18 cleanup were STX 74.4%–93.2%; dcSTX 64.3%–83.7%; GTX2,3 75.8%–88.2%; B-1/GTX5 75.8%–86.4%; and C1,2 73.5%–78.3%. Recoveries for N-1-hydroxylated PSP toxins using SPE-COOH ion exchange cleanup were NEO 53.1%–61.6%; GTX1,4 66.9%–78.5%; and C3,4 79.2%–80.5%.
5. Specificity	Specificity was not systematically assessed in the AOAC collaborative study; however, the method uses toxin-specific oxidation chemistry, chromatographic retention time, SPE cleanup/fractionation, and comparison with standards. The method also includes analysis of unoxidized extracts to identify naturally fluorescent coextractives.

6. Working and Linear Ranges	Working and linear ranges are not defined in the AOAC collaborative study or the OMA. Turner et al. 2009 and 2010 reported that PSP toxin calibration curves were linear over the working calibration range of 0–96 µg STX eq/100 g (0 to 9.6 µg STX eq/100 g for B-1/GTX5) and 0–120 µg STX eq/100 g (0 to 12 µg STX eq/100 g for B-1/GTX5), respectively.
7. Limit of Detection	In the collaborative study, Lawrence et al. noted that the limits of detection and quantitation varied for different PSP toxins and depended on the sensitivity of the fluorescence detectors used by the participating laboratories. LOD is described by the AOAC method applicability thresholds: "The method is applicable to quantifying saxitoxin, >2.2 µg/kg; GTX 2,3 together, >12.5 µg/kg (lowest concentration tested); B-1, >2.7 µg/kg; dcSTX, >0.8 µg/100 g; NEO, >4.0 µg/100 g; GTX 1,4 together, >5.0 µg/100 g; C1,2 together, >9.3 µg/100 g; and C3,4 together, >72.5 µg/100 g." Additional information on LOD from studies by Turner et al. 2009 and 2010 is provided in Appendix A Tables A3 and A5.
8. Limit of Quantitation / Sensitivity	Limits of quantitation (LOQ) are not stated in the OMA. Information on LOQ from studies by Turner et al. 2009 and 2010 is provided in Appendix A Tables A3 and A5.
9. Ruggedness	Ruggedness was not reported in the AOAC collaborative study; however, indirect support is provided through successful multi-lab implementation. Turner et al. 2009 and 2010 evaluated ruggedness using Plackett-Burman experimental designs for multiple PSP toxins. None of the tested parameters produced a statistically significant effect on method stability, as all calculated t-test values were below the critical value. See Turner et al. 2009 (Tables 14 and 15) and 2010 (Table 3).
10. Matrix Effects	Matrix effects were not evaluated as a separate validation parameter in the AOAC collaborative study. However, the study included multiple shellfish matrices — mussels, clams, oysters, and scallops — from geographically diverse sources and with varied PSP toxin profiles. The resulting recovery, reproducibility, and HorRat data therefore provide indirect evidence that the method performed acceptably across the shellfish matrices included in the official method scope. The authors reported that collaborators did not have difficulty with the shellfish types analyzed and that the method performed satisfactorily for all sample types studied.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Collaborative study samples were analyzed by mouse bioassay. These results were in very good agreement with HPLC-FLD results over the full concentration range studied. (Lawrence et al., 2005, Table 1) Additional comparative data in support of the OMA matrices are reported in Turner et al 2009 and 2010 and by the submitting laboratory (Appendix A, Table A7 and Figure A1).

D. Other Information	
1. Cost of the Method	The cost of chemicals and consumables including certified toxin standards, C18 columns, SPE cartridges, solvents/reagents, and plastic and glass consumables to run the method is estimated at $14-$25 per sample, depending on the toxin present. Other method cost categories include equipment and equipment maintenance costs (see D3) and labor.
2. Special Technical Skills Required to Perform the Method	Analysts must be trained in marine biotoxin sample handling, shellfish tissue extraction, SPE cleanup/fractionation, oxidation chemistry, HPLC-FLD operation, chromatographic integration, toxin calibration, toxicity equivalency calculations, and quality system documentation.
3. Special Equipment Required and Associated Cost	The estimated cost for an HPLC system with fluorescence detector and autosampler is $90,000-$130,000 depending on configuration and service options. Refurbished systems may be available for less. The estimated cost for an SPE vacuum manifold system, including vacuum pump, tubing, trap, fittings, and accessories is $4,000. An automated SPE system greatly reduces the method time/labor and is estimated at $70,000-$110,000. Additional standard lab equipment needs include a centrifuge, pH meter, vortex mixer, boiling water bath, and freezer/refrigerator storage for samples and standards.
4. Abbreviations and Acronyms Defined	AOAC = AOAC INTERNATIONAL; CRM = certified reference material; dcNEO = decarbamoyl neosaxitoxin; dcSTX = decarbamoyl saxitoxin; dcGTX2,3 = decarbamoyl gonyautoxins 2 and 3; FLD = fluorescence detection; GTX = gonyautoxin; HPLC = high-performance liquid chromatography; FLD = fluorescence detection; LOD = limit of detection; LOQ = limit of quantitation; LRM = laboratory reference material; MBA = mouse bioassay; NEO = neosaxitoxin; NSSP = National Shellfish Sanitation Program; PSP = paralytic shellfish poisoning; SPE = solid-phase extraction; STX = saxitoxin; TEF = toxicity equivalency factor.
5. Details of Turn Around Times (time involved to complete the method)	The method includes tissue homogenization, acetic acid extraction, SPE cleanup. SPE fractionation as needed, oxidation, HPLC-FLD analysis, chromatographic review, quantitation, quality control review, and reporting. Turnaround time for a batch of 10-20 samples is 24-48 hours depending on the profile present and number of injections required.
6. Provide Brief Overview of the Quality Systems Used in the Lab	This information is unavailable for the collaborative study, which is the primary reference for OMA 2005.06. For supporting comparative data generated by the Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research Institute's, the HAB Biotoxin Laboratory maintains and follows a Quality Assurance Program to ensure the precision, accuracy and reliability of all toxin analyses and for the production of scientifically sound, legally defensible data. Thorough documentation and standardization of laboratory processes, procedures and activities are required. Key practices include

	the use of Standard Operating Procedures, standard methods, training, quality control, QC acceptance criteria, corrective actions, sample integrity and chain of custody, and database record keeping and tracking. All QA practices are consistent with Good Laboratory Practices and all applicable safety, environmental and legal regulations and guidelines.
Submitters Signature	Date:
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. Comparability - The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose - The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value - HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection - The minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity - The minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range - The range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty - A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix - The component or substrate of a test sample.
12. Method Validation - The process of verifying that a method is fit for purpose.[1]
13. Precision - The closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability - The measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility - The measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery - The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness - The ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity - The ability of a method to measure only what it is intended to measure.[1]
18. Working Range - The range of analyte or measurand concentration over which the method is applied.

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Appendix A: PSP AOAC OMA 2005.06 Proposal

AOAC Official Method 2005.06: Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection

AOAC Official Method (OMA) 2005.06 is a High Performance Liquid Chromatography (HPLC) with fluorescence detection (FLD) method for the quantitative determination of paralytic shellfish poisoning (PSP) toxins in shellfish following prechromatographic oxidation with periodate and peroxide. The method was validated through an AOAC collaborative study and adopted as AOAC Official Method 2005.06, First Action, in 2005. The published collaborative study included 21 shellfish test samples representing mussels, clams, oysters, and scallops, with results obtained from 18 laboratories in 14 countries. The method scope includes STX, NEO, GTX2,3, GTX1,4, dcSTX, B-1/GTX5, C1,2, and C3,4. (B-2/GTX6 was included qualitatively in the original collaborative study, and Turner et al. 2009 validated the method for additional toxins dcNEO and dcGTX2,3. These additional forms are not included in the scope of this proposal, but will be the topic of a future method extension.)

In the ISSC Constitution Bylaws and Procedures, updated July 22, 2024, Procedure XV. Procedure for the approval of analytical methods for the NSSP, section vii(a) states that: ***AOAC approved methods that have undergone the AOAC Official Methods of Analysis (OMA) or FDA Office of Foods Level 3 or 4 validations may be accepted as an NSSP method without Single Lab Validation providing the AOAC or FDA multi-laboratory validation was performed in the raw molluscan shellfish matrix for which the Conference intends it to be used and is deemed by ISSC as fit for purpose.***

The European Union (EU) integrated OMA 2005.06 into its regulatory monitoring in 2006, and in 2019, officially adopted OMA 2005.06 as the EU Reference Method for the detection of PSP toxins in shellfish. This method has been rigorously vetted and validated and has been deemed to be fit for purpose across the EU.

The validation of this HPLC-FLD method is detailed in the 2005 Journal of AOAC INTERNATIONAL article by Lawrence et al., with the foundational development published in 2001 by Lawrence and Niedzwiadek. Since 2005, numerous publications have documented method refinements, modifications to improve recovery and detection of specific toxins, and validation of additional matrices. For the purposes of this ISSC application, however, the method specifics and the requested matrix scope are limited to those presented in the AOAC Official Method.

Supportive evidence: Selected statistics and information from some subsequent studies are referenced here only as additional supportive evidence. Turner et al. (2009) provides detailed single-laboratory performance statistics for mussels and additional toxins. Turner et al. (2010) provides supplemental validation evidence for OMA-relevant oyster and hard-clam matrices from a broader non-mussel bivalve validation study. The supplemental Turner statistics support method performance characteristics but do not expand the requested scope beyond the OMA matrices. Turner et al. (2012) is referenced as supplemental matrix-effects and comparability evidence for oysters. That study investigated why LC-FLD results in oysters were higher than mouse bioassay (MB) results and concluded that the discrepancy was attributable to suppression of the MB by oyster-associated metals, especially zinc and manganese, rather than enhancement of LC-FLD fluorescence. Paired HPLC/MB data generated by the submitting lab (Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute [FWRI]) are added as laboratory-specific supplemental comparative evidence].

Published Summary Statistics Supporting ISSC Acceptance

The tables below provide published summary statistics taken from Lawrence et al. (2005), Turner et al. (2009), Turner et al. (2010), and Turner et al. (2012) sources. Supplemental statistics are summarized only where relevant to the OMA-as-published application.

Table A1. Summary of results of spiked mussels presented in Lawrence et al. (2005). For N-1-hydroxylated toxins (NEO, GTX1,4) data from SPE-COOH cleanup is presented. For non-hydroxylated toxins (all others) data from SPE C18 cleanup is presented.

Analyte	Inferred Spike Level	number of labs	Mean Recovery (%)	Recovery RSD_R (%)	HorRat
NEO	200	8	57.3	26.6	1.20
	400	14	53.1	32.1	1.59
	800	14	61.6	32.4	1.82
GTX1,4	400	14	66.9	32.4	1.66
	800	15	78.5	26.6	1.55
STX	55	15	93.2	33.1	1.32
	220	15	76.8	25.4	1.22
	300	15	74.4	23.5	1.17
	600	15	74.5	25.2	1.33
dcSTX	100	15	83.7	49.2	2.12
	400	15	73.5	21.6	1.12
	600	14	64.3	10.5	0.57
GTX2,3	300	14	75.8	18.8	0.94
	600	16	88.2	31.6	1.80
	1200	16	79.5	30.8	1.91
B1	400	15	77.8	22.5	1.18
	800	15	86.4	32.3	1.91
	1600	15	75.8	19.8	1.27
C1,2	1000	13	73.5	14.8	0.89
	2000	14	78.3	14.5	0.97

Table A2. Lawrence et al. (2005) collaborative precision from blind duplicate treatments.

Cleanup / Table	Matrices represented	RSDr range (%)	RSDR range (%)	HorRat range
SPE-C18, Table 2005.06Q	Clams, mussels, oysters	6.03-31.69	13.74-38.00	0.73-1.69
SPE-COOH, Table 2005.06R	Clams, mussels, oysters	8.94-25.79	17.15-45.76	0.89-1.78

Table A3. Turner et al. 2009 mussel single-laboratory validation statistics

Criterion	Published statistic	Source table / section
Linearity	Working calibration range 0-96 µg STX eq/100 g for most toxins; r^2 values 0.902-0.996; F-test values all below F-critical = 1.98.	Table 4
LOD/LOQ	LODs ranged from 0.3 +/- 0.1 to 16.0 +/- 4.0 µg STX eq/100 g; LOQs ranged from 0.6 +/- 0.1 to 38 +/- 9 µg STX eq/100 g.	Table 7
Recovery	Triplicate mussel homogenate spikes at 40 and 16 µg STX eq/100 g showed recoveries by toxin listed in Table A4 below.	Table 10
Repeatability, naturally contaminated mussels	Total PSP intrabatch mean RSD 8% (range 2-12%; HorRat 0.82); total PSP interbatch mean RSD 14% (range 10-22%; HorRat 0.95).	Table 12
Measurement uncertainty	Combined expanded uncertainty (k = 2) for individual toxins ranged from 0.34 to 1.03.	Table 16
Comparability	HPLC versus MB comparison for mussels: n = 40, correlation coefficient 0.93, mean HPLC-FLD/MB result 101%, ratio RSD 30%, no significant statistical difference by two-tailed t-test (t = 0.76; t-critical = 2.02).	Parallel testing / Figure 5

Table A4. Turner et al. 2009 mussel recovery by toxin

Toxin	Recovery at 40 µg STX eq/100 g, % (RSD)	Recovery at 16 µg STX eq/100 g, % (RSD)	AOAC 2005.06 interlaboratory recovery range (both SPEs), %
GTX1,4[a]	81 (4) [a]	112 (4) [a]	67-79
dcNEO	53 (4) [a]	29 (4) [a]	NA
NEO	107 (6) [a]	136 (2) [a]	53-62
dcSTX	68 (8)	85 (1)	64-84
GTX2,3	66 (9)	94 (7)	76-88
GTX5	76 (7)	82 (1)	76-86
STX	68 (5)	122 (0.2)	74-93
dcGTX2,3	70 (18)	82 (1)	NA
C1,2	63 (10)	79 (6)	74-78

[a]Using modified SPE method.

Table A5. Turner et al. 2010 selected OMA-relevant oyster and hard-clam statistics

Criterion	Pacific/native oysters	Hard clams	Source table / section
Linearity	r^2 values across listed toxins generally 0.949-0.995; F-test values below F-critical = 2.08.	r^2 values 0.984-0.994 for listed toxins; F-test values below F-critical = 2.08.	Table 4
LOD	Oysters: approximately 0.2-16 µg STX eq/100 g across toxins and oyster species.	Hard clams: predicted values <16 µg STX eq/100 g; reported estimates approximately 0.2-14.9 µg STX eq/100 g for applicable toxins.	Table 6
LOQ	Oysters: approximately 0.6-40.0 µg STX eq/100 g across toxins and oyster species.	Hard clams: many values <16 µg STX eq/100 g; highest listed values 32 µg STX eq/100 g for GTX1,4 and NEO.	Table 7
Recovery (excluding N-1-hydroxylated toxins due to SPE method modification)	Mean recoveries averaged 92% in Pacific oysters and 81% in native oysters.	Mean recovery averaged 83%.	Recovery discussion and Table 8
Short-term repeatability	Mean short-term RSD across non-mussel validation was 5.3%, with most values <10%; exceptions were mainly N-hydroxylated toxins in oysters and hard clams.	Same study-wide interpretation; hard-clam exceptions mainly N-hydroxylated toxins.	Precision discussion / Table 10
Measurement uncertainty	Standardized uncertainty values: Pacific oyster 0.14-0.34; native oyster 0.11-0.33. Expanded uncertainty (k = 2) values: Pacific oyster 0.28-0.68; native oyster 0.22-0.66.	Standardized uncertainty values: 0.11-0.35. Expanded uncertainty (k = 2) values: 0.22-0.70.	Table 13

Table A6. Turner et al. 2012 oyster matrix-effects investigation supports that oyster LC-FLD/MB discrepancies reflect MB suppression by metals, not LC-FLD fluorescence enhancement.

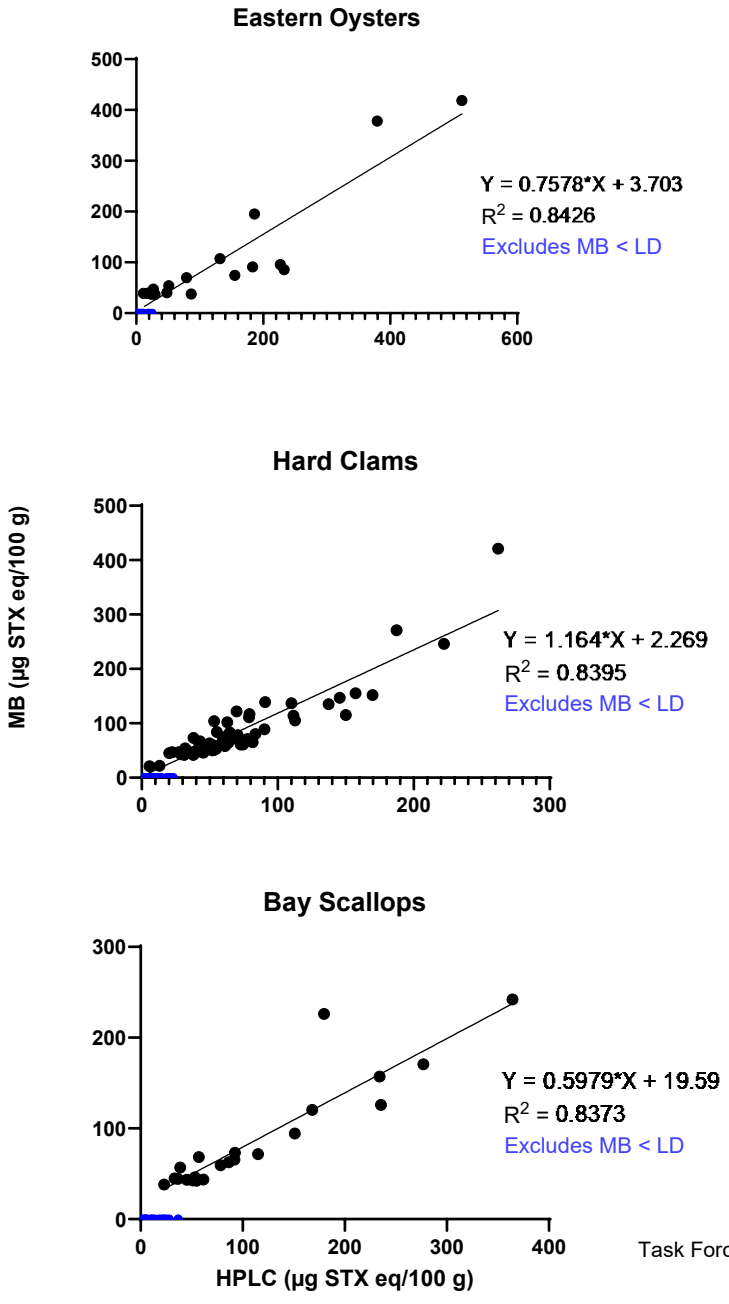
Study element	Result / interpretation
Purpose	Investigated why AOAC 2005.06 LC-FLD results in oysters were often 2-3 times higher than MB results.
Initial comparison	PSP-contaminated oyster, cockle, and mussel HCl extracts were analyzed by MB and LC-FLD; oyster samples showed the largest LC-FLD/MB differences.
Matrix component testing	Nutritional components and conductivity did not explain the discrepancy. Zinc and manganese identified as the strongest correlates with LC-FLD/MB toxicity ratio.
Correlation with metals	Correlation of LC-FLD/MB ratio with zinc was r = 0.81 and with manganese was r = 0.77; copper and cadmium correlations were lower.
Demetallation	Removing metals from oyster extracts reduced the mean LC-FLD/MB ratio from 277% in untreated extracts to 178% after pH 12 precipitation and 124% after PSA SPE demetallation.
Zinc fortification	Adding zinc to mussel and cockle extracts did not enhance LC-FLD results (LC-FLD untreated vs fortified correlation r = 0.94) but suppressed MB response; samples fortified above 180 microg/mL zinc had a mean LC-FLD/MB ratio of 306%.

Table A7. FWRI supplemental HPLC/MB comparative data

FWRI paired HPLC/MB results are included as supplemental laboratory-specific evidence. These data are not intended to replace the published validation statistics for AOAC Official Method 2005.06. Action-level agreement evaluated using 80 µg STX eq/100 g.

Matrix	n	Pearson r	Action-level agreement	HPLC >=80 / MB <80	MB >=80 / HPLC <80
Bay scallop	43	0.94	90.7%	4	0
Eastern oyster	28	0.87	92.9%	2	0
Hard clam	98	0.93	90.8%	2	7
All matrices	169	0.88	91.1%	8	7

Figure A1. FWRI supplemental HPLC/MB comparative data. Regressions exclude results where mouse bioassay was below the level of detection.



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Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection

After: AOAC International. (2005). AOAC Official Method 2005.06: Paralytic Shellfish Poisoning Toxins in Shellfish: Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection.

Principle

This method is applicable to determination of STX, NEO, GTX2,3, GTX1,4, dcSTX, B-1/GTX5, C1,2, and C3,4 in mussels, clams, oysters, and scallops. In this procedure, homogenized shellfish are extracted by heating with 1% acetic acid. Extracts are cleaned up using solid-phase extraction (SPE) C18 cartridges. Pre-chromatographic oxidation of the toxins present in extracts to fluorescent derivatives enables their detection by liquid chromatography. Extracts containing the toxins NEO, GTX1,4, and C3,4 must be further purified by using SPE-COOH cleanup/separation. PSP toxin concentrations are calculated by direct comparison with analytical standards.

Equipment and consumables required

- Electric blender
- Stainless steel No. 10 sieve
- Shucking knives
- Analytical balance
- Vortex mixer
- 2 mL volumetric flasks or graduated cylinders
- Polypropylene centrifuge tubes (graduated) with screw caps (50 and 15 mL)
- 5-10 mL graduated conical test tubes or equivalent
- Graduated cylinders (50 mL and 1-2 L)
- pH meter
- pH strips
- Boiling water bath capable of maintaining 100 °C
- Centrifuge capable of 3600 ×*g* and compatible with 50 mL tubes
- Glass beakers
- 5 mL cryovials
- Adjustable micropipettes and tips covering 5–500 µL
- Pipettes or dispensers covering 1–10 mL
- Timer
- Spatula
- Weigh boats
- Autosampler vials and inserts

- SPE C18 cartridges: Supelclean LC-18 cartridges (500 mg/3 mL volume) (Supelco) or equivalent.
- SPE COOH ion exchange cartridges: Bakerbond Carboxylic Acid silane (COOH) bonded to silica gel (500 mg/3 mL volume) (J.T. Baker) or equivalent.
- SPE manifold or automated SPE system capable of holding 3 mL cartridges.
- A high performance liquid chromatography system (HPLC) equipped with the following:
    - mobile phase system delivering a pulse-free flow of 2.0 mL/min
    - solvent degasser (recommended, not specified in OMA)
    - autosampler (preferably refrigerated to 6 °C) (recommended, not required in OMA)
    - injection loop suitable for 25-100 µL injections
    - temperature controlled column compartment capable of controlling temperature at 35 °C (recommended, not specified in OMA)
    - fluorescence detector able to achieve the required sensitivity with excitation set at 340 nm and emission at 395 nm
    - reversed phase C18 column (15 cm × 4.6 mm id, 5 µm particle size)

Optional for concentrating fractions:

- Rotary evaporator with water bath capable of maintaining 45 °C
- 50 mL round-bottom flasks
- Pasteur pipettes

Reagents

- Acetonitrile and methanol, HPLC grade.
- High-purity reagent water.
- Glacial acetic acid, analytical grade and its solutions: 1% (1 mL of glacial CH_3COOH to 100 mL with water) and 0.1 M (572 µL of glacial acetic acid to 100 mL with water).
- Sodium hydroxide (NaOH), 1 M (4 g to 100 mL with water); NaOH, 0.2 M (10 mL of 1 M NaOH diluted to 50 mL with water).
- Hydrogen peroxide (H_2O_2), 10% aqueous solution. Prepare fresh by mixing 1 volume of 30% H_2O_2 with 2 volumes of high-purity reagent water (1:2, v/v). Store the 30% H_2O_2 at 4 °C.
- Ammonium acetate, 0.01 M (0.77 g to 100 mL with water; mix to dissolve; dilute 10 mL of this solution to 100 mL with water to obtain 0.01 M).
- Sodium chloride (NaCl), 0.05 M (0.29 g to 100 mL with water) and 0.3 M (1.75 g to 100 mL with water).
- Ammonium formate, 0.3 M (4.73 g to 250 mL with water).
- Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$), 0.3 M (20.1 g to 250 mL with water). For anhydrous Na_2HPO_4, use 10.65 g to 250 mL with water.
- Periodic acid, 0.03 M (1.71 g to 250 mL with water). Store at 4 °C, protected from light.
- Periodate oxidant: prepared daily by mixing 5 mL each of 0.03 M periodic acid, 0.3 M ammonium formate, and 0.3 M Na_2HPO_4 and adjusting the pH to 8.2 with 0.2 M NaOH using a pH meter.
- Mobile phase: Prepare as required. A: 0.1 M ammonium formate (6.31 g ammonium formate dissolved in 1 L of water) and B: 0.1 M ammonium formate in 5% acetonitrile:water (6.31 g ammonium formate dissolved in 950 mL of water and 50 mL of acetonitrile added), both adjusted to pH 6 with 0.1 M acetic acid using a pH meter.

- Analytical standards of STX, NEO, GTX1,4, GTX2,3, C1,2, B-1 (GTX5) and dcSTX can be purchased from National Research Council Canada (Store as directed).

  Recommended analytical standard working stocks: Transfer 0.5 mL from each vial into a tared 2-mL volumetric tube and record the weight. Bring final volume to 2 mL with high purity water and record the final weight. Calculate stock concentration: µM in vial x wt. of transferred solution/final weight. Record the standard lot, date diluted, and stock concentrations. Store these stock solutions in plastic or deactivated glass vials at -20 °C (or as specified in NRC documentation). These are stable for at least a month.

  For convenience, standards may be further diluted and combined into three mixtures by appropriate dilution of the working stock solutions. AOAC OMA 2005.06 does not prescribe specific concentrations for these mixed standards. Working standard concentrations of approximately 0.05–0.4 µg/mL, depending on the toxin analog and detector response, may be convenient for routine analysis. Those solutions must be adjusted to about pH 4 with 0.1 M acetic acid. All further dilutions should be made using 0.1 mM acetic acid, so that the solutions are kept at about pH 4.

  - Mixture I: GTX1,4 and NEO
  - Mixture II: GTX2,3 , B1 (GTX5), STX , and dcSTX
  - Mixture III: C1,2 and C3,4

  Individual laboratory concentration targets will vary based on detector sensitivity and can be adjusted to achieve desired range of quantitation. Select round, standard volumes to maximize pipet accuracy, and calculate true final concentrations of each solution.
- Matrix modifier: An extract prepared from blank (PSP free) oysters, which have been extracted and cleaned up on an SPE C18 cartridge as described below. The extract is adjusted to pH 6.5 with 1 M NaOH and filtered using a 0.45 µm polyvinylidene fluoride (PVDF) filter.

Shellfish preparation

1. Collect at least 12 animals and a total mass of 100-120 grams of meat per sample.
2. Immediately after collection, shuck shellfish or place in dry storage between 0 and 10 °C. Store samples in a clean, waterproof, puncture resistant container and ship overnight for processing. Time from collection to extraction initiation should not exceed 24 hours.
3. Clean the outsides of the shellfish with fresh water.
4. Cut adductor muscles and open the shell. Rinse the inside of the shellfish with fresh water to remove sand and other foreign material.
5. Collect meat, in a single layer, on a number 10 sieve, and allow the sample to drain for 5 minutes. Discard any pieces of shell.
6. Blend the drained meats at high speed until homogenous (60-120 seconds).

Sample extraction

1. In a 50 mL polypropylene centrifuge tube mix 5.00 g (± 0.10 g) of shellfish sample with 3.0 mL of 1% acetic acid solution on a Vortex mixer for 2 min (European Union Reference Laboratory for Marine Biotoxins [EURLMB], 2020). Cap it loosely (to prevent pressure build up during heating) and then place

it in a boiling water bath (100 °C) so that the contents of the tube are below the water line. Heat for 5 min. Do not place so many tubes in the bath at once that the water bath stops boiling or the temperature drops for more than 30 sec.

2. Remove sample from the water bath, remix on a Vortex mixer, and cool by placing it in a refrigerator or a beaker of cold water for 5 minutes.

3. Centrifuge for 10 minutes at 3600 ×*g* and decant the supernatant into a 15 mL graduated conical test tube.

4. Add 3 mL of 1% acetic acid solution to the centrifuge tube containing the solid residue, mix well on a Vortex mixer for 2 min, and centrifuge again for 10 minutes at 3600 x*g*. Collect the supernatant into the same graduated conical test tube that contains the first portion of extract and make up the volume to 10.0 mL with water.

SPE clean-up

Ensure that SPE cartridges used give minimum 80% recovery with standards and the correct elution pattern according to steps below.

1. Condition a 3 mL SPE C18 cartridge with 6 mL of methanol followed by 6 mL of water. Add 1 mL (0.5 g shellfish equivalent) of the above crude extract to the cartridge. Keep the flow rate between 2-3 mL/min for all elutions. Collect the effluent into a graduated conical test tube (or equivalent). Do not let the cartridge run dry. Wash the cartridge with 2 mL of water and combine the washings with the effluent.

2. Adjust the extract to pH 6.5 with 1 M NaOH using pH indicator paper (calibrated against a pH meter) and then adjust the volume to exactly 4.0 mL with water. Use aliquots of this extract for oxidation with periodate and peroxide as described below.

3. **SPE-COOH ion exchange cleanup is required only for extracts that were found to contain N-1 hydroxylated PSP analogs after C18 cleanup (but can also be used to analyze non-hydroxylated analogs with peroxide oxidation if additional clean-up is needed).** Condition a 3 mL SPE-COOH cartridge by passing 10 mL of 0.01 M ammonium acetate through it. Keep the flow rate between 2-3 mL/min for all elutions. Do not let the cartridge run dry. Discard the effluent.

4. Fraction #1: Pass a 2 mL aliquot (0.250 g shellfish tissue equivalent) of shellfish extract from the SPE C18 cleanup through the cartridge and collect the effluent in a graduated conical test tube (or equivalent) marked as Fraction #1. Then pass 4.0 mL water through the cartridge and collect into the same tube. Ensure final volume is 6.0 mL in total. This fraction contains the C toxins.

5. Fraction #2: Pass 4.0 mL of 0.05 M NaCl solution through the same cartridge, collect in a graduated conical test tube (or equivalent) marked as Fraction #2. Ensure that the final volume is 4.0 mL. This fraction contains the toxins GTX1,4, GTX2,3, B1, B2 and dcGTX2,3.

6. Fraction #3: Then pass 5.0 mL of 0.3 M NaCl solution through the cartridge and collect in a graduated conical test tube (or equivalent) marked as Fraction #3. Ensure that the final volume is 5.0 mL. This fraction contains STX, NEO, and dcSTX.

Note: If problems with detector sensitivity are encountered each fraction can be concentrated. Collect each fraction from the ion exchange column into 50 mL round bottom flasks instead of graduated conical test tubes and evaporate to approximately 1 mL on a rotary evaporator with a water bath set at 45 °C.

Transfer the solution into a 5 mL graduated conical test tube using a Pasteur pipet. Rinse the 50 mL round bottom flask 3 times with about 0.2-0.3 mL of water each time, transferring the rinse into the graduated tube just so the final volume of extract is 2.0 mL. Note: A graduated 2 mL volumetric flask can also be used. Analyze fractions #1, #2, and #3 by LC after periodate and peroxide oxidations as described below.

Pre-column oxidation

All reagents and solutions used in the oxidation reactions are dispensed using autopipettes with disposable plastic tips.

Periodate oxidation: Add a 100 µL volume of standard solution or sample extract after SPE C18 or ion exchange cleanup to 100 µL of matrix modifier solution in an autosampler vial. Add 500 µL of periodate oxidant and mix well on a Vortex mixer. Allow the solution to react at room temperature for 1 minute, then add 5 µL of concentrated acetic acid and mix. Allow the mixture to stand for 10 minutes at room temperature before injecting 50-100 µL into the LC system. *Maintain consistent injection volumes across samples and standards.*

Peroxide oxidation: Add 25 µL of 10% (w/v) aqueous H_2O_2 to 250 µL of 1 M NaOH in an autosampler vial and mix. Then add 100 µL of standard solution or sample extract after SPE C18 or ion exchange cleanup. Mix and allow to react for 2 minutes at room temperature. Add 20 µL of concentrated acetic acid and mix the solution. Inject 25-50 µL of this solution into the LC system. (Note: Injecting more than 50 µL may cause peak broadening). *Maintain consistent injection volumes across samples and standard.*

Note: Oxidation reactions may be performed manually or by an automated pre-injection procedure using an HPLC autosampler capable of programmed reagent addition, mixing, reaction timing, and injection. When automated oxidation is used, the reagent proportions, order of addition, reaction times, and quenching conditions shall be equivalent to those specified in AOAC OMA 2005.06. Standards and sample extracts shall be subjected to the same automated oxidation procedure. Performance of the automated procedure shall be demonstrated to be equivalent to the manual oxidation procedure before routine use.

Calibration Curves

Perform a multipoint calibration (at least 6 points) of each of the analogs or standard Mix solutions during initial method verification and at least annually thereafter to confirm the established analytical working range and detector response for each analog. More frequent calibration may be performed as warranted by instrument performance. A new multipoint calibration shall also be performed following significant service or changes to the analytical system or procedure that may affect detector response or quantitation or with a new standard lot.

Routine sample quantitation may be performed using appropriate analytical standards in accordance with AOAC OMA 2005.06. The standards should be analyzed at the beginning of the batch and periodically thereafter at a frequency established by the laboratory to verify stability of detector response and retention time. Additional standards should be analyzed at the end of the batch.

HPLC analysis

The column should be a reversed phase C18 with dimensions of 15 cm × 4.6 mm id, 5 µm particle size. If a column of different dimensions is used, the gradient and flow rate conditions may have to be altered to retain the necessary separation of the PSP oxidation products within an approximately 20-minute chromatographic run.

The gradient used to elute the PSP oxidation products consists of two mobile phases (see Reagents above) under the following conditions: 0 to 5% mobile phase B in the first 5 min, 5 to 70% B for the next 4 min, and back to 0% B over the last 2 min. The system is permitted to run for another 3 minutes with 100% mobile phase A before the next injection. The flow rate is 2 mL/min.

For monitoring the LC effluent, a dual monochromator fluorescence detector, with excitation set to 340 nm and emission to 395 nm, is required. The detector must be sensitive enough to produce a peak with 10:1 signal/noise ratio for an injection of 400 pg of STX standard carried through the peroxide oxidation procedure under the chromatographic conditions employed.

The chromatographic system must be able to separate with baseline resolution the main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B1, and STX within a 20-minute chromatographic run.

Column temperature is not mentioned in the OMA, but maintaining a stable column temperature ensures reproducible and reliable chromatographic separations. Recommended column temperature is 35° C (EURLMB, 2020).

Equilibrate the system for at least 20 minutes with 100% mobile phase A before a run.

Prior to injecting samples, inject Mix I (periodate oxidation) and Mix II (peroxide oxidation) in duplicate, and Mix III (both oxidations, 1 injection each) at a single concentration each to verify system performance including peak separation.

Inject the matrix modifier (carried through periodate oxidation) to identify possible interferences.

Inject C18-cleaned sample extracts (periodate oxidation). If no peaks are observed, samples are negative. If extracts are found to contain N-1 hydroxylated PSP analogs, perform SPE-COOH ion exchange cleanup as described above and inject appropriate fractions (periodate oxidation). If non-hydroxylated PSP analogs are observed or suspected, inject the C18-cleaned extracts after peroxide oxidation*.

> **If PSP profiles typically contain only non-hydroxylated analogs, peroxide oxidations may be carried out first, but all samples should be analyzed with periodate oxidation as well for documentation.*

Analyze in separate sequences extracts oxidized with periodate and peroxide and include a chemical blank (oxidize 1% acetic acid solution with peroxide and periodate) with each sequence.

Include a negative control matched matrix as a matrix blank (periodate oxidation) for each matrix analyzed.

Include a positive control analyzed as appropriate with each run.

To verify that the PSP toxin peaks found in the chromatograms are indeed due to PSP and not to naturally fluorescent sample coextractives, a portion of each extract, after C18 cleanup, must be analyzed without oxidation. A volume of sample extract and matrix modifier are mixed with water instead of periodate oxidant before injecting it into the LC system. The resulting chromatogram will enable identification of peaks arising from naturally fluorescent coextractives. PSP toxins do not produce peaks under these conditions. These can be done at the end of the run.

Calculations

STX, GTX2,3, B-1, and C1,2 produce single oxidation products with both oxidation reactions, while dcSTX produces two oxidation products with both reactions. NEO, GTX1,4, and C3,4 each produce three peaks after periodate oxidation but only the second eluting peaks are used for quantitation (Figure 1). Because some PSP toxins (NEO and B-2; GTX1,4, and C3,4) give the same oxidation products, their quantitative determination can be made only after separation by COOH ion exchange chromatography as described above. If present in a high enough concentration, dcSTX will interfere with NEO quantification after periodate oxidation. NEO can be quantified by using mathematical ratios of peaks after periodate and peroxide oxidation as described below.

Quantitation requires a S/N >10 and is determined by direct comparison with an analytical standard at a concentration similar to that anticipated in the sample. If the toxin concentration is lower than the lowest concentration of the calibration curve for that analog, the sample is below detection limit for that analog. If it is higher than the highest concentration of the calibration curve for that analog, then do an appropriate dilution with water and rerun the sample to quantify that analog.

Concentration of individual toxin analogs in shellfish (µg/100 g) =

$$\frac{A_x \times C_s \times V_x \times D \times 100}{A_s \times M}$$

where:
A_x = peak area of the toxin in the extract analyzed
A_s = peak area of the nearest standard
C_s = concentration of standard (µg/mL)
V_x = final volume of extract analyzed (mL): 4 mL for C18 SPE; 6 mL for SPE-COOH Fraction 1; 4 mL for Fraction 2; and 5 mL for Fraction 3.
D = dilution factor (for those extracts where PSP toxins are above the highest standard and require dilution and reanalysis)
M = amount of test portion (g) carried through cleanup procedure: 0.5 g for SPE C18 and 0.25 g for SPE-COOH cleanup
100 = factor to convert results from µg/g to µg/100 g

The total toxicity in saxitoxin equivalents (STX eq) is calculated by multiplying each toxin analog concentration by its toxicity equivalent factor (TEF) as determined by Oshima (1995), corrected for its molecular weight (Table 1), and summing them.

Total toxicity in shellfish (µg STX eq/100 g) =

$$\sum TEF_i \times C_{xi}$$

where:
TEF_i = toxicity equivalent factor for each analog detected:
STX = 1; NEO = 0.88; dcSTX = 0.6; GTX1,4 = 0.63; GTX2,3 = 0.38; B1 = 0.051; C1,2 = 0.032; C3,4 = 0.022
For epimeric pairs, the mean of the two TEFs is used.

C_{xi} = concentration of each toxin analog measured in the shellfish sample (µg/100 g)

Example of Calculating NEO in the Presence of dcSTX (Refer to Figure 2)

Since the second peak from dcSTX is the same oxidation product as obtained from NEO, and these toxins are both in Fraction 3 after SPE-COOH, NEO must be calculated by difference when these two toxins are present together.

The peak area of NEO in a sample containing both NEO and dcSTX can be calculated in two ways.

Figure 2 shows chromatograms after periodate and peroxide oxidation of a dcSTX standard at 0.05 µg/mL and an unknown sample containing a mixture of NEO and dcSTX.

The peak areas are as follows:

dcSTX standard	A = 5.1470
	B = 0.9335
	E = 45.230
unknown sample	C = 9.4006
	D = 0.8347
	F = 43.795

Method 1. If the concentration of dcSTX in the sample containing a mixture of NEO and dcSTX is significant enough to produce a sensitive, well-integrated first peak (Fig.2, peak D) with periodate oxidation, then you can calculate the peak area corresponding to NEO (Fig. 2, peak $X_{(NEO)}$) from the ratio of the two dcSTX peak areas produced with periodate oxidation of the standard.

For the dcSTX standard analyzed with periodate oxidation, the ratio of the two peak areas is calculated as:

$$A / B = 5.1470 / 0.9335 = 5.5 = X_{(dcSTX)} / D \quad \Rightarrow \quad X_{(dcSTX)} = 5.5D$$

where: $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - 5.5D = 9.4006 - 5.5 \times 0.8347 = 4.8098$$

Method 2. If the concentration of dcSTX in the sample containing NEO and dcSTX is not significant enough to produce a well-integrated first peak (Fig, 2, peak D) with periodate oxidation, then you can calculate the peak area corresponding to NEO (Fig. 2, peak $X_{(NEO)}$) from the ratio of the dcSTX peak produced with periodate oxidation (Fig.2, peak A) and the dcSTX peak area produced with peroxide oxidation (Fig.2, peak E) in the standard.

For a dcSTX standard analyzed with periodate and peroxide oxidations, the ratio of the two peak areas is calculated as:

$$E / A = 45.230 / 5.1470 = 8.8 = F / X_{(dcSTX)} \quad \Rightarrow \quad X_{(dcSTX)} = F / 8.8$$

where: $X_{(dcSTX)}$ is the peak area of dcSTX in peak C.

The peak area of NEO can be calculated as follows:

$$X_{(NEO)} = C - X_{(dcSTX)} = C - F / 8.8 = 9.4006 - 43.795 / 8.8 = 4.4239$$

Method 1 yields a peak area estimation of 4.81 for NEO while method 2 yields a value of 4.42. The results differ by about 10%, which is within the experimental error of the entire method. The regression equation of the NEO calibration curve can then be used to calculate the concentration of NEO in the oxidized sample or fraction.

Quality Control Criteria

1. Only certified reference materials are used for standard solutions.
2. Calibration linearity $R^2 \geq 0.98$
3. Daily injection schedules must include the adequate frequency of injection standards and extraction blanks based on an assessment of individual standard toxin variability and lack of carry over.
4. Peak must have S/N > 10 and be above the lowest calibration curve point for quantitation.
5. The main oxidation products of GTX1,4 (together as one peak), C1,2 (together as one peak), NEO, GTX2,3 (together as one peak), B1, and STX must be separated with baseline resolution.
6. Peaks asymmetry is routinely monitored to evaluate the performance of the column.
7. The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3.
8. Identification retention time of sample vs standard must be within ±0.2 min.

References

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Figure 1 . PSP toxin peaks using periodate oxidation and peroxide oxidation. Arrows indicate peaks used for quantification.

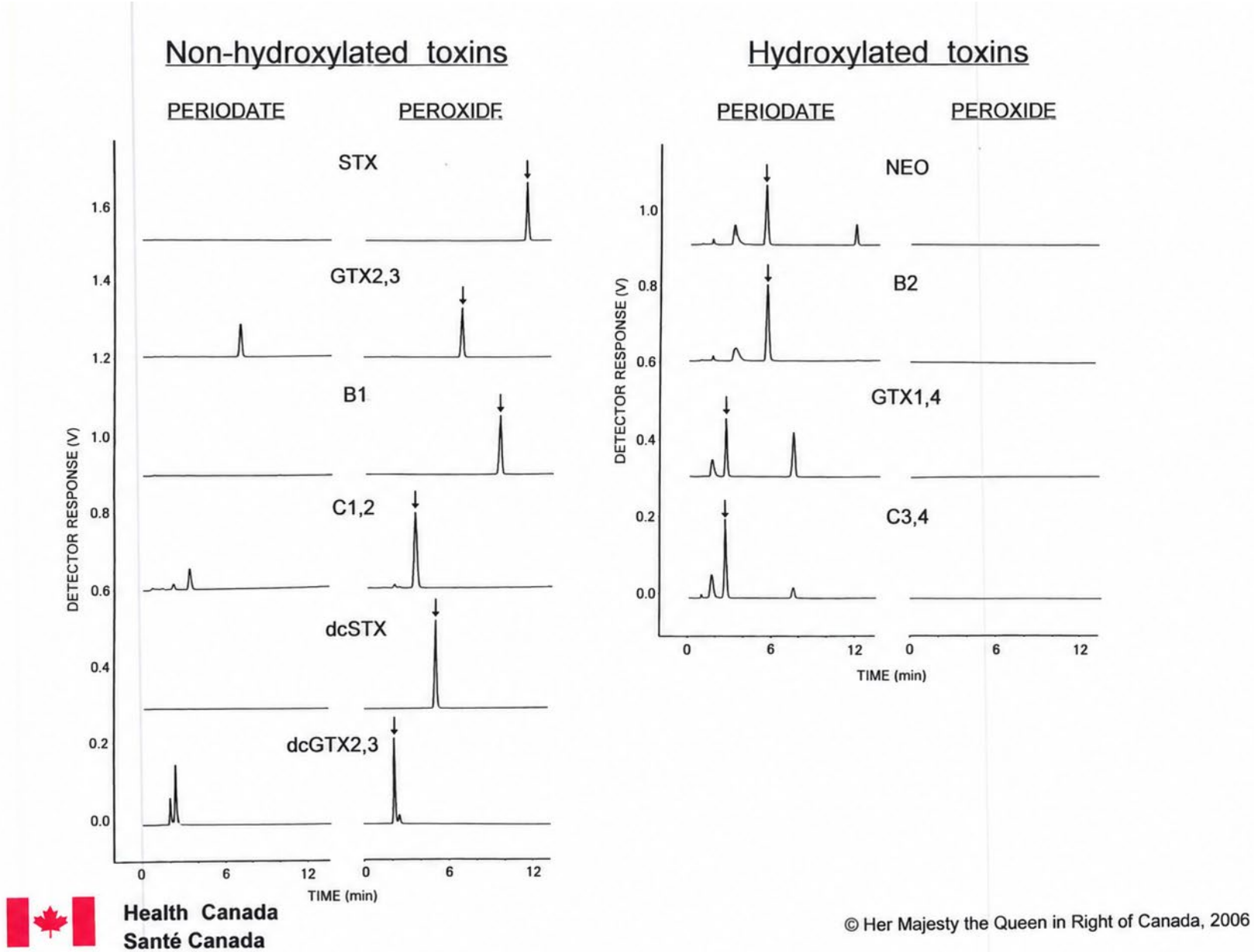
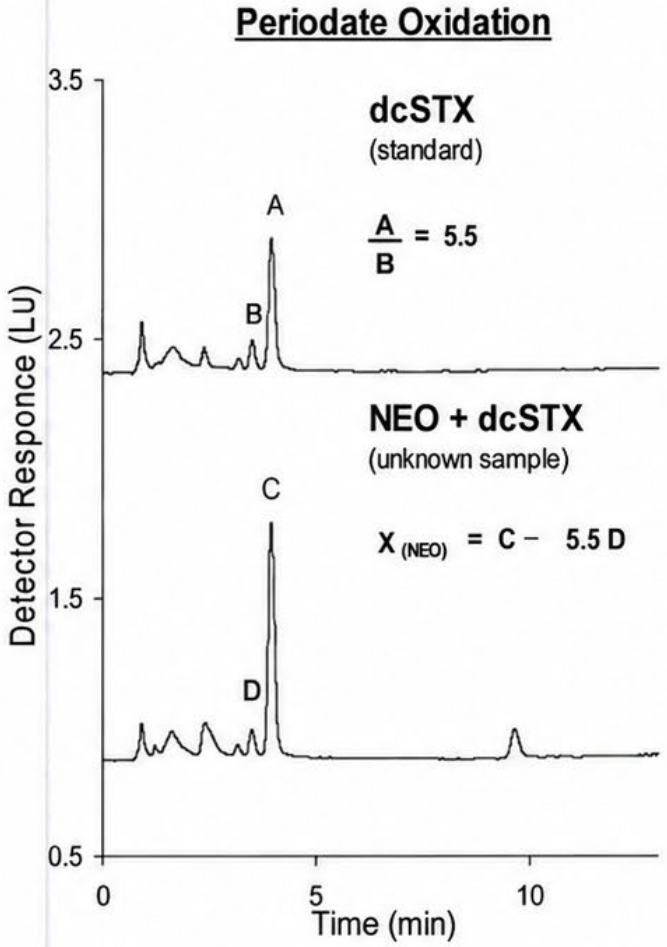
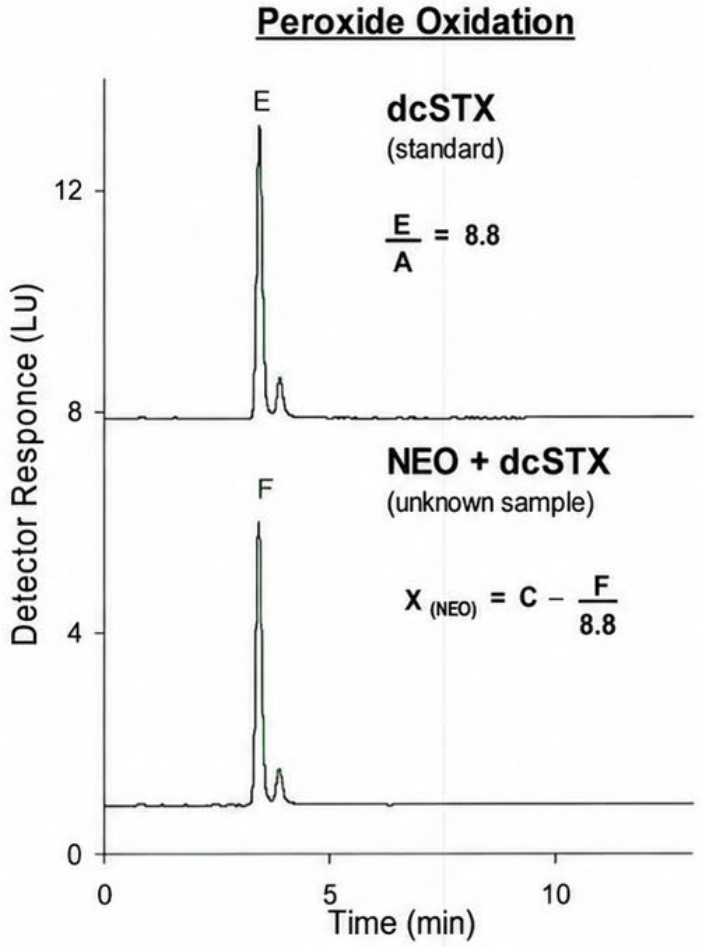


Figure 2: For use in example of calculating NEO in the presence of dcSTX.





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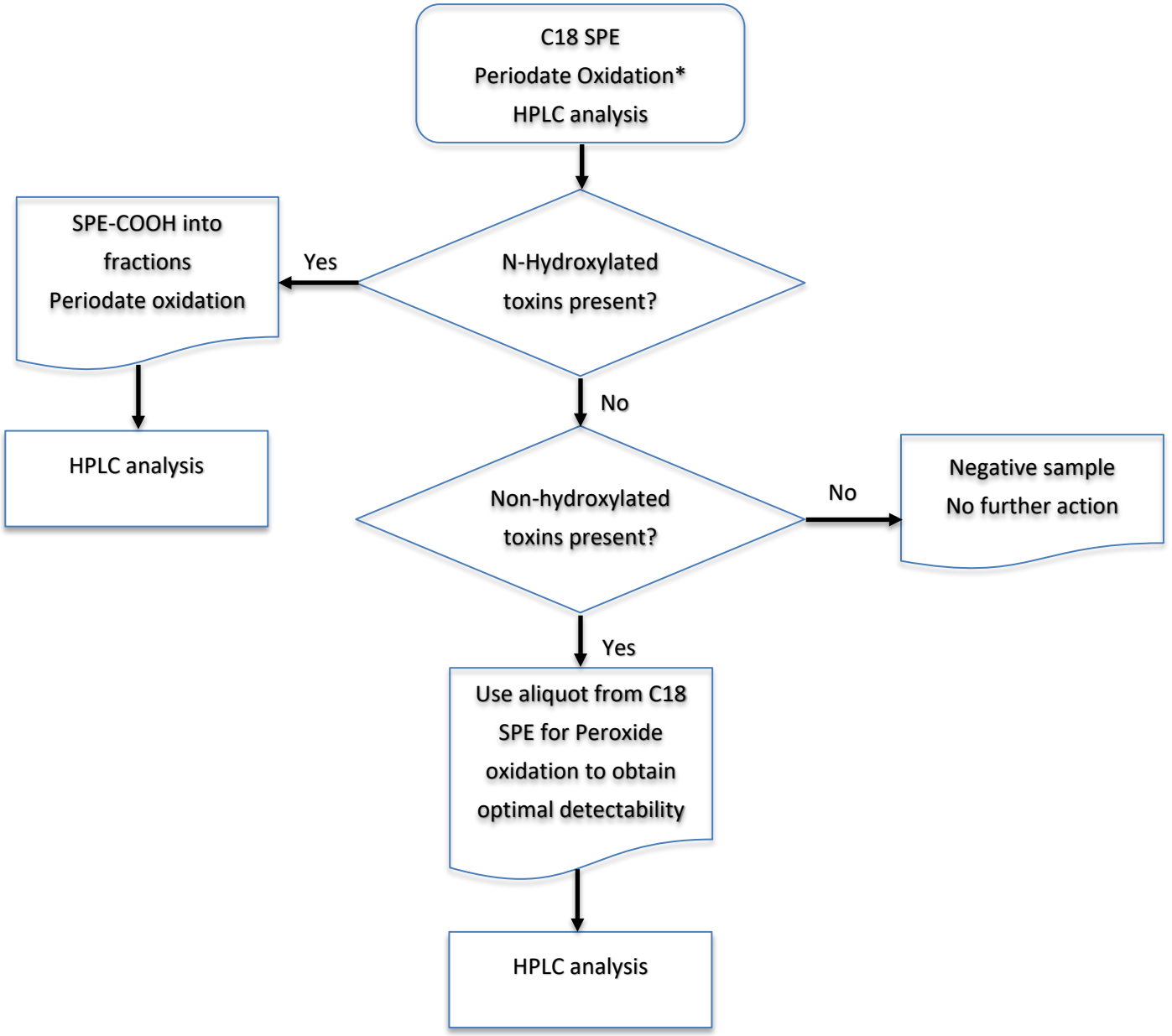
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Table 1. Toxicity of PSP toxin analogs relative to STX

	MU/µmol*	Relative toxicity	Molecular weight	MU/µg	Relative toxicity corrected for molecular weight
B1/GTX5	160	6.4%	379.4	0.42	5.08%
C1	15	0.6%	475.4	0.03	0.38%
C2	239	9.6%	475.4	0.50	6.06%
C3	33	1.3%	491.4	0.07	0.81%
C4	143	5.8%	491.4	0.29	3.51%
dcGTX2	1617	65.1%	352.3	4.59	55.3%
dcGTX3	1872	75.4%	352.3	5.31	64.1%
dcSTX	1274	51.3%	256.26	4.97	59.9%
GTX1	2468	99.4%	411.4	6.00	72.3%
GTX2	892	35.9%	395.35	2.26	27.2%
GTX3	1584	63.8%	395.35	4.01	48.3%
GTX4	1803	72.6%	411.4	4.38	52.8%
neoSTX	2295	92.4%	315.29	7.28	87.7%
STX	2483	100.0%	299.29	8.30	100%

*Oshima, Y. (1995). Postcolumn derivatization liquid chromatographic method for paralytic shellfish toxins. Journal of AOAC International, 78(2), 528–532.

Recommended Method Procedure



**If PSP profiles typically contain only non-hydroxylated toxins, peroxide oxidations may be carried out first for efficiency, but all samples should be analyzed with periodate oxidation as well for documentation.*

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. **X** Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
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9. Proposal Subject	Quantitative risk assessment of individual oyster harvests using real-time observations can expand Vibrio risk management options
10. Specific NSSP Guide Reference	@ .06 Vibrio vulnificus plan & @ .07 Vibrio parahaemolyticus plan
11. Text of Proposal/ Requested Action	Develop guidance through the Vibrio Management Committee to assist implementation of existing quantitative risk assessment models by state authorities and industry to manage vibrio risk of individual oyster harvests using real-time observed water and oyster temperatures as an alternative to historical monthly mean maximum water temperatures to achieve an equivalent level of consumer protection.
12. Public Health Significance	Quantitative risk assessment (QRA) for V. vulnificus (Vv) in raw oysters was released by the Food and Agriculture Organization/World Health Organization in 2005. Water temperature determined Vv levels in oyster at harvest and post-harvest growth on ambient air temperature and cooldown time to minimum growth temperature (<13°C). The US Food and Drug Administration developed an Excel risk calculator based on this QRA to facilitate scenario analysis by state authorities. The National Shellfish Sanitation Program (NSSP) adopted a Vv Control Plan based on this risk calculator in 2009. State can use average monthly maximum water temperature in the previous five years to implement controls that will minimize risk to: (<3/100,000 when >80°F; <2.5/100,000 when 75-80°F; and <1.75/100,000 when 70-75°F). The NSSP allows states other risk management options: "may implement alternative controls that will reduce the risk to a level comparable to the risk per serving identified above". States have generally adopted plans based on a single scenario analysis with rigid maximum harvest and cooling times. This "one size fits all" policy can result in excessive risk during heat anomalies and also restricts industry risk management options that could extent harvest periods by harvesting at cooler times or rapid cooling.

	A paradigm shift using observed harvest condition instead or relying on average historical temperatures is a more granular alternative control that improves QRA accuracy and expands compliance options. QRA models are available for V. parahaemolyticus and are amenable to real-time inputs by stakeholders to improve risk management using scenario analysis for emerging industry practices such as serving oysters during farm tours or to support a variance from re-mergence requirements.
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	Guidance for vibrio risk analysis using real time data for senario analysis

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☐ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
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9. Proposal Subject	Calibration and validation of hydrodynamic models used in shellfish growing area classification
10. Specific NSSP Guide Reference	Proposed changes are to Section IV Guidance Documents only (no change to the Model Ordinance): Section IV Guidance Documents – Chapter IV. Growing Areas @.01 Sanitary Survey .01 Sanitary Survey and the Classification of Growing Waters Components of a Sanitary Survey - (3) An evaluation of hydrographic factors that may affect distribution of pollutants throughout the area. (Pg 282) "Tracer dye studies provide site-specific dilution, dispersion and time of travel information, and can be used in calibration of site-specific hydrodynamic models." Section IV Guidance Documents – Chapter IV. Growing Areas @.03 Growing Area Classification .03 Classification of Shellfish Growing Waters Adjacent to Waste Water Treatment Plants, IV. Prohibited Classification, Guidance for Dilution Ratios (pg. 314)
11. Text of Proposal/ Requested Action	The Model Ordinance requires every sanitary survey to evaluate hydrodynamic characteristics (@.01 A.(1)(c)), and the guidance notes that tracer dye studies can be used to calibrate hydrodynamic models, but the NSSP Guide sets no requirements for how models used in classification are developed, calibrated or validated. Hydrodynamic models have become a widely used tool for predicting hydrodynamic factors. By combining data such as tidal amplitude/type and water circulation patterns with water depths and stratification caused by density (salinity and temperature) differences, prevailing wind patterns, and other external physical inputs, models predict dilution and time of transport of deleterious materials, e.g. WWTP outflows. The hydrodynamic model output on dilution and time of transport can then be applied to describe boundaries for shellfish growing area classification using the dilution criteria defined in *Section IV Guidance Documents – Chapter IV. Growing Areas @.03 Growing Area Classification .03 Classification of Shellfish Growing Waters Adjacent to Wastewater Treatment Plants. IV. Prohibited Classification. I. Establishment of Boundaries for the Prohibited Area, Guidance for Dilution Ratios* (pg. 314). However, hydrodynamic modelling is an inexact science that requires an iterative process of development, calibration and validation before the model output can be used as a predictive tool for dilution and dispersion and subsequently used in a regulatory environment to set the classification status of shellfish harvest areas. Without adequate validation, e.g. a tracer dye study as referenced in *Section IV Guidance Documents – Chapter IV. Growing Areas. Components of a Sanitary*

	Survey - (3) An evaluation of hydrographic factors that may affect distribution of pollutants throughout the area, model output cannot be shown to represent the actual environment it is intended to describe, and classification boundaries based on it have no demonstrated basis. This proposal requests two actions. First, add the guidance language shown below so that any hydrodynamic model used in a sanitary survey, or to set growing area classification boundaries, is calibrated and validated with site-specific field data, documented, and independently reviewed. Second, refer the proposal to the appropriate ISSC committee to develop more detailed guidance establishing minimum standards for model development, calibration, validation and review. The guidance (guardrails) would ensure hydrodynamic models that are used for regulatory decisions, such as defining growing area classification, are properly developed, calibrated and validated so that the model output is reflective of the environment it proposes to describe and accurate in its predictive capacity. It would establish what valid data are to be used for constructing the model, confirm the data inputs are properly calibrated, and ensure that validation of the model has been adequately verified. As a final step prior to regulatory use, the model, its output and its application should be reviewed by qualified reviewers independent of the model developer to ascertain its validity for the proposed specific application before it is used for growing area classification purposes. As is true of all procedures and protocols listed in the NSSP Guide, the identification and application of standards for shellfish sanitation and guidelines to protect human health are based on information that is peer reviewed and accepted in the scientific community. A standard for the application of hydrodynamic models for the purpose of shellfish area classification needs to be well defined and evaluated for the specific locale and situation where it is being applied and the NSSP Guide must carefully establish a standard set of guidelines for the application of modelling in shellfish management. **Proposed changes to Section IV Guidance Documents (new language underlined):** **Section IV Guidance Documents – Chapter IV. Growing Areas, @.01 Sanitary Survey, .01 Sanitary Survey and the Classification of Growing Waters** *[Add as a new paragraph immediately following item (3), An evaluation of hydrographic factors that may affect distribution of pollutants throughout the area]* Use of hydrodynamic models. Hydrodynamic models (computer models that simulate water circulation and the transport of pollutants in a growing area) are increasingly used to evaluate hydrographic factors and to estimate dilution and time of transport. Model results are only as reliable as the data used to build and test them. When a hydrodynamic model is used in a sanitary survey, or to establish the boundary of any growing area classification, the Authority should: (a) Calibrate the model using site-specific field data from the growing area and adjacent waters, such as tidal elevation, current velocity and direction,

	salinity, temperature and bathymetry; (b) Validate the model's predictions of dilution and time of travel against independent site-specific field measurements not used in calibration, such as a dye or other tracer study; (c) Document in the sanitary survey the model used, the input data and their sources, the calibration and validation methods and results, and the limitations of the model; and (d) Have the model, its calibration and validation, and its application reviewed by qualified personnel independent of the model developer before its results are used to establish classification boundaries. These recommendations are not intended to apply to simple dilution calculations, such as tidal prism or mass-balance estimates. **Section IV Guidance Documents – Chapter IV. Growing Areas, @.03 Growing Area Classification, .03 Classification of Shellfish Growing Waters Adjacent to Waste Water Treatment Plants, IV. Prohibited Classification, Guidance for Dilution Ratios** *[Add at the end of the introductory paragraph, before item (1)]* When a hydrodynamic model is used to estimate the dilution or time of transport used to set the boundary of a prohibited area, the model should be calibrated, validated, documented and reviewed as described in @.01 .01, Use of hydrodynamic models.
12. Public Health Significance	Applying a non-validated hydrodynamic model for shellfish growing area classification can result in incorrectly sized Prohibited Areas and incorrectly placed classification boundaries. The pendulum of damage can swing in both directions, either not closing areas when needed leading to enhanced human health risks or closing areas when not needed leading to economic damage to businesses dependent on shellfish harvest area classification for conducting their business. Requiring calibration and validation against site-specific field data gives model-based boundaries the same evidentiary footing the NSSP already expects of other data used in classification.
13. Cost Information	The proposal does not require any Authority to use a model; it applies only where models are used for classification. Costs would fall mainly on Authorities, or on permittees or consultants modeling on their behalf, for the field data needed to calibrate and validate a model (e.g., current measurements and dye or tracer studies). Many Authorities already conduct dye studies at WWTP outfalls, which would serve this purpose. Committee work to develop the guidance would rely on volunteer time and regular ISSC meeting costs.
14. Changes Needed to Guidance (if proposed change is adopted)	Add the guidance language shown in Item 11 to Section IV Guidance Documents, Chapter IV, @.01 .01 and @.03 .03. No change to the Model Ordinance. In addition, guidelines need to be developed that provide detailed standards for the development, calibration and validation of hydrodynamic models prior to their application for shellfish area classification. These guidelines must be included in the NSSP Guide. At a minimum, the guidance should address acceptable input data and sources; calibration procedures and performance criteria; validation methods (including tracer studies) and acceptance criteria; documentation to be included in the sanitary survey; independent technical review; and when re-

	calibration or re-validation is required (e.g., changes to an outfall location, discharge volume or bathymetry).

Proposal No. 27-133

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative

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Proposal Subject	Modification of NSSP Shellfish Laboratory Evaluation Checklist for Microbiology: Temperature Monitoring in the Laboratory
Specific NSSP Guide Reference	2023 NSSP Guide for the Control of Molluscan Shellfish NSSP Shellfish Section IV Guidance Documents – Chapter III. Laboratory Page | 265 Laboratory Evaluation Checklists Laboratory Evaluation Checklist for Microbiology PART 1 - QUALITY ASSURANCE 1.4 Laboratory Equipment
Text of Proposal/ Requested Action	1.4.9 Refrigerator temperature(s) are monitored at least once daily on workdays or when using continuous temperature monitoring systems daily active monitoring is not required so long as alarm thresholds can alert staff to temperature excursions affecting storage and sample integrity. Monitoring and alarm records are maintained. ~~Results are recorded and records maintained.~~
	1.4.11 The temperature of the incubator is maintained at 35 ± 0.5°C. When verified by continuous automated monitoring alarms triggered by excursions are investigated, with affected samples evaluated for validity. Monitoring and alarm records are maintained.
	1.4.14 Temperature of a working ~~the~~ waterbath is maintained at 44.5 ± 0.2°C ~~under all loading conditions.~~ When verified by continuous monitoring systems, alarms triggered by excursions are investigated, with affected samples evaluated for validity. Monitoring and alarm records are maintained.
	1.4.18 Air incubator/waterbath temperatures are taken twice daily on workdays or when using continuous temperature monitoring systems twice daily active monitoring is not required so long as alarm thresholds are addressed and samples are evaluated for validity for temperature excursions exceeding time/temperature limits for appropriate incubation periods. Monitoring and alarm records are maintained. ~~The results are recorded and records maintained.~~
1.4.23 New checklist item(s)	Temperature monitoring systems (wired/wireless) • Software must record temperature reading from each sensor/probe in the piece of equipment being monitored at the same or greater frequency as required by check list items.

	• When temperatures are out of acceptable temperature range and method specific time, event must be documented and corrective action taken. Monitoring and alarm records are maintained. Method specific time ranges:
	  ○ Methods using a 24 ± 2hr incubation period must not have temperature excursions exceeding 2 hours across all incubation steps
	  ○ Methods using a 48± 3hr incubation period must not have temperature excursions exceeding 3 hours across all incubation steps
	  ○ Methods with variable incubation times will not have temperature excursions greater than minimum of 2 hours scaled to total incubation period (ie 30-hour incubation period = 2 hours + 15 minutes, 18-hour incubation = 2 hours)
	• Temperature monitoring system records for each piece of equipment must be available for auditing.

Public Health Significance	Deviations from required waterbath and incubator temperatures are inevitable due to the nature of NSSP microbiology methods which require the transfer of cold samples and room temperature media into 35˚C incubators, and subsequently into 44.5˚C waterbaths (1.4.11 & 1.4.14). These fluctuations are not routinely captured in twice daily temperature monitoring as required by the NSSP checklist (1.4.9 & 1.4.18) but could greatly impact sample validity during equipment failure or malfunction. One solution to invalidating temperature deviations has been the implementation of electronic temperature sensing systems (i.e. data loggers). Data logging systems with built-in alarms and continuous monitoring close blind spots in temperature records that can occur over a workday or during times staff are away from the lab, allowing for close monitoring of storage and incubation steps which have temperature sensitive pieces. These systems, when set up correctly can quickly alert analysts to deviations that would invalidate samples as demonstrated by time/temperature laboratory assessments while liberating laboratory staff time from multiple audit points. Efforts to improve temperature monitoring during these procedures have run up against current NSSP guidance and checklist items due to the high-resolution data that is being produced by data logging systems and passive monitoring by staff. NSSP microbiology checklists should be updated to accommodate different temperature monitoring systems adopted by individual labs. Updated guidance on electronic temperature systems *Standard Methods for the Examination of Water and Wastewater* (24th Edition) 9-11: > Electronic temperature-sensing devices (i.e., data loggers) may be used so long as the laboratory's system for recording information from the devices also promptly notifies analysts of temperature violations so they can invalidate test samples, as appropriate, and request that new samples be collected. this system also must document the data such that time/temperature readings can be used to trace a sample and its testing conditions during laboratory assessments. Each data logger should be marked with any correction factor needed. Additionally, other areas overseen by FDA such as the National Conference on Interstate Dairy shipments Cultural procedures checklist contain language around temperature monitoring systems that allow for timed temperature excursions with documentation requirements for excursions specific to methods (Section 3: Temperature Measuring Devices). Guidance also includes requirements for software compatibility and records/auditing requirements. These, along with the updated

	Standard Methods for Examination of Water and Wastewater (24th Edition) provide updated guidance on temperature monitoring systems. In conclusion, laboratories that have implemented temperature monitoring systems have increased confidence in media productivity, sample integrity, and data confidence when appropriately implemented.
Cost Information	None- these changes include more appropriate practices for data logging devices for temperature monitoring. Laboratories would not be required to implement these systems unless desired.
References	American Public Health Association (APHA). 2022. *Standard Methods for the Examination of Water and Wastewater*, 24th Edition. APHA/AWWA/WEF, Washington, D.C. Standard Methods National Conference on Interstate Milk Shipments. Laboratory Form 2400 Cultural Procedures- General Requirements. March 2024

Proposed Task Force Consideration at the 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative
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Proposal Subject	DSP Alert™: Qualitative Detection of Diarrhetic Shellfish Toxins in Mussels and Oysters
Specific NSSP Guide Reference	Section IV Guidance Documents, Chapter III. Laboratory, @.02 Methods, .01 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	Request for approving DSP Alert™ as a qualitative screening tool for the detection of diarrhetic shellfish toxins in mussels and oysters.
Public Health Significance	Diarrhetic shellfish toxins (DSTs) are a group of heat-stable, lipophilic marine biotoxins produced primarily by certain dinoflagellates, including species of *Dinophysis* and *Prorocentrum*. DSTs, which include okadaic acid (OA), dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2), and their esterified derivatives, can accumulate in filter-feeding bivalve molluscs such as mussels, oysters, clams, and scallops. Consumption of contaminated shellfish can cause Diarrhetic Shellfish Poisoning (DSP), characterized by symptoms including diarrhea, nausea, vomiting, abdominal pain, and cramping. The toxic effects of DSTs are primarily associated with their inhibition of protein phosphatases, particularly protein phosphatases 1 and 2A, which disrupts normal cellular processes and intestinal function. DST-producing harmful algal blooms occur in many shellfish-growing regions worldwide, including coastal areas of North America, and can result in rapid increases in toxin concentrations in mussels and oysters. Such events may lead to harvesting restrictions or closures and can present significant risks to public health and the shellfish industry. Rapid and reliable detection of DSTs in mussels and oysters is therefore important for supporting routine monitoring, identifying potentially unsafe harvesting areas, and enabling timely management decisions.
Cost Information	Refer to “ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP” document attached for Sensoreal Inc.

Relevance of Okadaic Acid Determination

Diarrhetic shellfish toxins (DSTs), including okadaic acid (OA) and its analogues dinophysistoxins-1 and -2 (DTX1 and DTX2), are marine lipophilic toxins produced by certain dinoflagellates during harmful algal blooms (HABs). These toxins are the causative agents of Diarrhetic Shellfish Poisoning (DSP) in humans. Consumption of DST-contaminated bivalve mollusks can cause gastrointestinal symptoms, including diarrhea, nausea, vomiting, abdominal pain, and cramps. Due to these public health risks, regulatory authorities worldwide, including the United States, Canada, and Europe, require routine monitoring of bivalve shellfish. A maximum regulatory limit of 160 µg okadaic acid equivalents per kg of shellfish tissue is enforced for harvest closures and market access control.

Validation Data

Test Sensitivity and Threshold:	DSP Alert™ is engineered with a conservative decision threshold of 135 µg OA equivalent per kilogram of shellfish tissue (135 ppb) to provide an added safety margin relative to the regulatory limit.
Selectivity:	Okadaic acid: 100% DTX1: 25.5% DTX2: 71%
Comparability with LC-MS/MS:	In single-laboratory validation studies, the assay demonstrated 100% agreement with standard LC-MS/MS reference methods for samples containing DSP toxins at or above the regulatory action level (160 µg OA eq/kg) in mussels and scallops. No false negatives were observed at or above the regulatory limit.
General Limited Warranty:	The DSP Alert™ Rapid Test Kit is intended for research and pre-testing purposes only and is not a replacement for officially validated regulatory methods. **Users are responsible for determining product suitability. Sensoreal Inc. provides no expressed or implied warranties and is not liable for indirect, incidental, or consequential damages.**

To order or to learn more please contact:

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Instructions are effective as of (date): 2026-05-26

Version: 1.7

Sensoreal® DSP Alert™ Rapid Test for DSP toxins in Shellfish

Product ID: ALRT-DSP

1. General Description and Principle of the Method

Sensoreal DSP Alert™ is a rapid lateral flow immunoassay (LFA) designed for the qualitative detection of shellfish toxins which cause diarrheal shellfish poisoning (DSP), mainly okadaic acid (OA) in bivalve mollusks. The assay operates on a competitive binding format and has been evaluated on mussels, oysters, scallops, cockles and razorclams.

DSP toxins such as OA is extracted from homogenized shellfish tissue using a 100% methanol solution. The extract is then diluted and incubated with a lyophilized antibody–gold nanoparticle conjugate. If OA is present in the sample, it binds to the conjugate antibodies, reducing the amount of free antibody–gold complex available. The mixture is then applied to the lateral flow cassette and migrates across a membrane containing immobilized OA-protein at the test line. Consequently, the test line intensity is inversely proportional to the OA concentration (higher OA concentration = fainter test line). A control line will appear in all valid tests to confirm proper assay function. Results are objectively interpreted using the Sensoreal SensoRead™ scanner.

2. Safety

Treat all samples, extracts, and used test components as potentially contaminated with DSP toxins and biological material. Wear appropriate PPE, including gloves, safety goggles, and a laboratory coat. The DSP Alert™ protocol uses 100% methanol for extraction, which is hazardous and flammable, and includes a hydrolysis step with 2.5 M sodium hydroxide (NaOH) and 2.5 M hydrochloric acid (HCl) to release esterified DSP toxins. Handle all solvents and reagents in a well-ventilated area, follow institutional chemical safety procedures, and keep away from ignition sources.

3. Storage

-Lateral Flow Test Strips (Cassettes): Store at the temperature specified on the label. Do not freeze. Keep cassettes in their original, sealed pouches until immediately prior to use to protect them from moisture and humidity.
-Antibody Conjugate Vials (Lyophilized): This is the only kit component that may be frozen. Store frozen or as strictly specified on the product label. Keep vials sealed and do not open until ready to perform the assay.
-Diluent Powder Vials: Store at the temperature specified on the label. Do not freeze. Keep sealed to protect from excess heat and moisture.
-Buffer Solution: Store as directed on the label.
-General Stability: Protect all kit components from excess heat and direct sunlight. Do not utilize kit components past their printed expiration date or if they exhibit any signs of damage or deterioration.

4. Procedural Notes

-The DSP Alert™ test is optimized for analyzing freshly harvested bivalves, intact samples refrigerated for under 48 hours, or previously homogenized tissue kept frozen. Prior to beginning the extraction process, it is critical that all chilled or frozen samples are allowed to fully equilibrate to ambient room temperature.
-Unprocessed shellfish can be safely held under refrigeration (2–8°C) provided they will be tested within two days of harvest. If the testing timeline extends beyond this 48-hour window, the shellfish meat must be completely shucked, homogenized (as outlined in Section C), and stored at -20°C to ensure toxin stability prior to extraction.
-Please note that this protocol is exclusively validated for bivalve shellfish matrices. Any application to alternative sample types requires thorough independent validation by the user before routine testing is implemented.
-Follow the specified extraction, dilution, incubation, and read times exactly as detailed in Section C, D and E. Deviations from the protocol may adversely affect test performance.
- To avoid cross-contamination, use clean, disposable pipette tips and fresh extraction/dilution materials for each individual sample.
-Do not open the lateral flow cassettes or conjugate components until ready to initiate testing.
-Both visual and scanner-based interpretation are supported. Users may utilize the designated Sensoreal SensoRead™ scanner and its corresponding settings, or perform a manual visual interpretation of the results.
-Dispose of sample extracts, methanol-containing waste, and used kit components in strict accordance with institutional, local, and regulatory environmental requirements.
-As with any screening technique, positive results leading to regulatory action or market closure should be confirmed by a recognized alternative quantitative method (e.g., LC-MS/MS).

Protocol Instructions

A. Kit Components

The DSP Alert™ kit includes the following components:

1. Lateral Flow Test Strips (Cassettes)
2. Conjugate Vials (lyophilized antibody-gold nanoparticle)
3. Diluent Powder Vials ("D")
4. Buffer Solution ("B")

B. Materials and Equipment Required (Not Provided)

1. Sensoreal's SensoRead™ Scanner (used for result interpretation)
2. Shellfish shucking tools (knife, etc.)
3. Blender for tissue homogenization, Analytical weighing balance, Thermometer, Electronic timer
4. Centrifuge (compatible with 15 mL conical tubes and capable of 5000 rpm)
5. Adjustable pipettes covering volumes of 20–200 µL and 100–1000 µL and disposable pipette tips
6. 15 mL conical tubes (for extraction and collection), 2 mL microcentrifuge tubes, 5 mL glass vials with screwcap lid (for heating and hydrolysis step)
7. Water bath or Dry Block Heater
8. 100% Methanol, Sodium hydroxide (NaOH) pellets, Hydrochloric acid (HCl): 37% w/w, Deionised (DI) water
9. The use of test tube racks and a vortex mixer is recommended for operational ease; however, these components are not mandatory for the methodology.

C. Reagent Preparation, Sample Collection, and Processing

1. Preparation of 2.5M NaOH: Take 1g of NaOH pellets and dissolve in 10 mL of DI water in 15 mL conical tube. Shake vigorously to ensure all pellets dissolve.
2. Preparation of 2.5M HCl: Pour 7.9 mL of DI water in 15 mL conical tube. Carefully and slowly add 2.1 mL of 37% w/w HCl.
3. For each sample ID, shuck a representative population of 10 to 12 individual animals to obtain a minimum of 100 g of pooled shellfish tissue.
4. Thoroughly rinse the exterior of the shellfish with deionized or distilled water to remove extraneous debris, such as sand or mud.
5. Transfer the collected tissue to a sieve and allow it to drain to remove excess fluid.
6. Homogenize the tissue using an immersion blender (or equivalent mechanical blender) until a completely uniform, smooth consistency is achieved.

D. Toxin Extraction and Hydrolysis

1. Using a clean spatula and weighing balance, weigh exactly 2.5 g of the homogenized shellfish sample and transfer it into a 15 mL conical tube.
2. Add 10 mL of 100% methanol extraction solvent to the conical tube containing the sample.
3. Cap the conical tube tightly and shake vigorously (or utilize a vortex mixer) for 5 minutes.
4. Transfer the tube to a centrifuge and spin at 4000 rpm for 5 minutes to separate the particulate matter from the toxin-containing solvent.
5. Following centrifugation, carefully transfer the supernatant (the clear liquid) into a clean 15 mL conical tube.
6. Preheat a water bath or Dry Block Heater to **76°C**. Use a thermometer to monitor the temperature and ensure it stabilizes before proceeding.
7. Using clean pipettes, transfer 800 µL of the supernatant in a 5 mL glass vial. Add 100 µL of 2.5M NaOH solution. Close the screw cap, shake well and place into the water bath or Dry Block Heater. Heat at 76^0C for 40 minutes. This is the hydrolysis step.
8. After 40 minutes, take out the glass vial from the water bath/Dry Block Heater and allow the contents to cool to room temperature. To the cooled solution, add 100 µL of 2.5M HCl. This will stop the reaction. This is the hydrolyzed extract.

E. Test Strip Sample Analysis Procedure

Ensure the Sensoreal SensoRead™ scanner is turned on and the correct lot QR code has been scanned prior to initiating the test.

1. Locate the Diluent powder tube ("D"). Using a pipette fitted with a new tip, add 2 mL of Buffer "B". Shake vigorously until all powder dissolves. This is the diluent solution. With fresh pipette tips, transfer 800 µL of diluent solution into a new 2 mL microcentrifuge tube. To this, add 200 µL of the hydrolyzed extract. Mix thoroughly. This is the Diluted Sample.
2. Using a pipette fitted with a new tip, transfer 70 µL of the Diluted Sample into the Conjugate vial. Pipette the mixture up and down to achieve complete dissolution of the lyophilized pellet, ensuring that no red specks remain adhered to the interior walls of the tube. Once the pellet is fully dissolved and the solution is homogeneous, let the vial sit uninterrupted at room temperature for exactly 10 minutes.
3. After the 10-minute incubation, transfer the contents of the Conjugate mix into the sample port of the test cassette.
4. Leave the cassette on a flat, horizontal surface. Set a timer and allow the assay to develop for exactly 10 minutes.

F. SensoRead™ Scanner Analysis

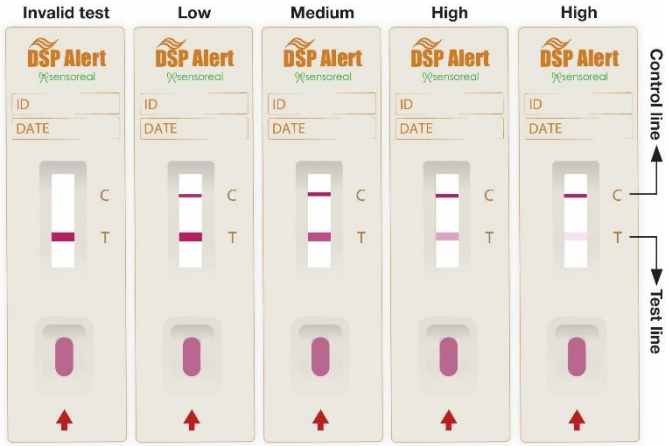
1. After exactly 10 minutes of development, immediately insert the test cassette into the scanner slot of the SensoRead™ device. Follow the on-screen prompts: Select Cassette -> DSP Alert
2. You will be prompted to identify the batch being used. Either select an existing Lot from the on-screen list, or for a new Lot, press "Add New Lot" and scan the batch-specific QR code located on the LFA test pouch or the Certificate of Analysis. This QR code provides the system with integral calibration parameters required for accurate interpretation. *(Note: Each Lot only needs to be scanned once; for subsequent tests with the same Lot, simply select it from the saved list).*
3. Once the Lot is confirmed, enter the unique Sample ID using the on-screen keypad and press "Run Test". The device will immediately initiate the analysis process to receive the qualitative result (T/C ratio and classification).

G. Interpretating the Results

Assay results can be interpreted visually or digitally using the SensoRead™ scanner, which calculates the Test-to-Control (T/C) line intensity ratio. While the absolute intensity of the control line may fluctuate between runs, a visible control line must be present for the assay to be considered valid; the absence of a control line indicates an invalid result requiring a retest.

The SensoRead™ scanner provides an objective determination of line intensities for consistent interpretation, as well as a digital photographic record of all test strips.

-High: The scanner has detected OA and other DSP toxins at or above the 135 µg OA eq/kg threshold. The scanner will flag this sample, indicating it has reached or exceeded the precautionary limit.
-Medium: The T/C ratio indicates concentrations between 80-135 µg OA eq/kg threshold, and the sample is considered compliant.
-Low: The T/C ratio indicates concentrations below 80 µg OA eq/kg, and the sample is considered compliant.



Test Line	Control Line	Interpretation
Present	Absent	Invalid Test (Repeat Test)
Faint or Non-Visible	Visible	High: ≥ 135 µg OA eq/kg
Moderate Intensity	Visible	Medium: 80-135 µg OA eq/kg
Equal to or More Intense than Control Line	Visible	Low: < 80 µg OA eq/kg

Note: It is essential to perform the reading at the designated 10-minute mark. Determinations made using strips that have dried for more or less than the required time may be inaccurate, as chromatographic line intensities vary with drying time. To ensure a rigorous safety margin against the 160 µg OA eq/kg *NSSP regulatory limit, the DSP Alert™ system is intentionally calibrated to a conservative early-warning threshold of 135* µg OA eq/kg.

For supplemental technical data or methodological assistance, please direct all inquiries to Sensoreal Technical Support at sales@sensoreal.com.

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
DSP Alert — LFA for DSP in Shellfish

LABORATORY:	
ADDRESS:	
TELEPHONE:	**FAX:**
EMAIL:	
DATE OF EVALUATION:	**DATE OF REPORT:**
LAST EVALUATION:	
LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	
SHELLFISH SPECIALIST:	
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: _____ Conformity is noted by a "√"

C- Critical K - Key O - Other N/A - Not Applicable

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

PART I – QUALITY ASSURANCE (QA)		
1.1 Quality Assurance (QA) Plan		
Code	**Ref**	**Item**
K	1,2	**1.1.1 Written QA Plan exists** (check √ those items which apply): ☐ Organization of laboratory ☐ Staff training requirements ☐ SOPs/Work Instructions ☐ Internal QC measures (equipment, calibration, maintenance, acceptance criteria) ☐ Laboratory safety (chemical safety applicable to methanol, hydrochloric acid and sodium hydroxide) ☐ Data integrity/record control ☐ Internal performance assessment ☐ External performance assessment (if applicable)
C	2	**1.1.2 QA Plan is implemented** (evidence of use, current version controlled, staff awareness).
1.2 Educational / Experience / Training Requirements		
Code	**Ref**	**Item**
C	1,2	**1.2.1 Supervisor/technical lead is qualified** per lab policy and applicable program requirements.
K	1,2	**1.2.2 Analysts are qualified and authorized** to perform DSP Alert testing; authorization list maintained.
C	3	**1.2.3 Method-specific training documented** (DSP Alert IFU/SOP, critical timings, pipetting, reader operation, invalid criteria, reporting).
K	2	**1.2.4 Competency is assessed and recorded** (initial + periodic).
1.3 Work Area		
Code	**Ref**	**Item**
O	2,3	**1.3.1 Work area adequate** for workload, sample prep, and storage.
K	2	**1.3.2 Work area clean and well lighted.**
K		**1.3.3 Work area has adequate temperature control** to support kit/storage requirements and stable testing conditions.
O	2	**1.3.4 Work surfaces nonporous/easily cleaned**; contamination prevention practices in place.
K	2,3	**1.3.5 Chemical safety controls in place** for 100% methanol, HCl and NaOH (labeling, SDS access, waste handling).
1.4 Laboratory Equipment (General + Method-Relevant)		
Code	**Ref**	**Item**
K	2	**1.4.1 Equipment inventory exists** (includes model/ID, location, calibration/verification needs).

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

C	2	**1.4.2 Variou Micropipettors used in method are calibrated/**verified for volumes used (e.g., 70 µL, 100 µL, 200 µL, 800 µL, 10 mL or appropriate devices). Records maintained..
K	3	**1.4.3 Timer(s) available and controlled** for 10-min conjugate incubation, 40-min hydrolysis reaction and 10-min cassette development.
K	2,	**1.4.4 Balance used for 2.5 g tissue is verified/calibrated** at relevant range; records maintained.
C	1,2	**1.4.5 Centrifuge performance is verified** (speed/time appropriate; maintenance/service records maintained).
C	3	**1.4.6 DSP Alert reader is installed and functional** (method/settings controlled; alignment/cassette insertion per IFU).
K	3	**1.4.7 Reader performance checks documented** (startup/self-test or verification check per IFU/SOP).
O	2	**1.4.8 Refrigeration/temperature monitoring where required** (for kits/reagents as specified). Records maintained.
1.5 Reagent / Kit Storage, Preparation, and Inventory Control		
Code	**Ref**	**Item**
C	2	**1.5.1 DSP Alert kits stored per IFU** (temperature range, protection from extremes).
K	3	**1.5.2 Lot # and expiry tracked** for kits, cassettes, conjugate vials, diluent (as applicable).
C	3	**1.5.3 Extraction solvent is prepared correctly (100% methanol)** and labeled (contents, date, preparer), **along with 2.5M HCl and 2.5M NaOH.**
K	2,3	**1.5.4 Reagents and samples clearly labeled** to prevent mix-ups; chain-of-custody maintained.
O	2,3	**1.5.5 Receiving inspection documented** (condition upon receipt, storage initiated promptly).
1.6 Records, Data Integrity, and Document Control		
Code	**Ref**	**Item**
C	3	**1.6.1 Controlled documents in use** (current IFU/SOP, version controlled, accessible to staff).

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

C	3	**1.6.2 Traceability is ensured** (sample ID → extraction → dilution → cassette → reader result → report).
K	2,3	**1.6.3 Raw data retention** (reader output files, run sheets, logs) per policy.
K	2	**1.6.4 Deviations/nonconformities documented** with corrective actions (CAPA or equivalent).
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR DSP TOXINS — DSP ALERT (LFA)		
2.1 Collection, Receipt, and Transportation of Samples (Laboratory Controls)		
Code	**Ref**	**Item**
C	3	**2.1.1 Representative sample is received and logged** (sample identifiers, species, harvest area/source, date/time).
K	2,3	**2.1.2 Sample condition assessed on receipt** (temperature/condition noted; acceptance/rejection criteria defined).
K	3	**2.1.3 Time-to-processing controls** defined (or contingency plan if delays occur).
O	3	**2.1.4 Storage conditions documented** prior to preparation/extraction (refrigerated/frozen as defined).
2.2 Preparation of Samples for Analysis — Homogenization		
Code	**Ref**	**Item**
C	2..3	**2.2.1 Shellfish are shucked and homogenized to uniform consistency** suitable for representative subsampling.
K	2.3	**2.2.2 Homogenization method is standardized** (equipment, time, cleaning between samples to prevent carryover).
K	2.3	**2.2.3 Cross-contamination controls in place** (clean tools, workspace cleaning, single-use consumables where appropriate).
2.3 Preparation of Samples for Analysis — Extraction and Clarification (DSP Alert)		
Code	**Ref**	**Item**
C	3	**2.3.1 Tissue mass for extraction is 2.5 g** (within defined tolerance).
C	3	**2.3.2 Extraction solvent is 100% methanol and 10 mL** is added per method (within tolerance).
K	3	**2.3.3 Sample is mixed thoroughly or** vortexed and **left to stand for 5 minutes to allow toxins** to be extracted ensure thorough mixing.
C	3	**2.3.4 Centrifugation is performed at 4000 rpm for 5 minutes** (or validated equivalent); supernatant is collected.
C	3	**2.3.5 800 µL of supernatant is transferred to a small tube and 100 µL** NaOH solution is added (within tolerance).

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

K	3	**2.3.6 Supernatant collection avoids pellet disturbance** (minimize particulates that can affect flow).
K	3	**2.3.7 Tube is vortexed/mixed well after NaOH addition.**
C	3	**2.3.8 Hydrolysis is performed at 76°C for 40 minutes** (within defined tolerance), using a monitored heating block or water bath.)
K	3	**2.3.9** Sample is allowed to cool to room temperature before neutralization.
C	3	**2.3.10 After cooling, 100 µL HCl solution is added** and mixed well to complete hydrolysis/neutralization.
C	3	**2.3.11** Dilution is performed as specified: **800 µL diluent + 200 µL hydrolyzed methanolic extract** (within tolerance).
K	2,3	**2.3.12** Extract/hydrolysate handling and storage are defined if not analyzed immediately (conditions/time limits documented).
2.4 Sample Assay — Dilution, Conjugate Incubation, Cassette Development, Reader		
Code	**Ref**	**Item**
C	3	**2.4.1 Dilution is performed as specified:** 200 µL supernatant into 800 µL diluent (within tolerance).
C	3	**2.4.2 70 µL of diluted sample is added to the conjugate vial** (within tolerance).
C	3	**2.4.3 Conjugate is fully resuspended** (aspirate/mix until uniform red solution; no visible clumps).
K	3	**2.4.4 Conjugate incubation is 10 minutes** (within defined window).
C	3	**2.4.5 Entire red solution is dispensed into cassette** (complete transfer; no leakage).
C	3	**2.4.6 Cassette development time is 15 minutes** (results read within defined window).
C	3	**2.4.7 Cassette is inserted into reader and result obtained** using controlled settings/method file.
C	3	**2.4.8 Invalid test criteria are defined and followed** (e.g., reader error/invalid flag; actions documented).
K	2	**2.4.9 Proper disposal practices followed** for methanol-containing waste and biohazard materials.
2.5 Quality Control (QC), Acceptance/Rejection, and Reporting		
Code	**Ref**	**Item**
C	3	**2.5.1 Internal validity check met** (control indicator per IFU/reader validity). Invalid results are not reported as valid.
K	3	**2.5.2 Reader check/verification performed** at defined frequency; failures addressed before reporting results.
K	2,3	**2.5.3 Run documentation complete** (operator, date/time, kit lot/expiry, sample ID, deviations).
C	2,4	**2.5.4 Reporting and notification procedures defined** for results requiring program action (as applicable).
K	2	**2.5.5 Data retention and traceability maintained** (reader outputs + run sheets retained per policy).
REFERENCES		
1. NSSP Model Ordinance / NSSP Guide for the Control of Molluscan Shellfish (FDA/ISSC) — current edition. 2. Good Laboratory Practice (GLP) and laboratory quality system/document control requirements.		

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

3. DSP Alert Method Documentation: DSP Alert IFU/Procedure Card; internal SOPs; reader user manual/method file ID; SLV/ISSC proposal documents (as applicable). 4. Laboratory sample receipt/chain-of-custody procedures and applicable program requirements (state/agency procedures).

LABORATORY:	**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**	

Page	Item	Observation	Documentation Required

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
DSP ALERR LFA ASSAY FOR DSP COMPONENT: (Part I-II)	
A. Results	
Total # of Critical (C) Nonconformities	______________________
Total # of Key (K) Nonconformities	______________________
Total # of Critical (C), Key (K), and Other (O) Nonconformities	______________________

SHELLFISH LABORATORY EVALUATION CHECKLIST

DSP Alert (Lateral Flow Assay) for Diarrheal Shellfish Poisoning (DSP)

B. Criteria for Determining Laboratory Status of ASP Alert assay for ASP:

1. **Conforms Status:** The DSP Aler for DSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The DSP Alert assay for DSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. The number of Critical nonconformities is ≥ 1 but < 4;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status**: The DSP Alert assay for DSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is ≥4;
   b. or total # of Key nonconformities is ≥ 6;
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____
LEO Signature:_____ Date:_____

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	DSP Alert
Name of the Method Developer	Sensoreal Inc.
Developer Contact Information	Hosseinali Safavieh 6674 Esplanade Avenue (Suite B), Montreal QC H2V 4L5

A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for a simple, rapid detection tool for reliably detecting diarrhetic shellfish toxins (DSTs) such as okadaic acid (OA) in shellfish, that can be used in laboratory settings.
2. What is the intended purpose of the method?		Qualitative screening of DSTs in mussels and oysters.
3. Is there an acknowledged need for this method in the NSSP?		There is a need for simple assays that deliver rapid and accurate results for limited-use screening in marine biotoxin testing.
4. What type of method? i.e. chemical, molecular, culture, etc.		Lateral flow immunoassays
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		DSP Alert
Method Scope		Qualitative screening and detection of DSTs such as OA, Dinophysistoxin-1 (DTX1) and Dinophysistoxin-2 (DTX2) , etc in mussels and oysters.
References		Study Report, kit insert/IFU and SDS and Quality Manual (QM) included in submission

Principle		Competitive lateral flow immunoassay for detection of DSTs using aqueous-based extraction.
Any Proprietary Aspects		Yes, commercial test kit
Equipment Required		Centrifuge, shucking tools, conical tubes, blender, timer, pipettes, Sensoreal Sensoread™ scanner.
Reagents Required		DSP Alert™ test devices and provided reagents inside the kit; Sodium hydroxide; Hydrochloric acid; 100% Methanol; Deionized water.
Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2–8°C prior to testing.
Safety Requirements		Used test devices, concical tubes containing slurry or shellfish extracts, and pipettes should be handled as though contaminated with DSTs and disposed of accordingly. Gloves and lab coats should be worn during testing.
Clear and Easy to Follow Step-by-Step Procedure		Step-by-step procedure card included in kit insert and submission
Quality Control Steps Specific for this Method		Each test device includes an internal control line to confirm proper function. A mussel certified reference material (CRM-DSP-Mus-c) from the National Research Council Canada can be used as positive control.
C. Validation Criteria		
1. Accuracy / Trueness (Probability of Detection)		A sensitivity of 100% for the detection of DSTs was achieved under the following conditions: ● All mussel and oyster samples in the POD study containing total DST concentrations at or above the regulatory limit of 160 µg OA eq/kg were correctly identified as positive, with 0% false negatives and 2.5% false positives. ● Across all study components, 100% of samples containing total DST concentrations below 74 µg OA eq/kg were correctly identified as negative. ● Sensitivity of 1.0 and specificity of 0.959 was obtained.
2. Measurement Uncertainty		Not applicable
3. Precision Characteristics (repeatability and reproducibility)		Not applicable.
4. Recovery		Not applicable
5. Specificity		Not applicable

6. Working and Linear Ranges		Not applicable
7. Limit of Detection		The positive threshold of the DSP Alert has been set at 135 µg OA eq/kg.
8. Limit of Quantitation / Sensitivity		Not applicable.
9. Ruggedness		No impact on performance was observed across different lots or with a ± 4-minute variation in LFA run time.
10. Matrix Effects		No significant matrix effect was observed between mussel and oyster samples.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		The assay demonstrated comparability to the LC-MS/MS reference method when testing naturally incurred shellfish samples, with 0% false negatives.
D. Other Information		
1. Cost of the Method		Approximately $32/test
2. Special Technical Skills Required to Perform the Method		None. Training on assay operations is provided by Sensoreal Inc..
3. Special Equipment Required and Associated Cost		Centrifuge. Sensoreal Sensoread™ scanner (approximately $4000)
4. Abbreviations and Acronyms Defined		All relative abbreviations and acronyms have been defined in the study report.
5. Details of Turn Around Times (time involved to complete the method)		The test can be operated in under 60 minutes, including sample preparation.
6. Provide Brief Overview of the Quality Systems Used in the Lab		
Submitters Signature 	Date: September 18, 2026	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	

Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between

results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.

14. **Quality System -** The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.

15. **Recovery –** The fraction or percentage of an analyte or measurand recovered following sample analysis.

16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]

17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]

18. **Working Range –** the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

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Date: September 18, 2026

Laboratory Methods Review & Quality Assurance Committee
Interstate Shellfish Sanitation Conference
209-2 Dawson Road
Columbia, SC 29223-1740

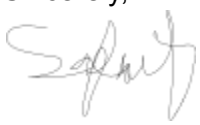
Please find enclosed the single laboratory validation (SLV) study report and supporting documentation for DSP Alert™, Sensoreal Inc.'s rapid qualitative assay for the detection of diarrhetic shellfish toxins (DSTs), including okadaic acid (OA), dinophysistoxin-1 (DTX1), and dinophysistoxin-2 (DTX2), in mussels and oysters. DSTs are responsible for diarrhetic shellfish poisoning (DSP) and represent an important public-health concern in areas affected by harmful algal blooms.

We respectfully request the Committee's review of this submission and consideration of DSP Alert™ for acceptance as an ISSC-approved screening method for the detection of relevant DSTs in mussels and oysters. The assay offers important advantages in rapid time-to-result, procedural simplicity, and ease of implementation in laboratory settings. Its acceptance by the ISSC could provide meaningful benefits to shellfish growers, testing laboratories, regulators, and public-health authorities by supporting timely screening and informed management decisions.

The SLV study included evaluation of assay performance using naturally contaminated mussel and oyster samples through comparability and probability of detection (POD) studies, assessment of selectivity and potential interference from other marine biotoxins, crossreactivity and a ruggedness evaluation. The resulting validation demonstrated reliable detection of samples containing total DST concentrations at or above the regulatory limit of 160 µg OA eq/kg, with no false-negative results. The assay also provided a conservative safety margin through its reporting threshold of 135 µg OA eq/kg, acceptable specificity, consistent performance across varied OA and DTX toxin profiles, and robust performance under reasonable procedural and environmental variations.

We would be pleased to answer any questions or provide any additional information that may assist the Committee in its review. Thank you for your time and consideration of this submission.

Sincerely,



Hosseinali Safavieh
CEO, Sensoreal Inc.

Enclosures:
- SLV Study Report
- DSP Alert™ IFU & Procedure card
- Safety Data Sheet
- DSP Alert-ISSC Proposal form
- ISSC Method Application Checklist
- DSP Alert Safety Data Sheet
- Sensoreal DSP Alert Laboratory Checklist

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Single Laboratory Validation Study of the Sensoreal DSP Alert Lateral Flow Assay for the Qualitative Detection of Okadaic Acid and other Diarrheal Toxins in Shellfish

Daud H Khan[1*], Andrew D. Turner[2], Ryan Alexander[2], Shamina Page[2], Karl Dean[2], Maarij Baig[1], Hosseinali Safavieh[1]

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- Address: 6674 Esplanade Avenue (Suite B), Montreal QC H2V 4L5 Canada
- Submitted: March 2026

1. Introduction

Okadaic acid (OA)-group toxins, including OA and the related analogues dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2), are an important food-safety concern in shellfish harvesting and aquaculture (1–5). These toxins, produced primarily by dinoflagellates of the genera Dinophysis and Prorocentrum, can accumulate in shellfish during harmful algal bloom events. Consumption of contaminated shellfish can cause diarrhetic shellfish poisoning (DSP), a gastrointestinal illness characterized by diarrhea, nausea, vomiting, abdominal pain, and cramping (4–7). The regulatory maximum permitted limit for OA-group toxins in shellfish is 160 µg OA equivalents per kilogram of shellfish tissue (µg OA eq/kg) (8,9).

LC-MS/MS, the reference method for official control testing of OA-group toxins, provides high analytical reliability but requires specialized equipment, trained personnel, and access to laboratory infrastructure (10–12). These requirements can limit timely testing and decision-making, particularly for shellfish harvesters, small producers, and remote communities (13).

To address this need, Sensoreal Inc. developed DSP Alert, a rapid, portable, lateral flow assay-based screening tool for the qualitative detection of OA in mussels and oysters. DSP Alert provides a qualitative positive or negative result using a reporting threshold of 135 µg OA eq/kg, thereby providing a conservative safety margin below the regulatory limit of 160 µg OA eq/kg. The assay is intended to complement centralized laboratory testing by supporting faster and more accessible screening.

2. Principle of the Method

Sensoreal DSP Alert™ is a rapid lateral flow immunoassay (LFA) designed for the qualitative detection of okadaic acid (OA) and the other DSTs dinophysistoxin-1 (DTX1) and dinophysistoxin-2 (DTX2) in bivalve mollusks. The assay uses a competitive binding format. DSTs are first extracted from homogenized shellfish tissue using 100% methanol extraction solution. The extract is then hydrolyzed using 2.5M sodium hydroxide (heating at 76°C for 40 minutes), followed by addition of 2.5M of hydrochloric acid. The hydrolysis reaction breaks down DST esters into free OA and DSTs. The hydrolyzed extract is then diluted and incubated with a lyophilized antibody–gold nanoparticle conjugate. We respectfully request your review of this submission and consideration of DSP Alert for acceptance as an ISSC-approved method for the determination of DSTs in mussels and Pacific oysters

When OA, DTX1 and DTX2 are present in the sample (following hydrolysis), they bind to the conjugate antibodies, reducing the amount of free antibody–gold complex available to bind at the test line. The mixture is then added to the lateral flow cassette, where it migrates across a membrane containing immobilized OA-protein at the test line. As a result, test-line intensity is inversely proportional to the total DST concentration (reported as µgOAeq/kg): higher DST levels produce a fainter test line. A control line appears in all valid tests to confirm proper assay performance. Because the antibody was raised against OA, its affinity varies across individual PSP congeners, with the strongest affinity observed for OA.

Results are read using the Sensoreal SensoRead™ scanner, which provides a numerical test/control (T/C) ratio and a qualitative classification and additionally semi-quantitative(High, Medium, or Low). In this validation study, High results were considered positive, while Low and Medium results were considered negative unless otherwise specified. The assay is intended for users with basic laboratory skills (e.g., shellfish handling, homogenization, and simple pipetting) and is designed as a screening tool to support harvest-area closure decisions and help prevent contaminated shellfish from reaching the market. Although the technology can be applied to bivalve mollusks more broadly, the current validation and approval effort is focused specifically on mussels and Pacific oysters only.

3. Intended Use

DSP Alert™ is intended for the qualitative screening of shellfish for OA, DTX1 and DTX2 in mussels and oysters only, with a positive result indicating samples at or above the toxic/regulatory threshold of 160 µgOAeq/kg. The test kit is designed for use by quality control personnel, laboratory staff, and other trained users familiar with handling shellfish samples and performing basic sample preparation and pipetting procedures.

To enable a safety buffer zone for shellfish growers and researchers and ensure that no negative toxic results appear, the toxic or positive threshold of the assay has been set at 135 µgOAeq/kg. This means ideally all samples above 135 µgOAeq/kg should be detected as positive or toxic by the assay, providing an early warning for users.

4. DSP Alert Method

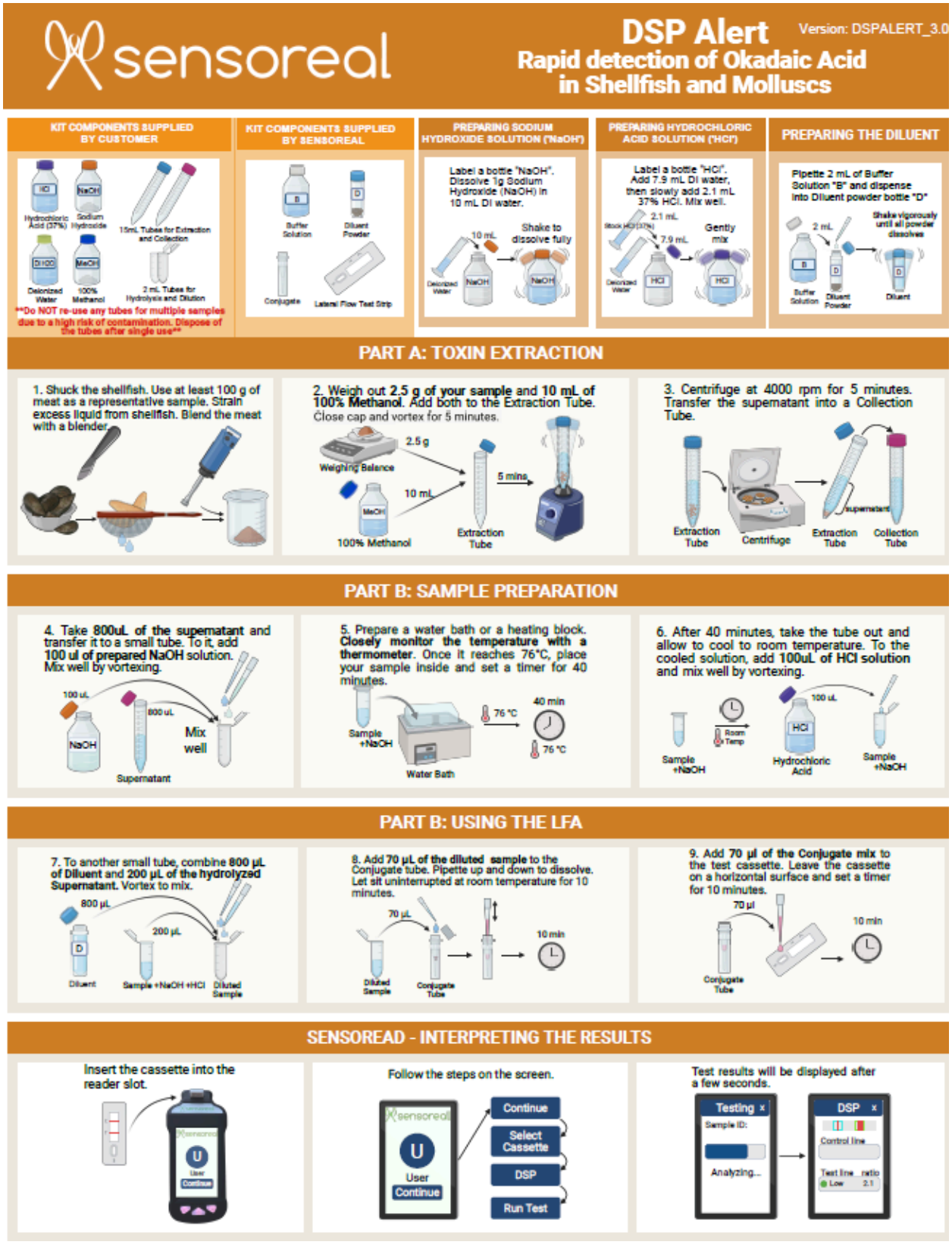


Figure 1: Procedure card illustrating the DSP Alert method.

4.1 Materials Provided

The DSP Alert kit includes the following components:

- Lateral Flow Test Strips (Cassettes)
- Conjugate Vials (lyophilized antibody-gold nanoparticle)
- Diluent Powder Vials ("D")
- Buffer Solution ("B")
- Sensoreal SensoRead™ scanner (required for interpreting results).

4.2 Additional materials required, but not provided

Equipment and consumables required for operation but not provided with the kit include:

- Shellfish-shucking tools, such as a shucking knife
- Blender for tissue homogenization
- Analytical balance
- Thermometer
- Electronic timer
- Centrifuge compatible with 15 mL conical tubes and capable of reaching 5,000 rpm
- Adjustable pipettes covering volume ranges of 20–200 µL and 100–1,000 µL
- Disposable pipette tips
- 15 mL conical tubes for sample extraction and collection
- 2 mL microcentrifuge tubes
- 5 mL screw-cap glass vials for the heating and hydrolysis steps
- Water bath or dry-block heater
- 100% methanol
- Sodium hydroxide (NaOH) pellets
- Hydrochloric acid (HCl), 37% w/w
- Deionized (DI) water
- Test-tube racks and a vortex mixer are recommended for operational convenience but are not required to perform the method.

4.3 Precautions

- Do not open cassette or conjugate components until ready to use. Keep all test components in their original packaging/containers until the time of testing.
- Do not use kit contents beyond the expiration date.
- Treat all samples, extracts, and used test components as potentially contaminated with domoic acid and biological material. Wear appropriate PPE (e.g., gloves, lab coat, eye protection) during testing.
- To avoid cross-contamination, use clean pipette tips/tubes and fresh extraction/dilution materials for each sample

- Follow the specified extraction, dilution, incubation, and read times exactly; deviations may affect test performance.
- Use only the designated Sensoreal reader/method settings for interpretation, as applicable.
- Dispose of sample extracts, methanol-containing waste, and used kit components in accordance with institutional, local, and regulatory requirements.
- Safety documentation (SDS) for relevant reagents/solutions should be available to users upon request / maintained by the laboratory.

4.4 Storage Requirements

- Store DSP Alert™ kit components according to the product IFU labeling (controlled storage conditions as specified by Sensoreal).
- Do not freeze kit components, except the antibody conjugate.
- Keep components in their original packaging until use.
- Protect kit components from excess heat, moisture, and direct sunlight.
- Do not use components that are expired or show signs of damage/deterioration.

4.5 Sensoread reader set-up

- Turn on the reader and scan the QR code to select the correct test for the lot number.

4.6 DSP Alert Method Protocol

- For LFA testing, homogenized whole flesh was prepared from 10–12 individual shellfish representative of the sampling area. This was equivalent to approximately upto 100 g of shellfish meat.
- The shellfish were shucked, and the whole flesh was removed from the shells, strained using a sieve, and pooled in a blender jar.
- The pooled tissue was thoroughly homogenized to produce a representative shellfish homogenate.
- A 2.5 g aliquot of the homogenized tissue was weighed into a polypropylene centrifuge tube.
- A 10 mL volume of 100% methanol was added to the tube, and the sample was mixed for 3 minutes.
- The sample was centrifuged at 4,000 rpm for 10 minutes.
- Following centrifugation, 800 µL of the methanolic extract was transferred into a 2 mL microcentrifuge tube or vial.
- A 100 µL volume of 2.5 M sodium hydroxide (NaOH) was added to the methanolic extract.
- The vial was securely capped, mixed thoroughly, and placed in a preheated water bath or dry-block heater maintained at 76°C.
- After 5 minutes of heating, the vial cap was checked to confirm that no leakage had occurred. The vial was then immediately returned to the heating unit.
- The sample was heated for a total of 40 minutes to complete the hydrolysis step.
- Following hydrolysis, the vial was removed from the heating unit and uncapped. The hydrolysis reaction was quenched by neutralizing the extract with 2.5 M hydrochloric acid (HCl).

- A 200 µL aliquot of the neutralized hydrolyzed extract was transferred into a test tube containing 800 µL of sample diluent buffer and mixed thoroughly.
- A 70 µL aliquot of the diluted extract was transferred into a conjugate vial.
- The contents of the conjugate vial were mixed thoroughly by repeated pipetting and incubated for 10 minutes.
- Following incubation, the mixture was applied to the sample port of the DSP Alert test cassette.
- The test cassette was allowed to develop for 10 minutes.
- After exactly 10 minutes of development, the test cassette was immediately inserted into the reader slot of the SensoRead device. ***Cassette*** → ***DSP Alert*** was selected using the on-screen prompts.
- The lot used for testing was then identified. An existing lot was selected from the on-screen list. For a new lot, ***Add New Lot*** was selected, and the batch-specific QR code located on the LFA test pouch or Certificate of Analysis was scanned. The QR code provided the lot-specific calibration parameters required for result interpretation. Each lot was scanned only once and was subsequently selected from the saved list for additional tests performed using the same lot.
- Once the lot was confirmed, the unique Sample ID was entered using the on-screen keypad, and ***Run Test*** was selected.
- The test was analyzed by the device, and the resulting test-to-control line ratio and qualitative positive or negative classification were recorded.
    - **Note:** To minimize the risk of false-negative results and provide a safety margin below the regulatory limit, the qualitative reporting threshold for DSP Alert was set at 135 µg OA eq/kg rather than the regulatory limit of 160 µg OA eq/kg. Samples with total OA-group toxin concentrations at or above 135 µg OA eq/kg were therefore classified as positive, supporting earlier detection before the regulatory limit was reached.

4.7 LC-MS/MS method analysis

- A 2.0 ± 0.01 g aliquot of homogenized shellfish tissue was weighed into a 50 mL polypropylene centrifuge tube.
- Samples were extracted using the triple-step methanol extraction procedure described in the European Union Reference Laboratory for Marine Biotoxins standard operating procedure (14).
- For the first extraction, 6.0 mL of 100% methanol was added to the homogenized tissue.
- The sample was vortex-mixed at 2,500 RPM for 3 minutes and then centrifuged at 4,500 RPM for 10 minutes.
- Following centrifugation, the supernatant was decanted into a 20 mL glass volumetric flask.
- The extraction procedure was repeated twice using successive 6.0 mL volumes of 100% methanol.
- The supernatants from all three extraction steps were combined in the same 20 mL volumetric flask.
- The final volume was adjusted to 20 mL using 100% methanol (14).

- Chromatographic analysis was performed using Waters Acquity and Acquity I-Class Ultra Performance Liquid Chromatography systems coupled to Waters Xevo TQ-A and TQ-S tandem mass spectrometers.
- Each system comprised a binary pump, autosampler, column oven, and tandem mass spectrometer.
- The mobile phases and chromatographic conditions were prepared and applied as described by Dhanji-Rapkova and colleagues (11).
- OA-group toxins in the hydrolyzed extracts were quantified against OA, DTX1, and DTX2 calibration standards prepared from certified reference materials.
- Two multiple reaction monitoring transitions were used for each analyte. The primary transition was used for quantification, and the secondary transition was used for confirmation.
- The individual concentrations of OA, DTX1, and DTX2 were quantified.
- Total DSP toxicity was calculated using the toxicity equivalence factors established by EFSA: 1.0 for OA, 1.0 for DTX1, and 0.6 for DTX2 (15).
- Final results were expressed as µg OA eq/kg of shellfish tissue.

5. Single Laboratory Validation study

A single-laboratory validation study was conducted to measure or evaluate the following parameters for PSP Alert:

- Probability of Detection(16)
- Comparability (16)
- Cross-reactivity (16)
- Ruggedness (16)
- Interference or Selectivity study (17)

For any naturally contaminated shellfish sample, DSP Alert results were compared to those of an accepted LC-MS/MS reference method (14). Matrices tested were mussels and oysters.

5.1 Comparability

Method

- Comparability is demonstrated when a new method intended as an alternative/substitute does not produce a significant difference in results compared with the officially recognized NSSP method for the same matrix and intended use.

- Naturally contaminated mussel and oyster samples were collected from different regions and seasons. For each Sample ID, whole flesh from 10–12 individual shellfish, equivalent to approximately 100 g of shellfish meat, was shucked strained, pooled, and homogenized.Then 3 aliquots were prepared. One aliquot was used to test with DSP Alert. The second aliquot was tested using the will be tested using the reference method

of LC/MS and the correct concentration (µg OA eq/kg) was obtained. The concentrations of OA, DTX1, and DTX2 were determined by LC-MS/MS and expressed as total µg OA eq/kg. The final aliquot was used in the following Probability of Detection Study.

- As per ISSC guidance, a total of 60 naturally contaminated samples, comprising 30 mussel and 30 oyster samples, were evaluated. False-positive, false-negative, true-positive, and true-negative results were recorded, and the sensitivity and specificity (18) of DSP Alert were calculated.

Results

- The individual sample results are presented in Table 1. DSP Alert correctly identified all samples containing OA-group toxin concentrations at or above the reporting threshold of 135 µg OA eq/kg.

- Based on the conservative reporting threshold of 135 µg OA eq/kg, no false-positive or false-negative results were observed for either mussel or oyster samples. All 19 mussel and 20 oyster samples containing concentrations below 135 µg OA eq/kg were correctly identified as negative. The remaining 11 mussel and 10 oyster samples, containing concentrations at or above 135 µg OA eq/kg, were correctly identified as positive. This corresponded to an overall sensitivity of 1.00 and specificity of 1.00.

- The DST profiles or toxin breakdown, including the individual concentrations of OA, DTX1, and DTX2 for each unique Sample ID, are also provided in Table 1.

Table 1. Results of the comparability study with naturally contaminated mussel and oyster samples tested with DSP Alert and reference method LC-MS/MS, along with toxin composition.

Species	Sample ID	OA (µg/kg)	DTX1 (µg/kg)	DTX2 (µg/kg)	Total DST Concentration (µg OA eq/kg)	Qualitative result
Mussels	M399	0.6	0.0	0.0	1	(-)
	M376	1.0	0.0	0.0	1	(-)
	M402	4.0	0.6	22.0	18	(-)
	M406	3.0	0.0	35.0	24	(-)
	M378	13.0	0.0	21.8	26	(-)
	M368	28.7	0.5	1.0	30	(-)
	M397	19.4	0.0	25.2	35	(-)
	M400	15.0	0.1	34.9	36	(-)

	M393	21.3	0.6	30.2	40	(-)
	M366	25.4	2.0	30.0	45	(-)
	M382	20.2	0.0	47.7	49	(-)
	M394	23.6	4.0	40.2	52	(-)
	M377	24.2	0.2	55.0	57	(-)
	M302	57.4	1.0	2.1	60	(-)
	M404	19.6	15.2	43.2	61	(-)
	M380	39.4	5.9	46.8	73	(-)
	M331	30	1	73	74	(-)
	M395	70.2	16.0	0.0	86	(-)
	M381	112.2	15.1	0.0	127	(-)
	M336	144	15.337	2.779	161	(+)
	M365	144	15.3	3	161	(+)
	M303	71.7	1.8	153.4	166	(+)
	M363	65	5.3	160	167	(+)
	M374	162.9	7.7	0.0	171	(+)
	M362	79	3.9	175	188	(+)
	M371	160.0	8.0	52.2	199	(+)
	M354	138	5	99	202	(+)
	M358	112	0.3	176	218	(+)
	M351	135	55	122	263	(+)
	M305	143.3	3.9	203.9	270	(+)
Oysters	O324	0.0	0.0	0.0	0	(-)
	O540	2.5	0.0	0.0	3	(-)
	O543	3.9	0.0	0.9	4	(-)
	O416	3.8	0.0	2.0	5	(-)
	O542	10.2	0.0	2.3	12	(-)
	O519	25.8	0.3	0.0	26	(-)
	O518	15.0	0.0	28.5	32	(-)
	O517	19.0	1.4	28.1	37	(-)
	O500	20.0	1.6	31.2	40	(-)
	O523	25.8	11.0	14.7	46	(-)
	O422	24.7	0.0	41.5	50	(-)
	O506	31.7	14.4	18.8	57	(-)
	O418	32.0	0.0	48.7	61	(-)
	O529	54.7	10.2	5.4	68	(-)
	O406	39.6	18.0	23.5	72	(-)

	O504	16.0	0.0	105.6	79	(-)
	O404	20.0	0.0	132.0	99	(-)
	O532	55.7	8.1	72.8	107	(-)
	O526	98.3	18.0	0.0	116	(-)
	O514	65.7	26.1	46.5	120	(-)
	O525	122.4	8.5	9.9	137	(+)
	O424	74.8	11.3	88.9	139	(+)
	O510	140.8	1.8	0.0	143	(+)
	O535	69.2	1.8	158.2	166	(+)
	O428	93	37	66	170	(+)
	O536	159.2	13.2	3.5	175	(+)
	O530	78.9	2.2	182.8	191	(+)
	O412	86.3	7.0	211.4	220	(+)
	O513	127.7	2.4	199.9	250	(+)
	O524	207.3	7.9	200.9	336	(+)

5.2 Probability of Detection

Method

- Probability of Detection (POD) is the probability that a qualitative method will produce a positive result at a given analyte concentration.

- The study included 120 naturally contaminated mussel samples and 120 naturally contaminated oyster samples from different geographical locations and sampling periods. These include separate aliquots from the samples tested in the comparability study.

- For each Sample ID, 10–12 individual shellfish, equivalent to approximately 100 g of shellfish meat, were shucked, pooled, and homogenized. Each homogenate was divided into two portions: one was tested using DSP Alert, and the other was analyzed by LC-MS/MS to determine the total OA-group toxin concentration.

- For each matrix, the samples were divided into six concentration ranges, with 20 samples per range:
    - <20 µg OA eq/kg
    - 20–59 µg OA eq/kg
    - 60–109 µg OA eq/kg
    - 110–159 µg OA eq/kg
    - 160–199 µg OA eq/kg

- ≥ 200 µg OA eq/kg

- False-positive and false-negative results were recorded, and the sensitivity and specificity of DSP Alert were calculated using a reporting threshold of 135 µg OA eq/kg.

Results

- The POD results are summarized in Table 2, and the increase in the percentage of positive results with increasing OA-group toxin concentration is presented in Figure 1.

- All mussel and oyster samples containing <110 µg OA eq/kg produced negative results, while all samples containing ≥160 µg OA eq/kg produced positive results.

- Within the transition range of 110–159 µg OA eq/kg, 8/20 mussel samples (40%) and 13/20 oyster samples (65%) produced positive results. The combined positive rate for this range was 21/40 (52.5%).

- All 95 samples containing OA-group toxin concentrations at or above the reporting threshold of 135 µg OA eq/kg were correctly identified as positive, with no false-negative results. Six samples below the reporting threshold produced false-positive results, representing 2.5% of the 240 samples tested.

- DSP Alert demonstrated an overall sensitivity of 1.000, specificity of 0.959. Matrix-specific specificities were 0.960 for mussels and 0.957 for oysters.

- The results of the POD study samples and the individual OA, DTX1, and DTX2 profiles for each Sample ID are provided in Appendix 1 (Table S1).



Figure 1. Dose–response curves for DSP Alert, showing the percentage of positive results with increasing total OA-group toxin concentration for (a) mussel and oyster samples separately and (b) both matrices combined.

Table 2: Results of the POD study on Mussels and Pacific Oysters with DSP Alert.

Species	DST Level (ug OA eq/kg)	Number of Samples	Number of Positives	Percentage Positive Results
Mussels	< 20	20	0	0%
	21-59	20	0	0%
	60-109	20	0	0%
	110-160	20	8	40%
	161-199	20	20	100%
	≥ 200	20	20	100%
Oysters	< 20	20	0	0%
	21-59	20	0	0%
	60-109	20	0	0%
	110-160	20	13	65%
	161-199	20	20	100%
	≥ 200	20	20	100%
All Matrices	< 20	40	0	0%
	21-59	40	0	0%
	60-109	40	0	0%
	110-160	40	21	53%
	161-199	40	40	100%
	≥ 200	40	40	100%

5.3 Ruggedness

Method

- Ruggedness is the ability of a method to tolerate minor, deliberate procedural variations without meaningfully affecting qualitative results. The parameter evaluated was LFA development time prior to reading.

- Following ISSC guidance, OA-free mussel and oyster tissues were used to prepare at least 10 independent composite samples, each comprising homogenized tissue from 10–12 animals of the respective species.

- Multiple aliquots of each homogenate were prepared and spiked at above the conservative threshold of 135 µg OA eq/kg, using a mixture of OA (CRM-OA-d; Lot# 20141119), DTX1 (CRM-DTX1-c; Lot# 20191010), and DTX2 (CRM-DTX2-c; Lot# 20221102) certified reference materials (CRMs) purchased from the National Research Council Canada (NRCC).

- For each sample, one aliquot was tested using the standard DSP Alert procedure and read at the nominal development time of 10 minutes. Additional aliquots from the same homogenate were processed identically but read at deliberately shortened or extended times to reflect realistic operator variation. Each run-time deviation was evaluated separately, with all other procedural conditions held constant.

- The LFA development times evaluated were:
    - 10 minutes — standard LFA development time
    - 6 minutes
    - 8 minutes
    - 12 minutes
    - 14 minutes

- Aliquots for each development-time condition were further subdivided to provide at least three replicate analyses.

Results

- Results of the DSP Alert ruggedness trials, including kit lot and LFA development time, are summarized in Table 3. No differences were observed in the number of positive results across the development times evaluated (6–14 minutes) at any spike level, indicating consistent assay performance within ±4 minutes of the standard development time.

- The DST profile or toxin breakdown of these spiked samples are detailed in Appendix 1 (Table S2).

Table 3: Ruggedness trials for DSP Alert – effect of LFA run time.	

Species	**DST Concentration (ug OA eq/kg)**	**Sample ID#**	**Number of Replicates**	**Number of Positives at 6 min**	**Number of Positives at 8 min**	**Number of Positives at 10 min (SOP time)**	**Number of Positives at 12 min**	**Number of Positives at 14 min**
Mussels	147	M1	3	3	3	3	3	3
	161	M2	3	3	3	3	3	3
	166	M3	3	3	3	3	3	3
	167	M4	3	3	3	3	3	3
	169	M5	3	3	3	3	3	3
	172	M6	3	3	3	3	3	3
	185	M7	3	3	3	3	3	3
	188	M8	3	3	3	3	3	3
	199	M9	3	3	3	3	3	3
	200	M10	3	3	3	3	3	3
Oysters	141	O1	3	3	3	3	3	3
	147	O2	3	3	3	3	3	3
	158	O3	3	3	3	3	3	3
	161	O4	3	3	3	3	3	3
	166	O5	3	3	3	3	3	3
	171	O6	3	3	3	3	3	3
	172	O7	3	3	3	3	3	3
	179	O8	3	3	3	3	3	3
	184	O9	3	3	3	3	3	3
	192	O10	3	3	3	3	3	3

5.4 Cross-reactivity

Method

- For LFA-based methods, cross-reactivity describes the relative assay response to individual analytes within the targeted toxin class. This study evaluated the response of DSP Alert to OA, DTX1, and DTX2, using OA as the reference toxin.

- Mussel tissue confirmed to be free of OA-group toxins was used to prepare representative homogenates, each comprising tissue from a minimum of 10–12 mussels. Aliquots of the homogenates were divided into three groups and fortified separately with OA, DTX1, or DTX2 CRMs obtained from NRCC. Calculated amounts of each toxin were added to produce a concentration series ranging from 0 to 250 nM. A non-fortified aliquot served as the corresponding blank for each group.

- The following CRMs were used:
    - OA: CRM-OA-d; Lot# 20141119
    - DTX1: CRM-DTX1-c; Lot# 20191010
    - DTX2: CRM-DTX2-c; Lot# 20221102

- Each fortified sample was further subdivided to provide three replicate analyses at each concentration. All samples were processed and tested according to the DSP Alert procedure.
- The T/C ratio obtained at each concentration was normalized relative to the corresponding blank sample (0 nM). Dose–response curves were generated by plotting the normalized assay response against the molar concentration of each toxin, allowing their responses to be compared on an equivalent molar basis.

- IC_{50} values were obtained from the dose–response curves (19). Cross-reactivity was calculated relative to OA using the following equation:
    - Cross-reactivity (%) = [IC_{50} (OA) / IC_{50} (toxin)] × 100

Results

- The DSP Alert cross-reactivity results are summarized in Table 4, and the corresponding dose–response curves are presented in Figure 2.

- Relative to OA (100% by definition), cross-reactivity was 25.5% for DTX1 and 71.0% for DTX2. These results demonstrate that DSP Alert responded to all three OA-group toxins, although the magnitude of the response differed among the analytes.

- DTX2 showed a response more closely comparable to OA than DTX1. The lower relative cross-reactivity of DTX1 indicates that a higher molar concentration was required to produce the same degree of inhibition as OA.

Table 4: Cross-reactivity data for DSP Alert using Okadaic acid, DTX1, and DTX2.

Group	Cross reactivity
OA	100.0%
DTX1	25.5%
DTX2	71.0%

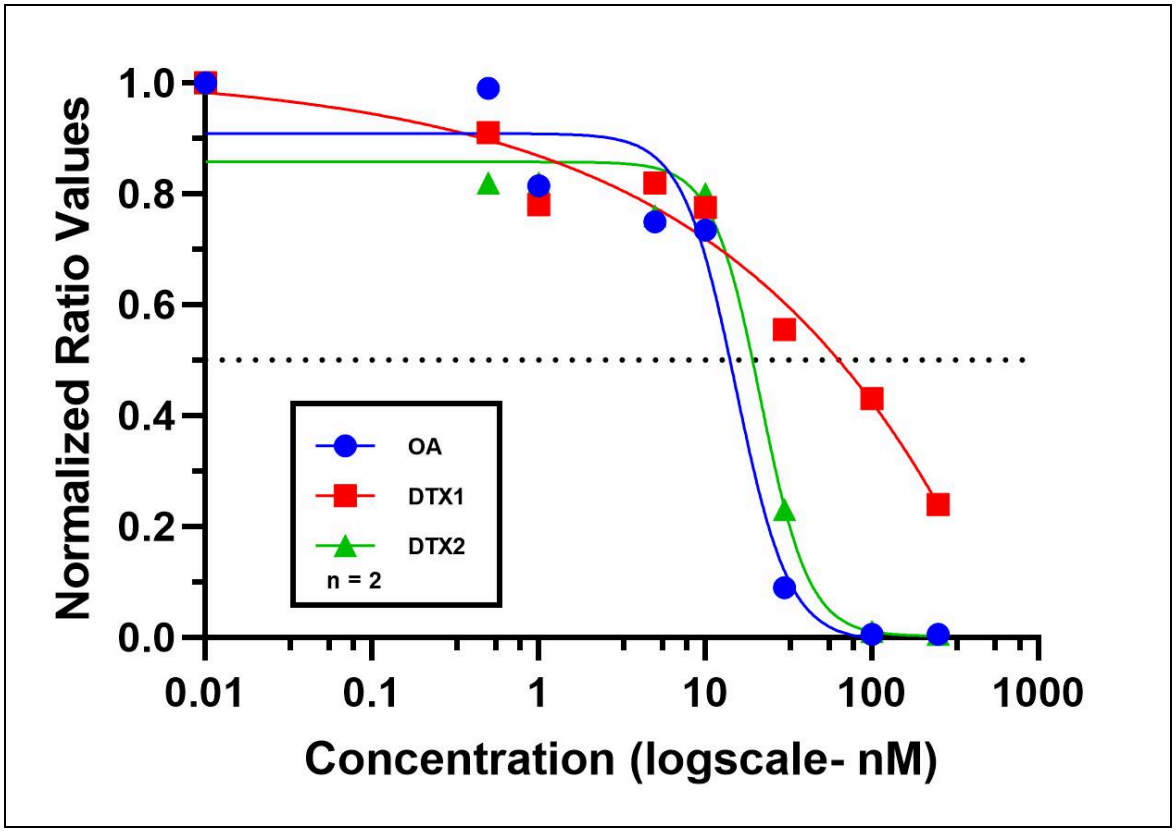


Figure 2: Cross-reactivity data obtained with various DSP toxins using DSP Alert.

5.5 Selectivity or Interference study with potentially interfering toxins

Method

Selectivity was evaluated to determine whether non-target marine and aquatic biotoxins produced positive responses in the DSP Alert LFA.

A total of 28 samples were prepared from 14 mussel and 14 oyster homogenates containing non-detectable levels of OA-group toxins, as confirmed by LC-MS/MS. These comprised 14 samples spiked with non-target toxins and 14 samples spiked exclusively with OA, with 7 mussels and 7 oysters in each group.

5.5.1 Preparation of samples containing potentially interfering toxins

- The following reagents were used for the interference/selectivity study.
    - OA toxin CRMs and calibration standards for OA (CRM-OA-d; Lot #20141119), DTX1 (CRM-DTX1-c; Lot #20191010), and DTX2 (CRM-DTX2-b; Lot #20150819) were obtained from NRCC.
    - Reference materials used for selectivity testing included an azaspiracid-1 (AZA1)-positive reference material (AZA Pos C; OEM #CRM-02-AZA1) obtained from Greyhound Chromatography, as well as a paralytic shellfish toxin Laboratory

Reference Material (PSP LRM), a cyanotoxin Laboratory Reference Material (Cyanotoxin LRM), and a domoic acid (DA) reference material (ASP K1) obtained from the Centre for Environment, Fisheries and Aquaculture Science (CEFAS).
    - The PSP LRM contained saxitoxin (STX), neosaxitoxin (NeoSTX), gonyautoxins 1–4 (GTX1–4), and the N-sulfocarbamoyl toxins C1 and C2 and also sourced from CEFAS. The Cyanotoxin LRM (CEFAS) contained a mixture of microcystin (MC) congeners, primarily leucine–arginine (LR), arginine–arginine (RR), tyrosine–arginine (YR), leucine–phenylalanine (LF), and leucine–tryptophan (LW).
    - The tetrodotoxin (TTX) CRM (CRM-TTX; Lot #20170328) was obtained from NRCC. Individual CRMs for several paralytic shellfish toxins (PSTs), including STX (CRM-STX-g; Lot #20201215), NeoSTX (CRM-NEO-d; Lot #20170411), GTX1&4 (CRM-GTX1&4-e; Lot #20211027), GTX2&3 (CRM-GTX2&3-d; Lot #20170419), and C1&2 (CRM-C1&2-c; Lot #20201208), were also obtained from NRCC.

- Samples S1–S7 consisted of mussel homogenates, and samples S8–S14 consisted of oyster homogenates. For each sample, 2.5 g of homogenate was weighed and spiked with a calculated volume of the designated reference material to achieve the required final concentration.

- Three PST preparations were evaluated, each at 1000 µg STX eq/kg: PSP LRM from CEFAS containing STX, GTX1–4, NeoSTX, and C1&2 (S1 and S8); a mixture of NRCC CRMs for STX, NeoSTX, GTX1&4, GTX2&3, and C1&2 (S2 and S9); and a mixture of NRCC standards for GTX1&4 and GTX2&3 only (S3 and S10). These preparations allowed evaluation across different PST profiles.

- The remaining samples contained 340 µg AZA1 eq/kg (S4 and S11), 430 µg MCs/g (S5 and S12), 2560 µg TTX/kg (S6 and S13), or 613 mg DA/kg (S7 and S14).

5.5.2 Preparation of OA controls

- Samples D1–D7 consisted of mussel homogenates spiked exclusively with OA, and samples D8–D14 consisted of oyster homogenates spiked exclusively with OA.

- Calculated volumes of CRM-OA-d (NRCC; Lot #20141119) were added to achieve concentrations of 115–667 µg OA eq/kg in mussels and 84–184 µg OA eq/kg in oysters.

- These target-analyte controls were analyzed alongside the samples containing non-target toxins using the DSP Alert LFA. Final toxin concentrations and qualitative results are presented in Table 5.

Results

- All 14 mussel and oyster samples containing non-target or potentially interfering toxins produced negative DSP Alert LFA results. No positive responses were observed with any of the three PST preparations or with AZA1, MCs, TTX, or DA at the concentrations evaluated.

- All eight OA-spiked samples containing ≥135 µg OA eq/kg produced positive results. Of the six samples containing <135 µg OA eq/kg, five produced negative results. One mussel sample containing 116 µg OA eq/kg (D4) produced a positive result, classified as a false positive relative to the 135 µg OA eq/kg reporting threshold.

- These findings support the selectivity of the DSP Alert LFA, with no cross-reactivity observed for the non-target toxins at the concentrations tested.

Table 5. Selectivity assessment of the DSP Alert™ LFA using mussel and oyster samples spiked with OA or non-target marine and aquatic biotoxins.

Species	Sample ID	Total DST Concentration (µg OA eq/kg)	Interfering Toxin	Interfering toxin concentration	Result
Mussels	S1	Non-detectable	PSP LRM: STX, GTX1–4, NeoSTX, C1&2	1000 µg STX eq/kg	Negative
	S2	Non-detectable	PSP mixture: STX, GTX1&4, GTX2&3, NeoSTX, C1&2	1000 µg STX eq/kg	Negative
	S3	Non-detectable	GTX mixture: GTX1&4, GTX2&3	1000 µg STX eq/kg	Negative
	S4	Non-detectable	AZA Pos C (90% AZA1)	340 µg AZA1 eq/kg	Negative
	S5	Non-detectable	Cyanotoxin LRM: MCs LR, RR, YR, LF, LW	430 µg MCs/g	Negative
	S6	Non-detectable	TTX CRM	2560 µg TTX/kg	Negative
	S7	Non-detectable	ASP K1	613 mg DA/kg	Negative
	D1	115	None	Not applicable	Negative
	D2	126	None	Not applicable	Negative
	D3	147	None	Not applicable	Positive
	D4	116	None	Not applicable	Positive
	D5	254	None	Not applicable	Positive
	D6	667	None	Not applicable	Positive

	D7	161	None	Not applicable	Positive
Oysters	S8	Non-detectable	PSP LRM: STX, GTX1–4, NeoSTX, C1&2	1000 µg STX eq/kg	Negative
	S9	Non-detectable	PSP mixture: STX, GTX1&4, GTX2&3, NeoSTX, C1&2	1000 µg STX eq/kg	Negative
	S10	Non-detectable	GTX mixture: GTX1&4, GTX2&3	1000 µg STX eq/kg	Negative
	S11	Non-detectable	AZA Pos C (90% AZA1)	340 µg AZA1 eq/kg	Negative
	S12	Non-detectable	Cyanotoxin LRM: MCs LR, RR, YR, LF, LW	430 µg MCs/g	Negative
	S13	Non-detectable	TTX CRM	2560 µg TTX/kg	Negative
	S14	Non-detectable	ASP K1	613 mg DA/kg	Negative
	D8	84	None	Not applicable	Negative
	D9	99	None	Not applicable	Negative
	D10	147	None	Not applicable	Positive
	D11	119	None	Not applicable	Negative
	D12	184	None	Not applicable	Positive
	D13	168	None	Not applicable	Positive
	D14	170	None	Not applicable	Positive

6. Quality Control

- Quality control (QC) testing for manufactured DSP Alert lots is performed at both the in-process and finished-product stages. For in-process QC, LFA cassettes are run using a blank diluent to confirm correct test and control line placement and acceptable line intensity.

- For finished-product release testing, DSP toxin–containing CRM mussel tissue (CRM DSP Mus-c, NRCC) is extracted, hydrolyzed and diluted to prepare equivalent DSP toxins levels of 0, 24, 40, 80, 135, 150, and 241 µg OA eq/kg. Each lot is then evaluated by testing the cassettes with these extracted QC samples (minimum of three replicates per level) to generate a dose–response curve. Lot acceptance criteria require that results between 0-100 µgOAeq/kg are negative and results at and above 160 µgOAeq/kg are all positive. Mixture of positive and negative results are expected between 100-135 µg OA eq/kg.

- QC standards are prepared as single-use aliquots and stored frozen to avoid repeated freeze–thaw cycles and to ensure consistent use of freshly prepared PSP toxin samples for each QC run.

7. Discussion

The present SLV study evaluated DSP Alert as a qualitative screening method for OA-group toxin detection in mussels and oysters. Performance was assessed through comparability, POD, ruggedness, selectivity, and cross-reactivity studies using naturally contaminated and spiked samples. The results characterized assay classification around the conservative reporting threshold of 135 µg OA eq/kg, as well as its tolerance to development-time variations and its selectivity.

Comparability and Probability of Detection

Comparability testing was performed using 60 naturally contaminated samples, comprising 30 mussel and 30 oyster samples, with OA-group toxin concentrations determined by LC-MS/MS. DSP Alert correctly classified all 21 samples containing concentrations at or above 135 µg OA eq/kg as positive and all 39 samples below this threshold as negative. No false-positive or false-negative results were observed, corresponding to an overall sensitivity and specificity of 1.00.

The POD study included 240 naturally contaminated samples, comprising 120 mussel and 120 oyster samples, including separate aliquots of samples evaluated in the comparability study. The proportion of positive results increased with OA-group toxin concentration. All samples containing <110 µg OA eq/kg produced negative results, while all samples containing ≥160 µg OA eq/kg produced positive results. Within the transition range of 110–159 µg OA eq/kg, positive results were obtained for 8/20 mussel samples (40%) and 13/20 oyster samples (65%), corresponding to a combined positive rate of 52.5%.

All 95 samples containing concentrations at or above the reporting threshold of 135 µg OA eq/kg were classified as positive, with no false-negative results. Six samples below the reporting threshold produced false-positive results, representing 2.5% of the complete POD dataset. Overall sensitivity, specificity, and agreement were 1.000, 0.959, and 0.975, respectively. Matrix-specific specificities were 0.960 for mussels and 0.957 for oysters. These findings demonstrated consistent detection above the reporting threshold, with mixed classifications confined to the concentration range approaching that threshold.

Ruggedness

Ruggedness testing assessed whether deliberate variations in LFA development time affected qualitative classification. Ten mussel and ten oyster homogenates were fortified with mixtures of OA, DTX1, and DTX2 at concentrations above the reporting threshold. Each sample was tested

in triplicate at development times of 6, 8, 10, 12, and 14 minutes, with 10 minutes representing the standard procedure.

All samples produced positive results at every development time evaluated. Consistent classifications were obtained across concentrations of 147–200 µg OA eq/kg in mussels and 141–192 µg OA eq/kg in oysters. These findings indicated that a ±4-minute variation from the standard development time was tolerated for the positive samples under the conditions tested. The assessment did not establish the effect of development-time variation on samples below the reporting threshold.

Interference or Selectivity and Cross-reactivity

Selectivity testing evaluated whether non-target marine and aquatic biotoxins produced positive DSP Alert results in mussel and oyster homogenates containing non-detectable levels of OA-group toxins. All 14 samples fortified with PST preparations, AZA1, MCs, TTX, or DA produced negative results. The three PST preparations represented different toxin profiles and were each tested at 1000 µg STX eq/kg. The remaining toxins were evaluated at 340 µg AZA1 eq/kg, 430 µg MCs/g, 2560 µg TTX/kg, and 613 mg DA/kg.

Among the OA-spiked controls, all eight samples containing ≥135 µg OA eq/kg produced positive results. Five of the six samples below this threshold produced negative results, while one mussel sample containing 116 µg OA eq/kg produced a false-positive result. These findings supported assay selectivity, with no cross-reactivity observed against the non-target toxins at the concentrations tested. Because these toxins were evaluated separately from OA, the study did not establish whether their presence could affect OA detection in samples containing both target and non-target toxins.

Cross-reactivity testing separately evaluated the relative response of DSP Alert to OA, DTX1, and DTX2 on an equivalent molar basis. Relative to OA at 100%, cross-reactivity was 25.5% for DTX1 and 71.0% for DTX2. The assay therefore responded to all three OA-group toxins, although higher molar concentrations of DTX1 and DTX2 were required to produce the same degree of inhibition as OA.

These differences indicate that toxin composition can influence assay response. Nevertheless, no false-negative results were observed among naturally contaminated samples at or above the reporting threshold in the comparability and POD studies. This supports performance across the toxin profiles represented in the evaluated samples, while additional evaluation of DTX1-dominant samples would help characterize the implications of its lower relative response.

Suitability for ISSC Adoption

DSP Alert provided rapid qualitative detection of OA-group toxins in mussels and oysters using a standard LFA development time of 10 minutes. All naturally contaminated samples containing ≥135 µg OA eq/kg were detected as positive in both the comparability and POD studies. The 240-sample POD study demonstrated a concentration-dependent response, with no

false-negative results, false positives representing 2.5% of samples, a sensitivity of 1.000, and a specificity of 0.959.

Ruggedness testing demonstrated consistent positive classifications across development times of 6–14 minutes. Selectivity testing showed no positive responses to the non-target toxins evaluated, while cross-reactivity testing confirmed responses to OA, DTX1, and DTX2, with differing relative sensitivities.

Based on the data presented, DSP Alert performed consistently under the conditions evaluated and may be suitable for consideration by ISSC as a rapid qualitative screening method for OA-group toxins in mussels and oysters.

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Appendix 1

Table S1: Summary of results of POD study with DSP Alert on mussels and oyster samples, along with DST profile or toxin breakdown.

Matrix	Sample ID	OA	DTX1	DTX2	Total DST Concentration (ug OA eq/kg)	Qualitative Result
Mussels	M312	0.0	0.0	0.0	0.0	(-)
	M512	0.0	0.0	0.0	0.0	(-)
	M513	0.0	0.0	0.0	0.0	(-)
	M514	0.0	0.0	0.0	0.0	(-)
	M524	0.0	0.0	0.0	0.0	(-)
	M324	0.0	0.0	0.0	0.1	(-)
	M341	0.0	0.0	0.0	0.1	(-)
	M399	0.6	0.0	0.0	0.6	(-)
	M390	0.7	0.0	0.0	0.7	(-)
	M376	1.0	0.0	0.0	1.0	(-)
	M511	3.8	0.0	0.0	3.8	(-)
	M324_2	4.5	0.0	0.0	4.5	(-)
	M341_2	5	0.0	0.0	4.9	(-)
	M526	5.4	0.0	0.0	5.4	(-)
	M-s1	5.4	0.0	0.0	5.4	(-)
	M525	6.7	0.0	0.0	6.7	(-)
	M340	16	0.0	0.0	16.0	(-)
	M340-2	17	0.0	0.0	17.2	(-)
	M402	4.0	0.6	22.0	17.8	(-)
	M392	7.9	0.0	16.8	18.0	(-)
	M406	3.0	0.0	35.0	24.0	(-)
	M378	13.0	0.0	21.8	26.1	(-)
	M401	12.0	0.0	28.0	28.8	(-)
	M368	28.7	0.5	1.0	29.8	(-)
	M403	16.0	0.5	29.0	33.9	(-)
	M397	19.4	0.0	25.2	34.5	(-)
	M396	12.1	0.0	39.2	35.6	(-)
	M400	15.0	0.1	34.9	36.1	(-)
	M391	15.8	0.1	36.7	37.9	(-)
	M393	21.3	0.6	30.2	40.0	(-)
	M367	16.2	0.0	40.1	40.3	(-)
	M366	25.4	2.0	30.0	45.4	(-)
	M382	20.2	0.0	47.7	48.8	(-)

	M314	29.5	0.0	34.2	50.0	(-)
	M394	23.6	4.0	40.2	51.7	(-)
	M339	36	0.0	28.1	53.1	(-)
	M383	23.9	0.6	51.1	55.2	(-)
	M527	31	0.0	42.3	56.0	(-)
	M377	24.2	0.2	55.0	57.4	(-)
	M528	38	0.0	34.9	59.0	(-)
	M302	57.4	1.0	2.1	59.6	(-)
	M339_2	45	0.0	26	60.6	(-)
	M404	19.6	15.2	43.2	60.8	(-)
	M379	29.3	0.9	54.4	62.8	(-)
	M321	60.0	2.4	6.5	66.3	(-)
	M327	53	0.0	23	66.9	(-)
	M380	39.4	5.9	46.8	73.4	(-)
	M331	30	1	73	73.7	(-)
	M349	64	0.0	17	74.2	(-)
	M321_2	59.2	3.9	19.2	74.6	(-)
	M331_2	30	0.0	79	77.0	(-)
	M301	35.6	0.0	76.3	81.3	(-)
	M369	35.9	0.9	76.7	82.8	(-)
	M395	70.2	16.0	0.0	86.2	(-)
	M300	50.8	0.0	64.4	89.4	(-)
	M333	59	36	0.0	95.0	(-)
	M327_2	84	0.0	19	95.0	(-)
	M405	69.1	0.0	48.1	98.0	(-)
	M319	45.2	0.0	101.2	105.9	(-)
	M333_2	79	29	0.0	108.7	(-)
	M600	47.8	1.2	102.3	110.3	(-)
	M610	51.2	3.3	98.3	113.6	(-)
	M608	110.0	4.7	0.0	114.7	(-)
	M612	45.4	3.7	111.2	115.9	(-)
	M615	49.4	2.5	109.5	117.5	(-)
	M325	40.5	0.0	128.4	117.5	(-)
	M613	113.0	12.3	2.4	126.8	(+)
	M381	112.2	15.1	0.0	127.2	(-)
	M606	112.2	15.1	0.0	127.2	(-)
	M313	49.2	0.0	132.3	128.6	(-)
	M315	64.2	0.0	113.7	132.4	(+)
	M609	59.8	3.9	114.7	132.5	(-)
	M616	56.5	2.8	125.1	134.3	(-)
	M360	72	1.9	102	134.8	(-)
	M602	71.7	1.9	102.0	134.8	(+)
	M325_2	52.4	0.0	148.2	141.3	(+)

	M614	129.2	14.1	2.7	144.9	(+)
	M607	128.2	17.2	0.0	145.4	(+)
	M316	89.3	5.0	89.2	147.8	(+)
	M370	71.7	1.9	143.2	159.5	(+)
	M336	144	15.337	2.779	161.3	(+)
	M365	144	15.3	3	161.3	(+)
	M330	156	6	0.0	161.4	(+)
	M388	107.6	2.3	0.0	162.0	(+)
	M330_2	158	0.0	9	163.8	(+)
	M303	71.7	1.8	153.4	165.5	(+)
	M363	65	5.3	160	166.8	(+)
	M364	71	3.5	158	169.2	(+)
	M611	149.9	18.2	3.8	170.4	(+)
	M374	162.9	7.7	0.0	170.6	(+)
	M605	162.9	7.7	0.0	170.6	(+)
	M603	95.6	2.6	136.0	179.7	(+)
	M361	73	5.9	178	185.4	(+)
	M318	181.3	3.0	3.7	186.5	(+)
	M362	79	3.9	175	188.0	(+)
	M320	78.4	0.0	183.2	188.3	(+)
	M398	102.3	0.0	43.2	193.0	(+)
	M350	148	5	69	194.5	(+)
	M371	160.0	8.0	52.2	199.4	(+)
	M604	160.0	8.0	52.2	199.4	(+)
	M359	85	7.6	179	199.9	(+)
	M601	85.1	7.6	178.6	199.9	(+)
	M354	138	5	99	201.7	(+)
	M352	145	5	104	211.8	(+)
	M358	112	0.3	176	217.7	(+)
	M372	197.2	24.2	5.7	224.8	(+)
	M332	85	5	230	227.9	(+)
	M348	160	5	114	233.5	(+)
	M326	70.4	0.0	274.2	234.9	(+)
	M357	133	17.6	145	237.6	(+)
	M355	122	50	110	238.6	(+)
	M389	218.3	12.4	16.7	240.7	(+)
	M356	139	44.4	97	241.0	(+)
	M317	228.0	16.0	0.0	244.0	(+)
	M328	245	0.0	4	247.5	(+)
	M353	128	53	116	250.5	(+)
	M328_2	209	18	45	254.0	(+)
	M351	135	55	122	263.0	(+)
	M523	223.3	4.3	65.8	267.1	(+)

	M305	143.3	3.9	203.9	269.6	(+)
Oysters	O430	0.0	0.0	0.0	0	(-)
	O432	0.0	0.0	0.0	0	(-)
	O434	0.0	0.0	0.0	0	(-)
	O324	0.0	0.0	0.0	0	(-)
	O341	0.0	0.0	0.0	0	(-)
	O431	1.4	0.0	0.0	1	(-)
	O546	1.9	0.0	0.0	2	(-)
	O435	3	0.0	0.0	3	(-)
	O540	2.5	0.0	0.0	3	(-)
	O545	2.8	0.0	0.0	3	(-)
	O438	3.9	0.0	0.0	4	(-)
	O543	3.9	0.0	0.9	4	(-)
	O541	4.9	0.0	0.0	5	(-)
	O416	3.8	0.0	2.0	5	(-)
	O437	4.7	0.0	2.4	6	(-)
	O542	10.2	0.0	2.3	12	(-)
	O544	12.3	0.0	0.0	12	(-)
	O436	5.7	0.0	12.8	13	(-)
	O433	17.2	0.0	0.0	17	(-)
	O340	18	0.0	0.0	18	(-)
	O519	25.8	0.3	0.0	26	(-)
	O502	26.4	0.4	0.0	27	(-)
	O518	15.0	0.0	28.5	32	(-)
	O501	14.4	0.0	29.6	32	(-)
	O402	33.0	0.5	0.0	33	(-)
	O517	19.0	1.4	28.1	37	(-)
	O442	18.5	0.0	31.6	37	(-)
	O443	23.4	1.8	21.4	38	(-)
	O401	18.0	0.0	37.0	40	(-)
	O500	20.0	1.6	31.2	40	(-)
	O523	25.8	11.0	14.7	46	(-)
	O422	24.7	0.0	41.5	50	(-)
	O400	25.0	2.0	39.0	50	(-)
	O440	38.2	0.0	20.6	51	(-)
	O314	26.0	0.0	43.6	52	(-)
	O441	28.8	12.3	23.8	55	(-)
	O506	31.7	14.4	18.8	57	(-)
	O439	43.5	0.0	23.7	58	(-)
	O339	39	0.0	30.56	58	(-)
	O520	22.8	0.6	59.0	59	(-)
	O418	32.0	0.0	48.7	61	(-)
	O503	27.2	0.7	65.6	67	(-)

	O529	54.7	10.2	5.4	68	(-)
	O406	39.6	18.0	23.5	72	(-)
	O448	49.6	0.0	43.7	76	(-)
	O521	16.9	0.0	99.2	76	(-)
	O504	16.0	0.0	105.6	79	(-)
	O527	29.3	18.6	56.8	82	(-)
	O403	34.0	0.9	82.0	84	(-)
	O445	50.6	0.0	59.1	86	(-)
	O446	49.8	0.0	65.9	89	(-)
	O444	70.3	0.0	38.2	93	(-)
	O449	95.3	0.0	2.0	97	(-)
	O404	20.0	0.0	132.0	99	(-)
	O447	68.1	0.0	56.5	102	(-)
	O450	76.9	0.0	44.2	103	(-)
	O531	53.8	20.0	49.4	103	(-)
	O532	55.7	8.1	72.8	107	(-)
	O533	58.3	0.0	83.6	108	(-)
	O421	46.1	0.4	104.5	109	(-)
	O109	45.2	0.0	109.0	111	(-)
	O313	48.5	0.4	110.0	115	(-)
	O526	98.3	18.0	0.0	116	(-)
	O325	44.6	0.0	119.7	116	(+)
	O509	103.2	14.4	0.0	118	(-)
	O423	55.6	1.7	103.3	119	(-)
	O514	65.7	26.1	46.5	120	(-)
	O417	55.0	0.4	108.0	120	(-)
	O515	65.3	12.7	75.0	123	(+)
	O315	58.5	1.8	108.8	126	(+)
	O525	122.4	8.5	9.9	137	(+)
	O424	74.8	11.3	88.9	139	(+)
	O522	119.4	5.7	28.5	142	(+)
	O510	140.8	1.8	0.0	143	(+)
	O316	78.8	11.9	93.6	147	(+)
	O409	129.0	18.0	0.0	147	(+)
	O508	137.6	4.8	9.6	148	(+)
	O451	152	0.0	0.0	152	(+)
	O452	139	0.0	29	156	(+)
	O453	142.3	0.0	23.9	157	(+)
	O538	161	0.0	0.0	161	(+)
	O505	137.6	6.4	34.4	165	(+)
	O535	69.2	1.8	158.2	166	(+)
	O427	90	0	129	168	(+)
	O428	93	37	66	170	(+)

	O415	162.0	9.0	0.0	171	(+)
	O318	165.0	7.1	0.0	172	(+)
	O536	159.2	13.2	3.5	175	(+)
	O429	93	18	107	175	(+)
	O410	176.0	2.3	0.0	178	(+)
	O425	98	39	69	179	(+)
	O426	98	19	112	184	(+)
	O408	172.0	6.0	12.0	185	(+)
	O530	78.9	2.2	182.8	191	(+)
	O456	179.2	0.0	19.4	191	(+)
	O537	78.9	5.8	179.2	192	(+)
	O511	174.6	9.9	13.4	193	(+)
	O455	176.5	0.0	28.5	194	(+)
	O539	80	0.0	189	194	(+)
	O454	179.9	0.0	23.6	194	(+)
	O405	172.0	8.0	43.0	206	(+)
	O512	194.4	15.7	9.8	216	(+)
	O412	86.3	7.0	211.4	220	(+)
	O413	95.0	4.7	201.0	220	(+)
	O528	164.6	8.9	78.3	221	(+)
	O350	152	5	109	222	(+)
	O534	95.0	0.0	214.7	224	(+)
	O326	90.8	7.4	222.5	232	(+)
	O414	203.0	32.0	0.0	235	(+)
	O411	218.3	12.4	16.7	241	(+)
	O513	127.7	2.4	199.9	250	(+)
	O317	224.4	30.1	0.0	254	(+)
	O419	243.0	19.6	12.2	270	(+)
	O524	207.3	7.9	200.9	336	(+)
	O420	190.6	3.6	298.3	373	(+)
	O507	241.6	8.8	226.4	386	(+)
	O323	307.3	4.8	233.9	453	(+)
	O407	302.0	11.0	283.0	483	(+)
	O339	569	34.6	498.2	903	(+)
	O337	798	26.576	571.65	1167	(+)

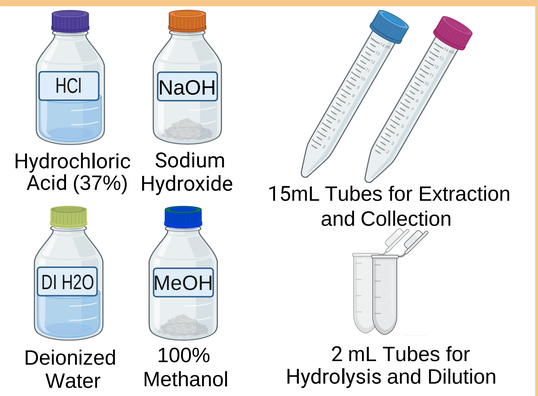
Table S2. DST profile or toxin breakdown of mussel spiked samples for Ruggedness study.					
Species	**Sample ID#**	**OA (ug /kg)**	**DTX1 (ug /kg)**	**DTX2 (ug /kg)**	**Total DST Concentration (ug OA eq/kg)**
Mussels	M1	78.8	11.9	93.6	147
	M2	144	15.337	2.779	161
	M3	71.7	1.8	153.4	166
	M4	65.4	5.3	160.2	167
	M5	71.1	3.5	157.6	169
	M6	165.0	7.1	0.0	172
	M7	72.7	5.9	178.0	185
	M8	79.0	3.9	175.1	188
	M9	89.7	5.8	172.1	199
	M10	85.1	7.6	178.6	200
Oysters	O1	50.8	0.0	150.4	141
	O2	78.8	11.9	93.6	147
	O3	149.0	8.5	0.0	158
	O4	161.2	0.0	0.0	161
	O5	69.2	1.8	158.2	166
	O6	162.9	7.7	0.0	171
	O7	165.0	7.1	0.0	172
	O8	98.0	39.0	69.4	179
	O9	97.5	19.0	112.0	184
	O10	78.9	5.8	179.2	192

DSP Alert

Version: DSPALERT_3.0

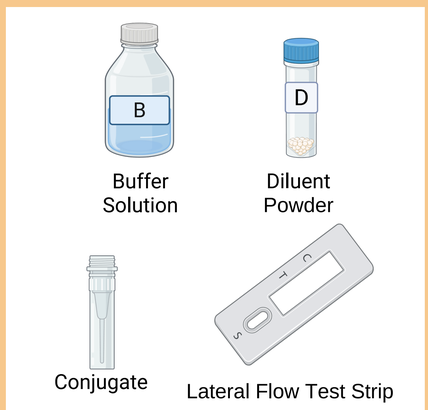
Rapid detection of Okadaic Acid in Shellfish and Molluscs

KIT COMPONENTS SUPPLIED BY CUSTOMER



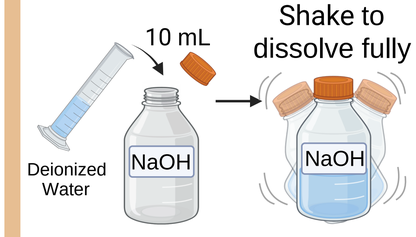
****Do NOT re-use any tubes for multiple samples due to a high risk of contamination. Dispose of the tubes after single use****

KIT COMPONENTS SUPPLIED BY SENSOREAL



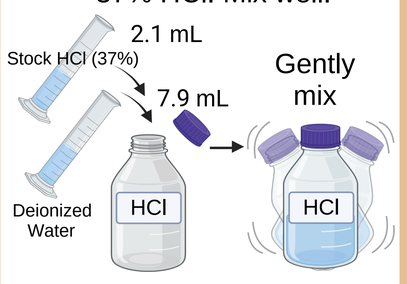
PREPARING SODIUM HYDROXIDE SOLUTION ('NaOH')

Label a bottle "NaOH". Dissolve 1g Sodium Hydroxide (NaOH) in 10 mL DI water.



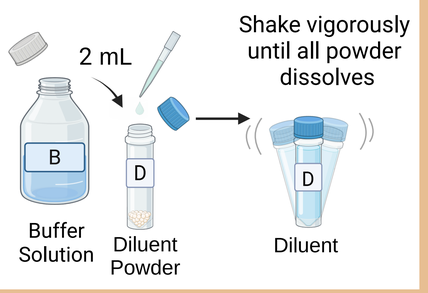
PREPARING HYDROCHLORIC ACID SOLUTION ('HCl')

Label a bottle "HCl". Add 7.9 mL DI water, then slowly add 2.1 mL 37% HCl. Mix well.



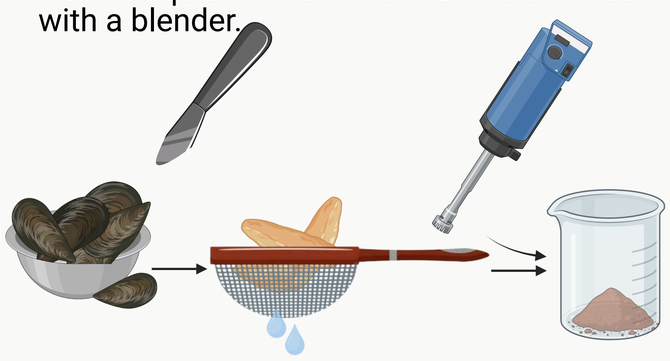
PREPARING THE DILUENT

Pipette 2 mL of Buffer Solution "B" and dispense into Diluent powder bottle "D"

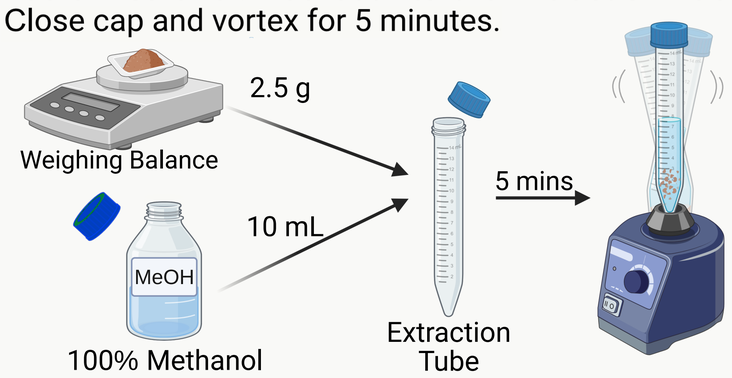


PART A: TOXIN EXTRACTION

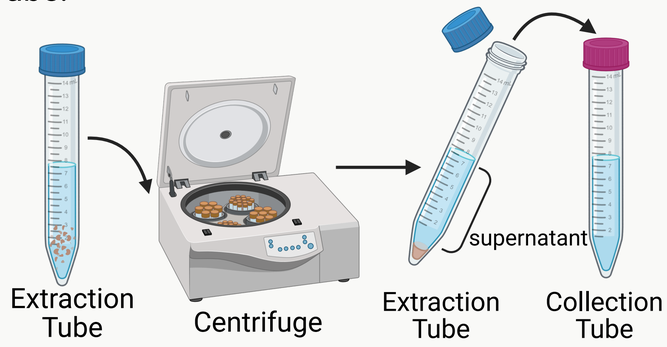
1. Shuck the shellfish. Use at least 100 g of meat as a representative sample. Strain excess liquid from shellfish. Blend the meat with a blender.



2. Weigh out **2.5 g of your sample** and **10 mL of 100% Methanol**. Add both to the Extraction Tube. Close cap and vortex for 5 minutes.

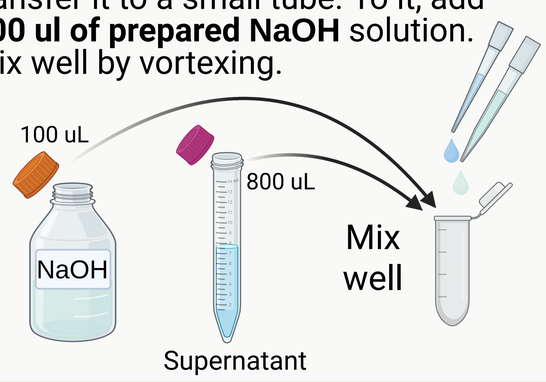


3. Centrifuge at 4000 rpm for 5 minutes. Transfer the supernatant into a Collection Tube.

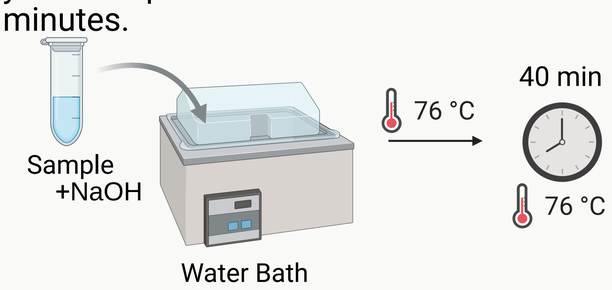


PART B: SAMPLE PREPARATION

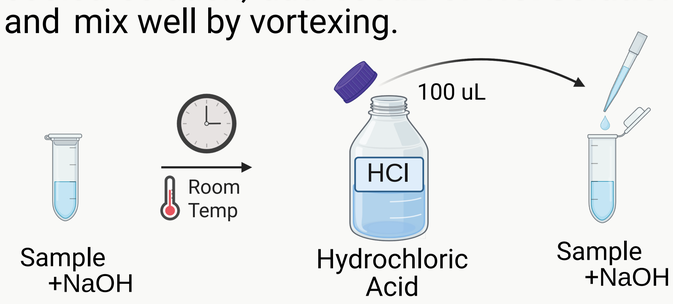
4. Take **800uL of the supernatant** and transfer it to a small tube. To it, add **100 ul of prepared NaOH** solution. Mix well by vortexing.



5. Prepare a water bath or a heating block. **Closely monitor the temperature with a thermometer**. Once it reaches 76°C, place your sample inside and set a timer for 40 minutes.

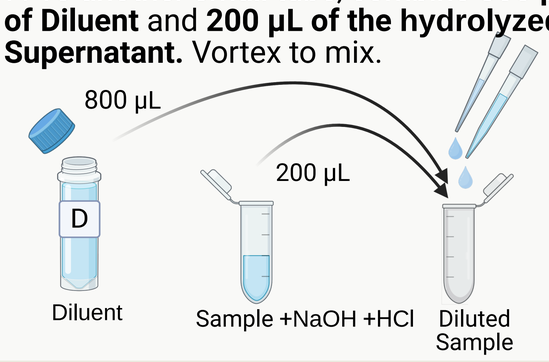


6. After 40 minutes, take the tube out and allow to cool to room temperature. To the cooled solution, add **100uL of HCl solution** and mix well by vortexing.

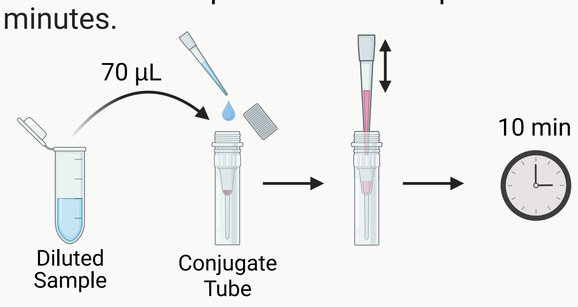


PART B: USING THE LFA

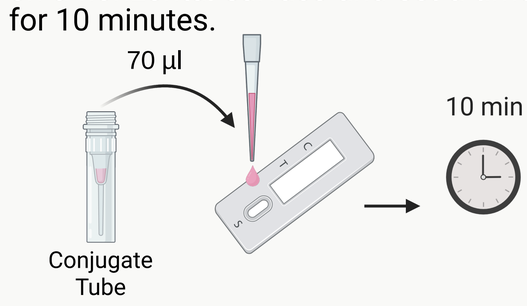
7. To another small tube, combine **800 µL of Diluent** and **200 µL of the hydrolyzed Supernatant.** Vortex to mix.



8. Add **70 µL of the diluted sample** to the Conjugate tube. Pipette up and down to dissolve. Let sit uninterrupted at room temperature for 10 minutes.

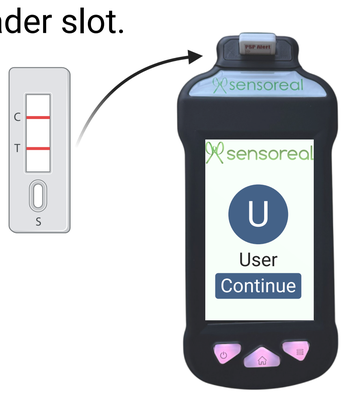


9. Add **70 µl of the Conjugate mix** to the test cassette. Leave the cassette on a horizontal surface and set a timer for 10 minutes.

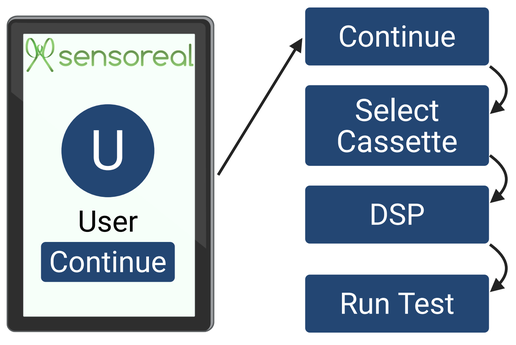


SENSOREAD - INTERPRETING THE RESULTS

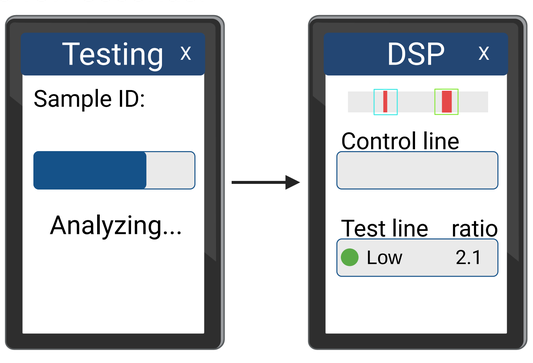
Insert the cassette into the reader slot.



Follow the steps on the screen.



Test results will be displayed after a few seconds.



Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Shannara Lynn
3. Affiliation	NOAA
4. Address Line 1	3209 Fredrick St
5. Address Line 2	
6. City, State, Zip	Pascagoula, MS 39567
7. Phone	(601)568-2524
8. Email	shannara.lynn@noaa.gov
9. Proposal Subject	Updating Glassware BTB Residue Testing Criteria to Follow Manufacturer Instructions.
10. Specific NSSP Guide Reference	Section IV. Guidance Documents – Chapter III. Laboratory @.01 Quality Assurance .01 (Laboratory Evaluation Checklist – Laboratory Quality Assurance Program) Specify Part I and line item 1.5.7 within that checklist.
11. Text of Proposal/ Requested Action	With each load of labware/glassware washed, ~~the contact surface of~~ several ~~dry~~ pieces from each load are tested for ~~residual~~ detergent residues (acid or alkali) with aqueous 0.04% bromothymol blue in accordance with manufacturer instructions. Results are recorded and records maintained.
12. Public Health Significance	Detergent residues remaining on laboratory glassware can inhibit microbial growth or interfere with chemical assays, potentially yielding false-negative or inaccurate analytical results in shellfish safety testing. Ensuring glassware is completely free of detergent residues is essential for public health
13. Cost Information	N/A
14. Changes Needed to Guidance (if proposed change is adopted)	No additional changes to the NSSP Guidance Documents are required. The proposed change in Field 11 directly updates line item 1.5.7 of the Laboratory Evaluation Checklist – Laboratory Quality Assurance Program in Section IV, Chapter III.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Growing Area Guidance Review and Update
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter IV. Growing Areas
11. Text of Proposal/ Requested Action	See supplemental document
12. Public Health Significance	Section IV. Chapter IV. Growing Area Guidance section has not been updated in some time. There are also sections of the guidance that are outdated and do not match the current NSSP MO. This proposal provides a suggested update for the Growing Area Guidance Chapter.
13. Cost Information	No change in cost.
14. Changes Needed to Guidance (if proposed change is adopted)	

Section IV. Chapter IV. Growing Areas

@.01 Sanitary Survey

.01 Sanitary Survey and the Classification of Growing Waters

NSSP Guidance Documents provide the public health principles supporting major components of the NSSP and its Model Ordinance, and summaries of the requirements for that component. NSSP Model Ordinance requirements apply only to interstate commerce although most ~~S~~states apply the requirements intrastate. For the most up to date and detailed listing of requirements, the reader should consult the most recent edition of the Model Ordinance.

~~Oysters, clams, mussels and scallops are filter feeders that pump large quantities of water through their bodies when actively feeding. During this process, molluscan shellfish can concentrate microorganisms, toxigenic micro-algae and poisonous or deleterious substances from the water column when they are present in the growing waters (Kennedy *et al.*, 1996). Concentrations in the shellfish may be as much as 100 times that found in the water column. If human pathogens are concentrated to an infective dose, and if the shellfish are consumed raw or partially cooked, human disease can result. If toxigenic micro-algae are present and producing toxin, human illness or death can occur, and cooking is not reliable as an effective barrier against intoxication.~~

~~The goal of the NSSP is to control the safety of shellfish for human consumption by preventing harvest from contaminated growing waters. In implementing this concept, the NSSP uses five (5) classifications for growing areas: approved, conditionally approved, restricted, conditionally restricted, and prohibited. The placement of a growing area in any one classification is based upon the growing area's conformance with the requirements established for that classification. Conformance with a classification's requirements is established through the sanitary survey.~~

~~The1ituate1e relationship between sewage pollution of shellfish growing areas and disease has been demonstrated many times (Rippey [a] and [b], 1994). Shellfish-borne infectious diseases are generally transmitted through a fecal-oral route (i.e., the shellfish become contaminated by sewage and are eaten by humans). The pathway can be quite circuitous. The cycle usually begins with fecal contamination of the growing waters. Feces deposited on land surfaces can release pathogens into surface waters via storm water runoff or collected wastes can be discharged directly into a waterway. The runoff or discharge may go directly into the growing area or indirectly, as is the case with wastes transported by freshwater streams to estuarine or marine waters. Information concerning the relationship between sewage pollution of bivalve shellfish growing areas and human disease is available in several good summaries (Hackney and Potter 1994 [a] and [b]; Jaykus *et al.*, 1994; Stelma and McCabe, 1990).~~

~~Epidemiological investigations of shellfish-caused disease outbreaks have found difficulty in establishing a direct cause and effect between a numerical correlation and pollution source strength, bacteriological quality of water, and the degree of hazard to health. Tidal fluctuations and pollution source variations contribute to a high degree of variability in water quality. Investigations made from 1914 to 1925 by the States and the Public Health Service, a period when disease outbreaks attributable to shellfish were more prevalent, indicated that typhoid fever or other enteric diseases would not ordinarily be attributed to shellfish harvested from water in which not more than fifty (50) percent of the one (1) cc (cubic centimeter) portions of water examined were positive for the coliform group (an MPN of approximately 70 per 100 ml), provided the areas were not subject to direct contamination with small amounts of fresh sewage which could not be detected by bacteriological examination.~~

~~Following the oyster-borne typhoid outbreaks during the winter of 1924-25 in the United States (Lumsden, 1925), the National Shellfish Certification Program, now the NSSP, was initiated by the States, the Public Health Service, and the shellfish industry (Frost, 1925). The 1925 criteria for safe growing areas were stated as: (1) the area is sufficiently removed from major sources of pollution so that the shellfish would not be subjected to fecal contamination in quantities which might be dangerous to the public health, (2) the area is free from pollution by even small quantities of fresh sewage, and (3) bacteriological examination does not~~

~~ordinarily show the presence of the coli- aerogenes group of bacteria in one (1) cc dilutions of the growing area water. The collective application of these criteria was known as the sanitary survey, which was used to determine if an area was safe for shellfish harvesting for human consumption. These criteria were adopted in the United States in 1925. Reliance on these criteria and others to measure excess variability in water quality were combined together with sanitary reconnaissance (shoreline survey), hydrographic and meteorological considerations, and patrol of closed harvest areas has generally proven effective in preventing major outbreaks of disease transmitted by the fecal-oral route. For a complete discussion of the history of the NSSP, see the historical overview by David Clem (1994) and the NSSP Guidance Document Guide Section IX., *History of the InterstateNational Shellfish Sanitation Program*.~~

~~The ability of shellfish to concentrate chemical pollutants from water and sediment can lead to accumulation of these poisonous and deleterious substances to levels that constitute a public health hazard (Kurland *et al*., 1960; Texas Dept. of Health, 1977). These poisonous or deleterious substances may enter shellfish growing areas through industrial or domestic waste discharges, seepage from waste disposal sites, agricultural land, geochemical reactions, or naturally occurring toxigenic micro-algae (O'Connor and Beliaeff, 1995; Liston, 1994). The degree to which these substances are concentrated depends upon such variables as the species of shellfish, water temperature and salinity, the level of contaminants in the waters, and the physiological conditions of the shellfish (Capuzzo, 1996; Roderick and Schneider, 1994; Rosijadi, 1996). The potential public hea'th hazard posed by these substances must also be considered in assessing the safety of shellfish growing areas.~~

~~For a full discussion of the public health risk associated with micro-toxigenic algae, see the NSSP Guidance Document, *Guidelines for Developing a Marine Biotoxin Contingency Plan Marine Biotoxin Control Guidance*.~~

Components of the Sanitary Survey

~~A review of epidemiological investigations of disease outbreaks attributable to the consumption of bivalve shellfish reveals that three (3) general situations occur in the contamination of growing areas placed improperly in the approved classification. First, improperly conducted or outdated sanitary surveys or misapplication of water quality data have unwittingly allowed harvesting from sewage contaminated growing areas. Second, fresh fecal material present and not diluted, diffused, or not detected by ordinary bacteriological sampling procedures caused shellstock contamination (Lumsden, 1925). Dr. Gurion recognized the possibility of chance contamination as early as 1902 in his report on a typhoid outbreak:~~

~~"There is a zone of pollution established by the mere fact of the existence of a populated city upon the banks of a stream or tidal estuary which makes the laying down of oysters and clams in these waters a pernicious custom if persisted in, because it renders these articles of food dangerous at times, and always suspicious (Gurion, 1917)."~~

~~Third, shellfish illnesses have been traced back to areas where an intermittent pollution source contaminated the shellfish. Some of these areas could have been placed in the conditionally approved classification and managed to avoid harvest of polluted shellstock, provided the occurrences of the sources of pollution could be predicted and the boundaries of their effects determined. For a full discussion of the use of the conditional classifications, see the NSSP Guidance Document, *Management Plans for Growing Areas in the Conditional Classifications* .~~

The first critical control point in preventing food-borne illness from shellfish consumption is identifying growing areas of acceptable sanitary quality. The completion of a sanitary survey is of paramount importance in making the distinction between acceptable and unacceptable growing areas and is the key to accurate growing area classification as approved, conditionally approved, restricted, conditionally restricted, or prohibited. A sanitary survey is not required to classify a growing area as prohibited but may help inform the decision to classify an area as prohibited. Under the NSSP Model Ordinance (MO), a sanitary survey is required for each growing area prior to its approval by the ~~S~~state as a source of shellfish for human consumption or as a source for shellfish to be used in a depuration or relay operation unless the shellstock are seed to be cultured as outlined in the NSSP MO Chapter VI. Shellfish Aquaculture @.02 Seed Shellstock. A sanitary survey is an in-depth evaluation of all environmental factors that have a bearing on the water quality in a shellfish growing area. The environmental factors include both actual and potential

pollution sources, whether natural or man-made, as well as meteorological, ~~and~~ hydrodynamic~~graphic~~, and geographic characteristics of the growing area. The principal components of a sanitary survey are (NSSP MO, Chapter IV. @.01 A.(1): (1) a shoreline survey to identify~~ication~~ and evaluate~~ion of the~~ pollution sources that may affect the areas, (2) ~~an evaluation of the meteorological factors, (3) an evaluation of hydrographic factors that may affect distribution of pollutants throughout the area,~~a survey of the microbiological ~~water~~ quality of the water, ~~and (4)~~(3) ~~an assessment of water quality~~ an evaluation of the effects of meteorological, hydrodynamic, and geographic characteristics on the growing areas, and (4) a determination of the appropriate growing area classification. ~~For a complete discussion of the sanitary survey, see *Sanitary Surveys of Growing Waters* (Garreis, 1994).~~

(1) ~~*An evaluation of the pollution sources that may affect the growing areas*~~ *A shoreline survey*.

A pollution source survey (also known as a shoreline survey) must be conducted of the growing area shoreline and watershed to locate direct discharges (e.g., municipal and private sewage and industrial waste discharges, sewage package treatment units, malfunctioning septic tanks and animal manure treatment lagoons) and non-point sources of pollution (e.g., storm water runoff, and runoff from agricultural and wildlife areas) that actually or potentially impact the growing area. Municipal and industrial wastewater treatment facilities should be evaluated in terms of actual loading versus design capacity, type and concentration of pollutants discharged, effectiveness of their treatment processes and pollution control devices. For additional information concerning sewage treatment plant discharges and their control, see the NSSP Guidance Documents Section IV. Chapter IV. @.03 .01, *Management Plans for Growing Areas in the Conditional Classification*~~s~~ and .03, *Classification of Shellfish Growing Waters Adjacent to Wastewater Treatment Plants* and the U.S. Environmental Protection Agency documents concerning increasing reliability of sewage treatment plants (USEPA [a] and [b], 1974).

The following survey procedures should be followed in the shoreline survey.

● Survey Assignment

Each shoreline survey area must be determined and assigned by the Authority. Each survey area must be identified by a unique designation, including all shoreline survey maps. All survey data must be identified by this unique designation that allows for tracking of all forms used in the survey. All shoreline survey data must be documented and filed promptly.

● Examination of Individual Properties for Pollution Sources

The boundaries of the shoreline survey area must be determined by an ~~in-field~~ investigation of the area topography and the proximity of individual properties to the growing area. Those properties ~~with the~~that have an actual or potential ~~to~~ impact to growing water quality must be included within the boundaries of the shoreline survey area. Once the boundaries of the shoreline survey area have been determined, all ~~businesses and residences must be examined and all potential discharges of wastes (raw sewage, kitchen wastes, laundry wastes, agricultural wastes, etc.) must~~ actual and potential pollution sources shall be evaluated in accordance with the requirements under NSSP MO Chapter IV. @.01 D.

Actual pollution source: A source found to be actively discharging, or that routinely and predictably discharges, into or adjacent to growing area waters. Examples include operating wastewater treatment plant outfalls, active stormwater discharge points, and occupied marinas or vessels that regularly discharge waste.

Potential pollution source: A source that is present in or near a growing area's watershed but is not currently discharging into or adjacent to growing area waters. Such sources have the capability to discharge under certain conditions, such as storm events, equipment failure, or increased human activity. Examples include inactive stormwater outfalls, unoccupied boat moorings, animal farms near the shoreline, and sewage lift stations subject to overflow during heavy rainfall.

Examples of both actual and potential pollution sources to evaluate may include, but are not limited to: wastewater discharges, sanitary, industrial, or agricultural sources, marinas, mooring areas, animal farms, flocks of waterfowl and populations of wild animals such as deer and muskrat (including an estimation of

the number of birds or animals), and drainage ditches. Also, include any other potential source of pollution which in the surveyor's opinion might influence water quality.

~~* The location of each property with a pollution source adversely impacting the growing area must be provided.~~

To meet the requirements under NSSP MO Chapter IV. @.01 D.(2)(d)(ii) to determine whether the pollution source has a direct or indirect impact, include one of the two notations listed below in the survey:

~~* If the property has a pollution source adversely impacting a growing area, one (1) of the two (2) notations listed below must be made concerning its impact on water quality.~~

a) Direct Impact: A pollution source having direct impact is defined as any waste discharge which has immediate impact on the growing area. An attempt should be made to quantify the volume of the discharge.
b) Indirect Impact: A pollution source having an indirect impact is defined as any waste discharge which reaches the growing area in a roundabout way. An attempt should be made to quantify the volume of the discharge.

~~* All sanitary, industrial, or agricultural pollution sources must be located on a map of the survey area.~~

~~* All animal farms must be evaluated. Evaluation must include the number and type of animals.~~

~~* All marinas must be evaluated in accordance with the requirements of the Model Ordinance.~~

~~* Notations must be made of any flocks of waterfowl and an estimation of their number given. Populations of wild animals such as deer and muskrat should be noted and where possible an estimation of their number given.~~

~~* Drainage ditches must be evaluated.~~

~~* Any other potential source of pollution, which in the surveyor's opinion might influence water quality, must be noted.~~

At the end of each shoreline survey, the ~~surveyor~~Authority must write a summation~~ The surveyor must also~~and provide a comprehensive map of the survey area identifying the location of each pollution source found. Use of a letter, number, and/or symbol to easily identify the pollution source on the map, such as an anchor symbol and the number "1" for the first marina listed in the survey, is recommended to ensure that the location of the pollution source on the map can be easily associated with the survey data for that source.

Note: For each growing area, the central file shall include "The sanitary survey reviewed and signed by the Sanitary Survey Officer" in accordance with NSSP MO Chapter IV. @.01 A.(3)(a). The Sanitary Survey Officer who reviews and signs the sanitary survey, including any shoreline surveys, can be different than the officials who performed the shoreline survey(s) and/or wrote the summation report(s).

The level of surveillance for poisonous and deleterious substances in a shellfish control program may vary widely. The intensity of the surveillance is frequently driven by a history of marine biotoxin contamination, sanitary survey findings, or findings from investigations by other ~~S~~state or ~~F~~federal agencies or academia. Review of existing background data derived from national and international monitoring programs can also be useful (O'Connor, 1996; Beliaeff *et al.*, 1997). An assessment of possible sources in the sanitary survey should enable shellfish control program managers to determine if a potential problem exists and whether a need for a ~~4ituater~~ future field study exists. Sampling for specific chemical contaminants in shellfish is recommended only when the pollution source survey or information from a reliable source reveals a potential problem, or if there is concern because of a lack of information.

When poisonous or deleterious substances are found in shellstock, the Authority must evaluate the levels that may be present against known tolerance levels in human foods or other appropriate information, and determine what action, if any, should be taken. Additional information concerning this topic can be found in the NSSP Guidance Documents: ~~*FDA Action Levels, Tolerances and Other Values*~~*Guidance Levels for Poisonous or Deleterious Substances in Seafood*; *Shellstock Relay*; and ~~*Guidance for Developing Marine Biotoxin Contingency Plans*~~ *Marine Biotoxin Control Guidance*. In the absence of specific poisonous or deleterious tolerance or action levels, decisions ~~must~~should be made on a case-by-case basis using the best available knowledge.

~~(2) *An evaluation of meteorological factors.* Climate and weather can affect the distribution of pollutants or can be the cause of pollutant delivery to a growing area. Prevailing winds can determine the distribution of pollutants in a growing area. Rainfall patterns and intensity can affect water quality through pollutant delivery in runoff or cause flooding which can affect the volume and duration of pollutant delivery. An example of the effects of meteorology occurred in 1982. In the late fall, the arrival of cold fronts caused strong winds, abnormally low tides and high rainfall which resulted in raw sewage bypasses from overloaded sewage treatment plants. This combination of meteorological events resulted in raw sewage reaching a growing area causing shellfish-borne illness in 471 persons (Casper, 1982).~~

~~(3) *An evaluation of hydrographic factors that may affect distribution of pollutants throughout the area.* Examples of hydrographic factors are tidal amplitude and type, water circulation patterns, and the amount of fresh water. These factors, along with water depths and stratification caused by density (salinity and temperature) differences, and wastewater and other waste flow rates are used to determine dilution, and time of transport. Tracer dye studies provide site-specific dilution, dispersion and time of travel information, and can be used in calibration of site-specific hydrodynamic models.~~

(2) ~~*An assessment of water quality.*~~ *A survey of the microbiological quality of the water.*

Microbiological water quality refers to both bacterial and viral pathogens and indicators. In general, microbial reduction in seawater occurs by two (2) different processes – physical dilution by advection and diffusion, and a process of biological inactivation. Dilution factors are physical and predictable with a direct relationship between pollution loads and dilution water available. The inactivation process is more variable and appears to be associated with the following factors: sunlight and solar radiation, absorption and sedimentation, temperature, predation, antibiosis, action of inorganic salts, nutrient deficiencies, the action of heavy metals and other substances, and effects of specific bacteriophage. Kator (1994) has provided a good summary of current knowledge concerning inactivation of bacteriological and chemical indicators caused by the effects of environmental factors.

~~Field and laboratory studies have demonstrated that enteric viruses can survive in marine water and shellfish from a few days to several months (Jaykus, 1994). In general, viruses survive longer at lower temperatures, at low salinity and when bound to sediments.~~

~~Evidence from many field studies indicates that a constant relationship does not exist between the bacterial pathogen, viral pathogen or coliform group levels in shellstock and the presence of these organisms in the overlying water column (Kator, 1994; Jaykus *et al*, 1994). Experience in the NSSP, however, has shown that shellstock from waters meeting the water quality standards for the approved classification are unlikely to be involved in shellfish-associated disease outbreaks attributed to fecal contamination of the growing area. In part, this is because the coliform group (total coliform) water quality standard of 70 MPN per 100 milliliters of growing water is equivalent to the fecal material contributed from one (1) person diluted in about 2.27×10^8 liters (8 million cubic feet) of water free from the coliform group. Such a small amount of sewage reaching the growing area is likely to have been so treated, diluted, or aged that it will be of negligible public health significance.~~

The NSSP ~~in its~~ M~~odel~~ O~~rdinance~~ allows for the Authority to classify a growing area using either a total coliform group or fecal coliform MPN standard as part of its sanitary survey. The two (2) standards are believed to afford the same level of public health protection (Hunt and Springer, 1974). The NSSP MO~~odel Ordinance~~ further allows the application of either standard to different water bodies within the S~~s~~tate. The NSSP MO~~odel Ordinance~~ also recognizes two (2) distinct water quality ~~monitoring strategies~~sampling

approaches to collect the total coliform group or fecal coliform monitoring data for application of the standards: the adverse pollution conditions sampling strategy and the systematic random sampling strategy are the two (2) alternative approaches. Either ~~Adverse pollution conditions are to be established~~ strategy can be used for initial classification~~, but if no point source pollution source impact is found the systematic random sampling monitoring strategy can be used for monitoring~~. The Authority may adopt the use of both the total coliform group and fecal coliform standards and both monitoring strategies, if applicable, for each standard.

The difference between the adverse pollution condition monitoring strategy and the systematic random sampling monitoring strategy is determined by ~~three (3)~~two (2) factors:

~~(1) The presence or absence of point source impact in the growing area;~~
(1) The timing of water sample collection; and
(2) The way in which the ~~MPN data~~sample statistics are calculated for comparison to the standard.

An adverse pollution condition (APC) is a state or situation, caused by meteorological, hydrological, or seasonal events or point source discharges that has historically resulted in elevated total coliform group or fecal coliform levels in a particular growing area. In using this monitoring strategy, sample collection ~~must~~should be timed to be representative of the major pollution impacts, since the number of microorganisms in shellfish ~~respond~~ rapidly ~~to an~~ increase with~~in~~ the number of microorganisms in their surrounding waters. The APC monitoring strategy ~~must be~~may be used in initial growing area classification to assess the impact by sewage treatment facilities, combined sewer overflows, or other point source discharges and to evaluate the impact of nonpoint pollution. The results of ~~bacteriological~~microbiological sampling must be correlated with sewage treatment plant operation and evaluated in terms of treatment and nonpoint pollution contributions at the time of sampling. These results, combined with considerations for malfunctions, overloads, poor operation, and nonpoint triggering conditions ~~are used in the initial classification~~can be used in classifying a growing area. It may be beneficial to periodically conduct a statistical analysis of the evaluation of effects, such as impacts from rainfall, increased river stage, temperature changes, salinity changes, etc. on fecal coliform or total coliform group levels to determine if, in fact, a correlation exists and if certain adverse pollution conditions tend to have a significant impact on a growing area. Where statistically significant correlations are weak, or are not strongly correlated, the Systematic Random Sampling (SRS) sampling strategy is preferred.

~~The systematic random sampling monitoring strategy can be used in approved or restricted growing areas except those that are affected by point source pollution. This~~ The SRS strategy assumes that monitoring conducted on a pre-established schedule at an adequate frequency will capture weather or rainfall conditions that trigger ~~nonpoint~~ pollution contribution. For a full discussion of this strategy, see the NSSP Guidance Document, *Systematic Random Sampling Monitoring Strategy*. This strategy is recommended in cases where APCs are not known to have an impact on a growing area or where the Authority would like to gather as much information as possible about impacts on a growing area under a variety of different conditions. In cases where states collect more than six (6) samples a year and/or have an extensive dataset using the SRS sampling strategy, it may be possible to extract data collected under adverse pollution conditions from the larger data set and to perform both an APC and an SRS analysis on the data to determine which strategy may be most appropriate for the area.

Total coliform group or fecal coliform data collected under either the APC or the ~~systematic random sampling~~ SRS monitoring strategy ~~are~~ may be reported as an MPN (i.e., a statistical estimate of the number of bacteria per unit volume of water) as determined from the number of positive results in a series of fermentation tubes used in a particular laboratory test. A complete discussion of the MPN test can be found in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1985). Data may also be reported using the membrane filter (MF) (membrane-Thermotolerant *Escherichia coli* [mTEC]) of the water sample results. In the APC monitoring strategy, the application of the two (2) part water quality standards for both total coliform group and fecal coliform involves use of a median or geometric mean and a "percentage factor". The "percentage factor" corrects for the inherent variation of the MPN or MF (mTEC) analytical method.~~when used with a normally distributed data set~~ In the ~~systematic random sampling~~SRS strategy, the application of the two (2) part water quality standards for both the total coliform group and fecal coliform involves use of a median or geometric mean and an estimated 90th percentile as the statistic to measure the variance of the data set. The use of the strategy requires that the times of samples be

scheduled in advance, so monitoring runs are made with no consideration for meteorological conditions. ~~For a more in-depth explanation,~~ Also, see the NSSP Guidance Document, *Systematic Random Sampling Monitoring Strategy*.

(3) An evaluation of the effect of any meteorological, hydrodynamic, and geographic characteristics on the growing area. ~~factors.~~

(a) *Meteorological*: Climate and weather can affect the distribution of pollutants or can be the cause of pollutant delivery to a growing area. Prevailing winds can determine the distribution of pollutants in a growing area. Rainfall patterns and intensity can affect water quality through pollutant delivery in runoff or cause flooding which can affect the volume and duration of pollutant delivery. An example of the effects of meteorology occurred in 1982. In the late fall, the arrival of cold fronts caused strong winds, abnormally low tides, and high rainfall which resulted in raw sewage bypasses from overloaded sewage treatment plants. This combination of meteorological events resulted in raw sewage reaching a growing area causing shellfish-borne illness in 471 persons (Casper, 1982). Areas susceptible to pollution during certain meteorological conditions may need larger prohibited or restricted areas adjacent to actual or potential pollution sources or may be conditionally managed. ~~*An evaluation of hydrographic factors that may affect distribution of pollutants throughout the area.*~~

(b) Hydrodynamic: Examples of hydrodynamic factors are tidal amplitude and type, water circulation patterns, and the amount of fresh water. These factors, along with water depths and stratification caused by density (salinity and temperature) differences, and wastewater and other waste flow rates are used to determine dilution, and time of transport. Tracer dye studies provide site-specific dilution, dispersion, and time of travel information, and can be used in calibration of site-specific hydrodynamic models.

(c) Geographic: Examples of geographic factors are land use, soil type, topography, impervious surfaces, runoff impacts, and septic systems. In areas known for run-off of pollutants from animal farms or impervious surfaces during rainfall events or known for tidal flooding (particularly in areas susceptible to failing septic tanks or sewage collection systems), consider using more restrictive classifications or conditional management strategies.

(4) A determination of the appropriate growing area classification.

A written sanitary survey report is needed to integrate the data from the shoreline ~~pollution source~~ survey, the survey of the microbiological quality of the water, and the ~~hydrographic and~~ meteorological, hydrodynamic, and geographic~~investigations~~ evaluations~~and the water sampling~~ into a comprehensive information analysis. The purpose of this analysis is to determine the appropriate classification for the growing area and the geographic boundaries of the classification. ~~This report must~~ ~~i~~Include a compilation of relevant data, ~~a water sample data analysis using appropriate data sorting to determine adverse pollution conditions and recognized statistical techniques~~ an appropriate analysis of water sampling data to evaluate the effects of various factors on the growing area, conclusions as to the appropriate growing area classification, and recommendations for necessary follow-up actions in the report. The report may also consider relevant resource management issues, legal statutes and regulations, patrol and enforcement considerations, and jurisdictional boundaries ~~social, economic, or political factors~~ that may influence the establishment of the classification boundaries and the time periods for the open and closed status when conditionally approved and conditionally restricted classifications are proposed. Pollution conditions that cause closure and conditions and time periods for seasonal openings must be included in ~~the~~ conditional area management plans.

Keeping the sanitary survey current consists primarily of routinely evaluating major pollution sources, collecting water quality data from sampling stations under the selected NSSP water quality monitoring strategy, and analyzing the data to assure that the classification continues to represent current sanitary conditions in the growing area. The sanitary survey must be repeated fully every twelve (12) years in accordance with NSSP MO Chapter IV. @.01 C.(1). In the interim, review the sanitary quality of each growing area ~~must be reviewed~~ as often as is necessary to ensure that the classification is appropriate. Certain sanitary survey components are required by ~~the Model Ordinance~~ NSSP MO Chapter IV. @.01 C. to be updated annually and triennially (every third year) but may be updated even more frequently, such as in cases where the Authority is alerted to a change in sanitary conditions. ~~The growing area must be~~

~~subjected promptly to a more intense and comprehensive sanitary survey reevaluation when monitoring or other information reveals a substantial change in the sanitary conditions. A reevaluation report is required and must include a determination as to whether a change in growing area classification is necessary~~

The Authority is required to collect and maintain survey data and information for each growing area in a centrally located file. This central file can be maintained in a computer system and/or in paper format as long as, all required files can be made readily available for review. Experience with the sanitary survey program for determining the appropriate classification for each growing area indicates a tendency to omit or de-emphasize some components of the sanitary survey unless a central ~~S~~state file of all sanitary survey reports, update information, and reevaluation reports is maintained. This is particularly true when responsibility for shellfish sanitation is divided between two (2) or more ~~S~~state agencies. Maintenance of a central ~~S~~state file also simplifies the appraisal of ~~S~~state programs by the FDA and prevents loss of useful historical data.

~~Minimum Requirements of~~ Recommended Outline for the Sanitary Survey Report

The following outline contains the minimum requirements and recommendations for the written growing area sanitary survey report required in the NSSP Model Ordinance.

I. Executive summary (recommended)

II. Description of growing area (recommended)

A. Description of area and its boundaries with map or maps (required, but can be stored in separate file)

B. History of growing area classification

III. Shoreline survey (required)

A. Written summary of the shoreline survey findings

B. Summary of sources and locations

1. Location of each actual or potential pollution source site on comprehensive map of the survey area

2. Table of sources cross-referenced to the survey area map

3. The boundaries, based on area topography, of each shoreline survey area which identifies only the properties with the potential to impact shellfish growing waters along with a unique designation for the area

NOTE: For each actual or potential source described in III. B – G, include evaluation of impact or potential impact on growing area, determination of distance to growing area, and designation as having direct or indirect impact on growing area. Reference III.A.2 table of sources.

C. Sewage treatment systems – Assessment of reliability and effectiveness of each system

1. Municipal sewage treatment systems

2. Smaller sewage treatment systems (individual property, subdivision, etc.)

3. Septic tanks

D. Poisonous or deleterious substances

1. Industrial sites (include any sampling data)

2. Agricultural sources (include any sampling data)

3. Other sources (include any sampling data)

4. Sampling data not associated with specific known sources

E. Animal sources

1. Domestic animals

2. Wildlife

F. Hydrological features

1. Stormwater drainage

2. Streams

G. Vessel related sources

1. Marinas

2. Mooring areas

3. Other vessel issues

H. Other sources

IV. Survey of the microbiological quality of the water (required)

A. Map of sampling stations

B. Sampling plan/approach (include an example of advanced scheduling for systematic random sampling – recommended)

C. Summary of sampling activity, including table showing total numbers of observations collected at and frequency for each station

V. Evaluation of effects of meteorological, hydrodynamic, and geographic characteristics (required) – see guidance above

VI. Classification determinations (required) (include references to II.A maps)

A. Harvest restriction based on considerations other than microbiological sampling results

1. Marinas, mooring areas, and other vessel issues (refer to III.F)

a. Conditionally approved area (include results of cleansing studies or plan for conducting cleansing studies on a case-by-case basis)

b. Restricted area to be used as a source of shellstock for relay only (include results of cleansing study or plan for conducting cleansing studies on a case-by-case basis)

c. Conditionally restricted area to be used as a source of shellstock for relay only (include results of cleansing studies or plans to conduct cleansing studies on a case-by-case basis)

d. Prohibited area

2. Wastewater treatment systems (refer to III.B)

a. Conditionally approved area (include results of cleansing studies or plans to conduct cleansing studies on a case-by-case basis)

b. Restricted area to be used as a source of shellstock for relay only (include results of cleansing study or plan for conducting cleansing studies on a case-by-case basis)

c. Conditionally restricted area to be used as a source of shellstock for relay only (include results of cleansing studies or plan to conduct cleansing studies on a case-by-case basis)

d. Prohibited area

3. Poisonous or deleterious substances (refer to III.C)

a. Conditionally approved area (include results of cleansing studies or plans to conduct cleansing studies on a case-by-case basis)

b. Restricted area to be used as a source of shellstock for relay only (include results of cleansing study or plan for conducting cleansing studies on a case-by-case basis)

c. Conditionally restricted area to be used as a source of shellstock for relay only (include results of cleansing studies or plan to conduct cleansing studies on a case-by-case basis)

d. Prohibited area

4. Other restrictions (refer to III.D, G, and/or E)

a. Conditionally approved area (include results of cleansing studies for reopening, if warranted)

b. Restricted area to be used as a source of shellstock for relay only (include results of cleansing study or plan for conducting cleansing studies on a case-by-case basis)

c. Conditionally restricted area (include results of cleansing studies or plan for conducting cleansing studies on a case-by-case basis).

d. Prohibited area

B. Classification associated with microbiological sampling

1. Approved area

a. Table showing application of microbiological standards to water quality sampling results

2. Conditionally approved area

a. Descriptions of conditions under which each conditionally approved area is expected to meet approved area microbiological standards along with closure criteria

b. Tables showing application of microbiological standards to data collected for conditions under which each conditionally approved area is expected to meet approved area microbiological standards

c. Results of cleansing studies or plans to conduct cleansing studies on a case-by-case basis along with re-opening criteria

3. Restricted area to be used as a source of shellstock for depuration

a. Table showing application of microbiological standards to water quality sampling results

b. Results of cleansing studies or plans to conduct cleansing studies on a case-by-case basis

4. Conditionally restricted area restricted area to be used as a source of shellstock for depuration when in open status

a. Descriptions of conditions under which each conditionally restricted area is expected to meet restricted microbiological standards along with closure criteria

b. Tables showing application of microbiological standards to data collected for conditions under which each conditionally restricted area is expected to meet restricted area microbiological standards

c. Results of cleansing studies or plans to conduct cleansing studies on a case-by-case basis

~~A. Executive Summary~~
~~B. Description of Growing Area~~
~~(1) Location map or chart showing growing area~~
~~(2) Description of area and its boundaries~~
~~(3) History of growing area classification~~
~~* Date of last sanitary survey~~
~~* Previous classification(s) map(s)~~
~~C. Pollution Source Survey~~
~~(1) Summary of Sources and Location~~
~~* Information gathered under the shoreline survey procedures outlined above.~~
~~* Map or chart showing the location of major sources of actual or potential pollution in the survey area.~~
~~* Table of sources of pollution cross-referenced to the survey area map.~~
~~(2) Identification and evaluation of pollution sources~~
~~* Domestic wastes (discussion and maps)~~
~~* Storm water~~
~~* Agricultural waste (farms, feedlots, & slaughterhouse operations)~~
~~* Wildlife areas~~
~~* Industrial wastes~~
~~D. Hydrographic and Meteorological Characteristics~~
~~(1) Tides (type and amplitude), and currents (velocity and direction)~~
~~(2) Rainfall~~
~~* Amount~~
~~* When (e.g. time of year)~~
~~* Frequency of significant rainfalls~~
~~* Winds (Seasonality and effects on pollution dispersion)~~
~~(3) River discharges (volume and seasonality)~~
~~(4) Discussion concerning effects of pollution distribution and hydrographic factors (dilution, dispersion, and time of travel) on water quality throughout the growing area~~
~~* Salinity, depth, and stratification characteristics~~
~~* Computer model verification if used for classification.~~
~~E. Water Quality Studies~~
~~(1) Map of sampling stations~~
~~(2) Sampling plan and justification~~
~~* Adverse condition sampling~~
~~* Random sampling~~
~~(3) Sample Data Analysis and Presentation: Tables containing the basic NSSP statistics (number of samples, median or geometric mean, and the respective variability factors)~~
~~* Station by station monitoring data array collected under the adverse condition or systematic random sampling monitoring strategy~~
~~* Daily sampling results and number of samples collected for survey~~
~~* Overall compliance with NSSP criteria~~

~~* Sorting of data by environmental pollution condition~~
~~* Classification assigned to each station~~

~~F. Interpretation of Data in Determining Classification to Be Assigned to Growing Area: A discussion of how actual or potential pollution sources, wind, tide, rainfall, etc. affect or may affect water quality, that will address the following:~~

~~(1) Effects of meteorological and hydrographic conditions on bacterial loading~~
~~(2) Variability in the bacteriological data and causes~~

~~G. Conclusions~~

~~(1) Map or chart showing classification assigned to growing area(s) (closure lines, boundary lines separating various classifications)~~
~~(2) Legal description of growing area boundaries~~
~~(3) Management plan for growing area if in the conditionally approved or conditionally restricted classification~~
~~(4) Recommendations for sanitary survey improvement~~
~~* Changes in monitoring schedules, addition of sampling stations or station relocation, etc.~~
~~Comments~~

Growing Area Classifications

As a result of the information gathered during the sanitary survey, the Authority should be able to distinguish those growing areas suitable for harvest of shellstock for direct human consumption, those growing areas where the shellfish will require treatment to make them safe for human consumption, and those growing areas unsuitable to harvest for human consumption unless they are seed to be cultured as outlined in the NSSP MO Chapter VI. Shellfish Aquaculture @.02 Seed Shellstock. The probable presence or absence of pathogenic microorganisms, marine biotoxins, or other poisonous or deleterious substances in growing area waters is important to the Authority in deciding how the shellfish obtained from the growing area should be used. The Authority's decision, based on the sanitary survey information, will place all actual and potential growing areas in one (1) of the five (5) possible NSSP growing area classifications.

The five (5) growing area classifications are approved, conditionally approved, restricted, conditionally restricted and prohibited. Except for an emergency situation such as conditions following a hurricane when a growing area in the approved classification may be placed temporarily in the closed status, a growing area in the approved classification is always in the open status. The remaining four (4) growing area classifications all place some type of restriction on shellstock harvesting. For more information concerning the enforcement of these restrictions, see the NSSP Guidance Document, *Growing Area Patrol and Enforcement~~of Growing Area Restrictions~~*.

Growing areas are placed in the approved classification when the sanitary survey information and marine biotoxin surveillance data indicate that fecal material, pathogenic microorganisms, poisonous, or deleterious substances are not present in the growing area in unacceptable concentrations. Shellstock harvested from these growing areas may be sold directly to the public for consumption raw or cooked.

Use of the conditionally approved and conditionally restricted classifications by the Authority is optional. The conditional classifications are designed to address growing areas that are subject to intermittent microbiological pollution, poisonous or deleterious substances, and/or marine biotoxins. These classifications offer the Authority an alternative to placing the area in the restricted or prohibited classification year-round when, during certain times of the year or under certain conditions, the shellstock from the growing area may be safely harvested. The concept also applies to situations where conditions are acceptable for harvest when wastewater treatment plant operation is satisfactory, but not when a malfunction occurs. A management plan is required that describes the controls to provide public health protection in the use of the conditional classifications. For a full explanation of the conditional classifications and their use, see the NSSP Guidance Document, *Management Plans for Growing Areas in the Conditional Classification*.

A growing area may be placed in the restricted classification instead of the prohibited classification when the sanitary survey indicates a limited degree of pollution. This option may be used when the sanitary survey for the growing area indicates that the levels of fecal material or poisonous or deleterious substances

in the growing area are such that additional treatment through depuration or relay can render the shellstock safe for human consumption. A common situation in the use of the restricted classification occurs when a growing area is affected by non-point source pollution from either urban or rural sources. In this situation, the water quality fluctuates unpredictably or with sufficient frequency that the use of the conditionally approved classification is precluded. The Authority should use the restricted classification only when sufficient depuration or relay studies have been conducted to establish raw product quality requirements at the harvest level; and when the Authority has sufficient administrative and technical resources to properly administer this classification. These resources include monitoring of pollution sources; providing coordination between ~~S~~state, local, and industry officials; issuing special harvesting permits; and supervising the harvesting and transport of shellstock to relay sites or depuration facilities.

For a complete discussion of the supervision requirements at the harvest level, see the NSSP Guidance Document, *Shellstock Relay*.

Use of the restricted classification requires the Authority to develop the controls necessary to assure that the shellfish are relayed or depurated prior to consumption. ~~Bacteriological~~ Microbiological water quality standards are applied on a growing area specific basis when a restricted area is used as a source of shellstock for depuration. The criteria may vary according to the use to be made of the shellstock and the effectiveness of the relay or depuration process used to cleanse the shellstock. Process effectiveness is determined through a study, which establishes the fecal coliform density in the shellstock at the time of harvest, and the density that can be achieved at the completion of the process. Effectiveness of the process is likely to vary between growing areas used for natural cleansing treatment in relay operations and between depuration facilities used for controlled cleansing treatment. The species of shellstock may also affect the effectiveness of the relay or depuration process. For a complete discussion of relay, see the NSSP Guidance Document, *Shellstock Relay*.

A growing area is placed in the prohibited classification when the sanitary survey or marine biotoxin surveillance program indicates that fecal material, pathogenic microorganisms, poisonous or deleterious substances, marine biotoxins, or radionuclides are present or may reach the harvest area in excessive concentrations. The NSSP M~~odel O~~rdinance also requires that a growing area for which there is no sanitary survey be placed in the prohibited classification as a precautionary measure. If an area or areas are unclassified, they shall be treated as prohibited for NSSP purposes. Taking shellstock from a prohibited area for any human food purpose is not allowed except for the gathering of seed as outlined in the NSSP Model Ordinance Chapter VI. Shellfish Aquaculture @.02 Seed Shellstock. ~~or nursery culture for aquaculture or resource enhancement or the depletion of the areas classified as prohibited~~.

~~Depletion of prohibited areas can be an effective deterrent to illegal harvesting as it provides a safeguard against contaminated shellfish reaching the market and eliminates a temptation for harvesters. Depletion may be more economical and effective than patrol of prohibited areas in protecting public health. In a depletion operation, all market sized shellstock and as many of the smaller sized shellstock as can be gathered by reasonable methods are removed from the growing area. To be effective, depletion operations should be conducted at sufficient intervals to keep growing areas free of commercial quantities of market sized shellstock. When commercial harvesters conduct depletion operations, the Authority or the Patrol Authority must provide effective supervision.~~

The NSSP M~~odel O~~rdinance also requires that an area in the prohibited classification ~~(closed safety zone)~~ must be established between any sewage treatment plants or other waste discharges of public health significance and any growing area placed in the approved, conditionally approved, restricted, or conditionally restricted classification. The size of the prohibited area should be based on the effectiveness and level of sewage treatment; the location of the shellstock resource that would be affected; the classification of adjacent waters;~~,~~ the travel time of the discharge based on its velocity and the total response time (as an example: the time it would take for the person responsible for the operation of the sewage treatment facility to detect a failure and notify the Authority~~;~~ plus, the time it would take the Authority to issue a notice to stop shellstock harvesting);~~,~~ and the degree of effluent dilution and dispersion. Due consideration should be given to the possibility that emergency actions might be necessary on holidays or at night. For more information, see the NSSP Guidance Document, *Classification of Shellfish Growing Waters Adjacent to Wastewater Treatment Plants*.

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@.02 Microbiological Standards

~~.01 Total Coliform Standards~~

~~Note: The NSSP allows growing areas to be classified using either a total or fecal coliform standard. The NSSP further allows the application of either standard to different water bodies within the State. Once properly classified applying either standard for classification, the NSSP allows the use of the adverse pollution condition or the systematic random sampling strategy for routine classification monitoring as appropriate to the situation in the growing area. For maximum flexibility, a State may wish to adopt the use of both standards and both monitoring strategies as appropriate with each standard. At the Interstate Shellfish Sanitation Conference's annual meeting in 1992, Task Force II recommended that this portion of the *Model Ordinance* be codified according to the standard used and the monitoring strategy employed. The *Model Ordinance* has subsequently been recodified in this manner. This codification represents the delineation of the standards based on total coliforms. The division of the standards based on fecal coliforms is outlined in the main body of the *Model Ordinance* (Chapter IV.).~~

~~A. General. Either the total coliform or fecal coliform standard shall be applied to a growing area.~~
~~B. Sampling Stations. The Authority shall ensure that the number and location of sampling stations is adequate to effectively evaluate all pollution inputs into the growing area.~~
~~C. Exceptions.~~
~~(1) Except for growing areas classified as prohibited, in any growing area where there are nonpoint pollution sources which impact the water quality, a minimum of thirty (30) [illegible]~~

collected under various environmental conditions, shall be required to classify a growing area not previously classified under Chapter IV. @.03.

(2) Except for growing areas classified as prohibited or when systematic random sampling is applied in growing areas where there are no pollution sources having an effect on the water quality, a minimum of fifteen (15) samples shall be required to classify any growing area not previously classified under Chapter IV. @.03 when there are no pollution sources impacting the water quality.

(3) The Authority is not required to apply the total coliform standard if a detailed study verified by laboratory findings demonstrates that the coliforms recovered from the growing area are not of direct fecal origin and do not indicate a public health hazard.

D. Standard for the Approved Growing Area Classification in the Remote Status.

(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the total coliform standard below.

(2) Total Coliform Standard for the Remote Status. The total coliform geometric mean MPN of the water sample results for each sampling station shall not exceed 70 MPN per 100 ml; and not more than ten (10) percent of the samples shall exceed an MPN of:

(a) 230 MPN per 100 ml for a 5-tube, decimal dilution test;

(b) 330 MPN per 100 ml for a 3-tube, decimal dilution test; or

(c) 140 MPN per 100 ml for the 12-tube, single dilution test.

(3) Required Sample Collection.

(a) A minimum of two (2) samples per sampling station shall be collected annually.

(b) A minimum of the most recent fifteen (15) samples collected per sampling station shall be used to calculate the geometric mean and ten (10) percent criteria of the data to determine compliance with the standard established for the approved classification of remote growing areas.

E. Standard for the Approved Classification of Growing Areas when Evaluated for Adverse Pollution Conditions.

(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the total coliform standard in E Section (2).

(2) Total Coliform Standard for Adverse Pollution Condition Monitoring. The total coliform geometric mean MPN of the water quality sample results for each sampling station shall not exceed 70 MPN per 100 ml, and, not more than ten (10) percent of the samples shall exceed an MPN of:

(a) 230 MPN per 100 ml for a 5-tube, decimal dilution test;

(b) 330 MPN per 100 ml for a 3-tube, decimal dilution test; or

(c) 140 MPN per 100 ml for the 12-tube, single dilution test.

(3) Required Sample Collection.

(a) A minimum of five (5) samples shall be collected annually under adverse pollution conditions from each sample station in the growing area.

(b) A minimum of the most recent fifteen (15) samples collected under adverse pollution conditions from each sample station shall be used to calculate the geometric mean and ten (10) percent criteria of the data to determine compliance with this standard.

(c) Sampling station locations shall be adjacent to actual or potential sources of pollution.

F. Standard for the Approved Classification of Growing Areas when Evaluated for Nonpoint Source Pollution.

(1) Exception. If the tidal stage increases the total coliform concentration, the Authority shall use sample results collected during that tidal stage to classify the area.

(2) Pollution Sources. Harvest waters shall be:

(a) Impacted only by randomly occurring, intermittent environmental events; and,

(b) Not impacted by discharges from sewage treatment facilities or combined sewer overflows.

(3) Water Quality. The bacteriological quality of every station in the growing area shall meet the total coliform standard in Section F (4) or Section F (6) as appropriate to the monitoring strategy being used.

(4) Total Coliform Standard for Systematic Random Sampling. The total coliform geometric mean of the water sample results for each sampling station shall not exceed 70 MPN per 100 ml

and the estimated ninetieth (90th) percentile shall not exceed an MPN of:

(a) 230 MPN per 100 ml for a 5-tube, decimal dilution test;

(b) 330 MPN per 100 ml for a 3-tube, decimal dilution test.

(5) Estimated ninetieth (90th) Percentile. The estimated ninetieth (90th) percentile shall be calculated by:

(a) Determining the geometric mean and logarithmic (base ten [10]) standard deviation for the sample result from each sampling station; then

(b) Multiplying the log standard deviation in (a) by 1.28; and

(c) Adding the product from (b) to the log mean of sample results, and;

(d) Taking the antilog of the results in I to get the estimated ninetieth (90th) percentile.

(e) MPN values that signify the upper or lower range of sensitivity of the MPN test used in the ninetieth (90th) percentile calculation shall be increased or decreased by one (1) significant digit.

(6) Total Coliform Standard for Adverse Pollution Condition Monitoring. The total coliform geometric mean MPN of the water sample results for each sample station shall not exceed 70 MPN per 100 ml and not more than ten (10) percent of the samples shall exceed an MPN of:

(a) 230 MPN per 100 ml for a 5-tube, decimal dilution test; or

(b) 330 MPN per 100 ml for a 3-tube, decimal dilution test; or

(c) 140 MPN per 100 ml for a 12-tube, single dilution test.

(7) Required Sample Collection.

(a) Adverse Pollution Condition Monitoring. The Authority shall collect samples at the same frequency as described in Section E. (3) for application of the standard under Section E. (2).

(b) Systematic Random Sample Monitoring. The requirement for systematic random sample monitoring shall be met when:

(i) Sample station locations are adequate to produce the data to effectively evaluate all nonpoint sources of pollution;

(ii) Sample collection is scheduled sufficiently far in advance to support random collection with respect to environmental conditions. Compliance requires that prior to implementation; the schedule for random sampling shall be documented in the master file for the growing area and adhered to. If conditions at the time of scheduled sample collection are hazardous to the safety of the individuals assigned to collect samples, sample collection shall be rescheduled in accordance with provisions in the sampling schedule;

(iii) A minimum of six (6) random samples shall be collected annually from each sampling station in the growing area; and

(iv) A minimum of two (2) random samples shall be collected annually from each sample station in the growing area while in the inactive status. The sample collection frequency of six (6) random samples per station per year specified under @.02 F. (7) (b) (iii) must resume at least six (6) months before an area is reactivated; and

(v) A minimum of the thirty (30) most recent randomly collected samples from each sampling station shall be used to calculate the geometric mean and ninetieth (90th) percentile to determine compliance with this standard.

(c) Transition from Adverse Pollution Condition Monitoring to Systematic Random Sample Monitoring. If the Authority:

(i) Does not have thirty (30) recent randomly collected sample results from each station, then the previous fifteen (15) samples collected under adverse pollution conditions may be used with the most recent random samples to meet the minimum thirty (30) sample requirement for a transition period not to exceed three (3) years; and

(ii) Uses the transition period described in (i), as additional random samples are collected, the random samples shall chronologically replace the samples collected under adverse pollution conditions (e.g., sample thirty-one [31] replaces sample one [1])

G. Standard for the Restricted Classification of Growing Areas when Evaluated for Adverse Pollution Conditions and Used as a Shellfish Source for Shellfish Depuration.

(1) Water Quality. The bacteriological quality of every sample station in the growing area shall

meet the total coliform standard in Section G. (2).

(2) Total Coliform Standard for Adverse Pollution Conditions Monitoring. The total coliform geometric mean MPN of the water sample results for each station shall not exceed 700 per 100 ml and not more than ten (10) percent of the samples shall exceed an MPN of:

(a) 2,300 MPN per 100 ml for a 5-tube, decimal dilution test; or

(b) 3,300 MPN per 100 ml for a 3-tube, decimal dilution test; or

(c) 1,386 MPN per 100 ml for a 12-tube, single dilution test.

(3) Required Sample Collection. Samples shall be collected in accordance with Section E. (3).

H. Standard for the Restricted Classification of Growing Areas when Evaluated for Nonpoint Sources and Used as a Shellfish Source for Shellfish Depuration.

(1) Exception. If the tidal stage increases the total coliform concentration, the Authority shall use samples collected under that tidal stage to classify the area.

(2) Pollution Sources. Growing areas shall meet the requirements in Section F. (2).

(3) Water Quality. The bacteriological quality of every sample station in the growing area shall meet the total coliform standard in Section H. (4) or Section H. (6) as appropriate to the monitoring strategy being used.

(4) Total Coliform Standard for Systematic Random Sample Monitoring. The total coliform geometric mean MPN of the water sample results for each sample shall not exceed 700 per 100 ml and the estimated ninetieth (90^{th}) percentile shall not exceed:

(a) 2,300 MPN per 100 ml for a 5-tube, decimal dilution test; or

(b) 3,300 MPN per 100 ml for a 3-tube, decimal dilution test.

(5) Estimated ninetieth (90^{th}) percentile. The estimated ninetieth (90^{th}) percentile shall be calculated by the same method described in Section F. (5).

(6) Total Coliform Standard for Adverse Pollution Condition Monitoring. The total coliform geometric mean MPN of the water sample results for each station shall not exceed 700 MPN per 100 ml and not more than ten (10) percent of the samples shall exceed an MPN of:

(a) 2,300 MPN per 100 ml for a 5-tube, decimal dilution test; or

(b) 3,300 MPN per 100 ml for a 3-tube, decimal dilution test; or

(c) 1,386 MPN per 100 ml for a 12-tube, single dilution test.

(7) Required Sample Collection.

(a) Adverse Pollution Condition. The Authority shall collect samples at the same frequency as described in Section E. (3) for application of the standard under Section H. (6).

(b) Systematic Random Sample Monitoring. The Authority shall collect samples in the same manner and at the same frequency as specified in Section F. (7) (b) for application of the standard under Section H. (4).

.02 Systematic Random Sampling Monitoring Strategy

NSSP Guidance Documents provide the public health principles supporting major components of the NSSP and its Model Ordinance, and summaries of the requirements for that component. NSSP Model Ordinance requirements apply only to interstate commerce although most States apply the requirements intrastate. For the most up to date and detailed listing of requirements, the reader should consult the most recent edition of the Model Ordinance.

The first critical control point in preventing food-borne illness from shellfish consumption is identifying shellfish growing areas of acceptable sanitary quality. The completion of a sanitary survey is of paramount importance in making the distinction between acceptable and unacceptable growing areas, and is the key to accurate growing area classification as approved, conditionally approved, restricted, conditionally restricted, or prohibited. A sanitary survey is required under the NSSP Model Ordinance for each growing area prior to its approval by the State as a source of shellfish for human consumption or as a source for shellfish to be used in a depuration or relay operation. The principal components of a sanitary survey are: (1) identification and evaluation of the pollution sources that may affect the areas; (2) an evaluation of the meteorological factors; (3) an evaluation of hydrographic factors that may affect distribution of pollutants throughout the area; and (4) an assessment of water quality. For an in depth discussion of the sanitary survey, see the NSSP Guidance Document, *Sanitary Survey and the Classification of Growing Waters*.

~~The NSSP in its Model Ordinance allows the Authority to classify a growing area using either a total or fecal coliform standard as part of its sanitary survey. The two (2) standards are believed to afford the same level of public health protection. The NSSP Model Ordinance also recognizes two (2) distinct water quality monitoring strategies to obtain total coliform or fecal coliform monitoring data: the adverse pollution condition strategy to be used for initial classification and for monitoring; and the systematic random sampling strategy that can be used only for monitoring if no input from point source pollution is present.~~

~~Total coliform or fecal coliform monitoring data collected under either the adverse pollution condition or the systematic random sampling strategy and the data collected for initial classification are reported as MPN values. An MPN or most probable number is a statistically derived estimate of the number of bacteria per unit volume of water sampled. The value of the MPN is determined from the combination of positive and negative results obtained from a series of fermentation tubes used in a particular laboratory test. A complete discussion of the MPN test can be found in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1985).~~

~~NSSP water quality standards for growing area classification have two (2) components. The first component establishes a median MPN value. The second component, intended for use with data collected under uniform conditions, represents the variability inherent in the testing procedure and a small allowance for some additional variability peculiar to the changing conditions in the water being sampled. The original NSSP "variability factor" for the total coliform group, an MPN of 230 per 100 milliliters of sample was developed to include 90% of the samples collected under uniform conditions in which the only sources of variability operational are due to the test procedure and the allowance for some additional variability arising from changing conditions in the water being sampled. Therefore, if only these two (2) sources of variability are active in the sample data, then no more than ten (10) percent of the samples derived under these conditions will exceed an MPN value of 230 per 100 milliliters of sample when the 5- tube, decimal dilution MPN procedure is used. This is referred to in the NSSP as the ten (10) percent criteria where no more than ten (10) percent of the samples should exceed the variability factor established for the standard and the testing procedure being used.~~

~~This same type of reasoning has been applied to both the total and fecal coliform groups and appropriate ten (10) percent criteria developed for the MPN test employed (330 and 140 MPN per 100 milliliters for the total coliform group tested by the 3-tube, decimal dilution and the 12-tube, single dilution MPN procedures, respectively, and 43, 49 and 28 MPN per 100 milliliters for the fecal coliform group tested by the 5-tube and 3-tube, decimal dilution and 12-tube, single dilution MPN procedures, respectively).~~
~~Because these variability factors were derived for use with data sets collected under uniform conditions, they do not address wide swings in water quality that result from changing environmental conditions driven by random pollution events such as runoff carried pollutants following rainfall. Therefore, the ten (10) percent criteria is not considered sufficient to protect public health when shellfish are taken from growing area waters adversely affected by known meteorological or hydrological events that occur intermittently and are shown to degrade water quality.~~

~~While many growing area waters may meet the NSSP median value and ten (10) percent criteria, some shellfish growing area sampling stations still display a considerable level of variation in the MPN sample results. Sampling data of this type may indicate that the shellfish growing areas are intermittently polluted during adverse pollution conditions and pose a risk to the shellfish consuming public. The NSSP has never intended to place a growing area that is polluted ten (10) percent of the time in the approved classification. The dilemma facing the Authority, therefore, is how to distinguish between the inherent variation of the MPN test and the variability resulting from intermittent environmental conditions that degrade water quality. When environmental events (such as rainfall) produce unfavorable effects on water quality, the data may contain data points that vary widely from the median value of the established classification. Such a data set would probably contain upper outliers that represent periods when the shellfish may be exposed to significantly greater quantities of pollution. In this situation, the determination of NSSP conformity to the established classification standard for a set of growing water samples from a particular station may become an arbitrary function of the mechanics of sampling (timing and/or frequency) rather than an actual characteristic of the growing area. Use of a statistical method, the estimated 90th percentile, will detect these random pollution events that may cause a data set to be skewed because of a few high MPN values.~~

When shellfish water sampling data collected following intermittent pollution events are combined with data collected under normal conditions, variability is increased. The estimated 90th percentile will reflect this increased variability. Therefore, use of the estimated 90th percentile will protect against the potential public health problems that may result when shellfish are consumed from growing waters that are adversely affected by intermittent pollution events and improperly classified.

The method for calculating the ninetieth 90th percentile for use in evaluating growing water bacteriological data was suggested by the Georgia Department of Natural Resources, as an addendum to Interstate Shellfish Sanitation Conference (ISSC) Issue 8109. The ISSC adopted the systematic random sampling monitoring strategy and the method recommended for calculating the 90th percentile at its 1989 ISSC Annual Meeting.

Water Quality Assessment

In the adverse pollution condition monitoring strategy, the water quality standards for both total and fecal coliforms use the ten (10) percent criteria, the variability portion of the standard to adjust for the inherent variability of the MPN testing procedure in data with uniform bacterial densities. In the systematic random sampling strategy, the application of the water quality standard employs the variability portion of the standard to detect the impact of intermittent environmental events on water quality above and beyond those attributed by the MPN testing procedure alone.

A field sampling and data analysis design that employs a systematic random sampling plan for routine monitoring assumes that a statistically representative cross section of all meteorological, hydrographic, or other pollution events will be included in the data set. Therefore, all shellfish growing area data collected under the systematic random sampling plan are used to determine compliance with the appropriate total coliform or fecal coliform water quality standard. This sample collection and data analysis design may be applied only to growing areas that are affected by randomly occurring pollution events triggered by rainfall and runoff and that meet the standard for the approved or restricted classification. This sampling strategy may also be used to monitor growing areas where water quality is influenced by seasonal water uses or where harvesting is controlled by seasonal resource management restrictions. In this situation, monitoring must be done during the season when the growing waters are open. Systematic random sampling is not intended to nor should it be applied to areas impacted by point source pollution.

The systematic random sampling monitoring strategy and data analysis design presumes that if intermittent, unfavorable changes in water quality occur, they will be revealed in the bacteriological sampling results. These unfavorable sampling results will contribute to the variability of the data set. Data sets displaying high levels of variability will consequently exhibit an elevated estimated 90th percentile. The Authority's option to use the systematic random sampling strategy is, therefore, contingent upon acceptance of the estimated 90th percentile as the statistic used to measure the variability of the data set. Also required is that timing of monitoring runs be pre-planned far in advance so that effects of random non-point pollution events will be captured if they occur. This statistic, along with the geometric mean of the data set, can be used when evaluating each sampling station for compliance with the NSSP water quality standards.

An example of an acceptable systematic sampling plan is one that documents a pre-established sampling schedule in the growing area central file. Monthly or bimonthly sampling regimes are acceptable and the schedule is maintained so there is no avoidance of unfavorable conditions. A reasonable attempt must be made to collect samples on the pre-established days regardless of navigational conditions. Field sampling crews, however, are not required to take unnecessary risks to sample on any particular day. The sampling plan must address unsafe sample collection (boating) conditions by designating an alternate sampling day or by allocating extra sampling days in the schedule that may be used when needed.

If the growing area is to be used year-round for harvesting, the random sampling plan should stipulate the collection of samples throughout the year. If the growing area is intended to be approved for direct harvest for only part of the year, the random sampling plan would need only to address that period when the area is available for harvest. The only exception to this obligation in a random sampling regime is that the Authority will require sampling during a particular tidal condition, if that condition unfavorably impacts the water quality of the growing area.

Estimating the 90th Percentile

Use of the SRS ~~systematic random sampling~~ strategy involves calculating the estimated 90th percentile of the data~~. This statistic measures variability in the data and should not be exceeded by random pollution events if the growing area is properly classified.~~ When the Authority elects to employ the SRS ~~systematic random sampling~~ strategy, the following guideline must be used to calculate the estimated 90th percentile.

The estimated 90th percentile must be obtained using the following equation: Est.

90th percentile value = Antilog $[(S_{log})1.28^{A} + x_{log}]$

Where

S_{log} = base 10 logarithmic standard deviation. x_{log}

= base 10 log mean

^{A} The value 1.28 is obtained from the standard normal distribution

Other

* For the purpose of mathematical calculations, MPN values that ~~signify~~ fall in the upper or lower range of sensitivity for that test shall be increased or decreased 1 significant number. ~~(MPN counts are reported in the form of two (2) significant numbers.)~~ For example, an MPN value of 'less than 2.0' shall be decreased by 1 significant number to 1.9 to indicate the lower level of sensitivity of the 5-tube, decimal dilution MPN test. In a similar manner, 2.9 shall be used to indicate the MPN value of 'less than 3.0' for the 3-tube, decimal dilution MPN test. ~~Therefore, it would follow that a~~An MPN value of 1700 shall be used to indicate the MPN value 'greater than 1600' for the 5-tube MPN test.

* Logarithms may be rounded to 3 decimal places.

* Antilogs of log MPN calculations may be rounded to the ***next lower integer*** (zero decimal places) [example - antilog (0.556) = 3]

* The standard deviation of the log MPN data shall be calculated in the following manner:

$$S_{log} = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}}$$

Application of the Guideline

Example 1

(1) Convert MPN values to base 10 logarithms.

Obs	MP	Log_{10}	Obs	MPN	Log_{10}
1	2.9	0.462	16	3.6	0.556
2	2.9	0.462	17	3.6	0.556
3	2.9	0.462	18	3.6	0.556
4	2.9	0.462	19	9.1	0.959
5	2.9	0.462	20	9.1	0.959
6	2.9	0.462	21	9.1	0.959
7	2.9	0.462	22	9.1	0.959
8	2.9	0.462	23	9.1	0.959
9	3.6	0.556	24	9.1	0.959
10	3.6	0.556	25	23	1.362
11	3.6	0.556	26	23	1.362
12	3.6	0.556	27	23	1.362
13	3.6	0.556	28	43	1.633
14	3.6	0.556	29	43	1.633

15	3.6	0.556	30	460	2.663

(2) Calculate Geometric Mean and Standard Deviation.

Median - 3.6
Percentage greater than 43 - 3.3 %
Geometric Mean (Antilog x_{log}) - (Antilog 0.834) or 6.8
Log Standard Deviation (S_{log}) - 0.506

(3) Calculate Estimated 90th Percentile using above equation.

Est. 90^{th} = Antilog $[(S_{log})1.28 + x_{log}]$
= Antilog $[\underline{(0.506)}1.28 + \underline{0.834}]$
Est. 90^{th} = Antilog [1.482] or 30

(3) Interpret.

The geometric mean of the data set is less than 14 and the estimated 90^{th} percentile is less than 49 (3-tube, decimal dilution test). This station meets the NSSP fecal coliform water quality standard for the approved classification.

Example 2

(1) Convert MPN values to base 10 logarithms.

Obs	MPN	Log_{10}	Obs	MPN	Log_{10}
1	1.9	0.279	16	2.0	0.301
2	1.9	0.279	17	4.5	0.653
3	1.9	0.279	18	4.5	0.653
4	1.9	0.279	19	7.8	0.892
5	1.9	0.279	20	7.8	0.892
6	1.9	0.279	21	7.8	0.892
7	1.9	0.279	22	11	1.041
8	1.9	0.279	23	11	1.041
9	2.0	0.301	24	23	1.362
10	2.0	0.301	25	23	1.362
11	2.0	0.301	26	23	1.362
12	2.0	0.301	27	23	1.362
13	2.0	0.301	28	33	1.519
14	2.0	0.301	29	540	2.732
15	2.0	0.301	30	1700	3.230

(2) Calculate Geometric Mean and Standard Deviation.

Median - 2.0
Percentage greater than 43 - 6.6 %
Geometric Mean (Antilog x_{log}) - (Antilog 0.788) or 6 Log Standard Deviation (S_{log}) - 0.737

(3) Calculate Estimated 90th Percentile using above equation - Est. 90th =

Antilog $[(S_{log})1.28 + x_{log}]$
= Antilog [(0.737)1.28 + 0.788]
Est. 90th = Antilog [1.731] or 53

(4) Interpret.

~~While this station's geometric mean is less than14, the standard deviation that resulted from the high values in this data set would lead one to conclude that water quality may have been adversely affected by storm water runoff or another intermittent pollution event.~~ The estimated 90th percentile was 53 (greater than 43 for the 5-tube, decimal dilution MPN test). Therefore, this station ***would not meet*** the NSSP fecal coliform water quality standard for the approved classification.

References

1. U.S. Food and Drug Administration (FDA). 1965. National Shellfish Sanitation Program Manual of Operations, Part I. FDA, Washington, D.C., p. 11, footnote 6.
2. Interstate Shellfish Sanitation Conference. Sanitary Survey and the Classification of Growing Waters. *In* ISSC (ed.), NSSP Guide for the Control of Molluscan Shellfish. Interstate Shellfish Sanitation Conference, Columbia, S.C.
3. American Public Health Association. 1985. *Standard Methods for the Examination of Water and Wastewater*, 16th Ed. American Public Health Association, American Water Works Association, Water Pollution Control

Federation, Washington D.C.

@.03 Growing Area Classification

.01 Management Plans for Growing Areas in the Conditional Classification

~~NSSP Guidance Documents provide the public health principles supporting major components of the NSSP and its Model Ordinance, and summaries of the requirements for that component. NSSP Model Ordinance requirements apply only to interstate commerce although most States apply the requirements intrastate. For the most up to date and detailed listing of requirements, the reader should consult the most recent edition of the Model Ordinance.~~

~~The goal of the NSSP is to control the safety of shellfish for human consumption by preventing its harvest from contaminated growing areas so contaminated that the shellfish would be considered adulterated. In implementing this goal, the NSSP uses five (5) classifications for growing areas: approved, conditionally approved, restricted, conditionally restricted, and prohibited. The placement of a growing area in any one (1) classification is based upon the growing area's conformance with the requirements established for that classification. For a full explanation of this concept, see the public health explanation in NSSP Guidance Document, *Sanitary Survey and the Classification of Growing Waters* .~~

~~The conditional classifications are designed to address growing areas that are subject to intermittent microbiological pollution. These classifications offer the Authority an alternative to placing the area in the restricted or prohibited classification year round when during certain times of the year or under certain conditions, the shellstock from the growing area may be safely harvested. Public health protection from unsafe shellfish in the use of the conditional classifications is afforded through the use of a management plan. Using a thorough investigation conducted as part of the sanitary survey, the Authority determines that the growing area will be in the open status of its conditional classification for a reasonable period of time; that the factors which determine this period of time are known, predictable and are not so complex that the factors cannot be reasonably managed; and that the bacteriological water quality can be correlated with the factors affecting the distribution of pollutants in the growing area. The management plan for each growing area placed in a conditional classification is based on the information gathered during the investigation. The plan establishes a strict set of criteria, which must be met for the growing area to remain in the open status. Failure to meet the criteria automatically places the growing area in the closed status, with immediate notice to the public, the affected industry, and the plan's participants. Two (2) of the most important components of the management plan are the acceptance of and the agreement to the conditions of the management plan by the one (1) or more Authorities involved, other local, State and Federal agencies which may be involved, the affected shellfish industry, and the persons responsible for the operation of any treatment plants or other discharges that may be involved; and the annual reevaluation of the compliance with the plan to assure public health protection.~~

~~The criteria for the approved classification of the NSSP require that the growing area not be subject to human or animal fecal matter at levels that present an actual or potential public health risk, and not be contaminated with pathogenic organisms, poisonous or deleterious substances or marine biotoxin. From the review of growing area classifications and sanitary surveys conducted by national and international Authorities, it appears that a common misuse of the approved classification is the placement of a growing area in the approved classification when the use of the conditionally approved classification would have been more appropriate. Critical investigation usually reveals that the growing area is improperly classified because it is subject to intermittent pollution events, which is a contravention of the criteria for the approved classification.~~

~~Intermittent pollution events have been identified as a significant cause of shellfish-borne infectious disease outbreaks worldwide. As an example, in the fall of 1982, at least 471 persons developed gastroenteritis after consumption of~~

~~sewage contaminated oysters from a growing area that had been placed in the approved classification (Casper, 1982). An investigation into the outbreak demonstrated that the growing area could probably be safely open to harvesting in the summer when the prevailing winds are southerly and tides are high. In the late fall, the arrival of cold fronts can cause high rainfall, strong winds and abnormally low tides and raw sewage bypasses from overloaded sewage treatment plants. Under these conditions, sewage reached the growing area causing the outbreak. As a result of the investigation, the Authority learned that it should have placed the growing area in the conditionally approved classification and developed a management plan to automatically close the area in the late fall through spring when climatic conditions were likely to render the growing area unsafe. Under the management plan, the area would be reopened to harvesting in the summer when favorable conditions prevailed and would be intensively monitored to ensure that the summer conditions were met. In this instance, application of the conditionally approved area concept probably could have prevented the outbreak.~~

Use of the Conditional Classification

Use of the conditional classifications is a voluntary option for the Authority. There are two (2) types of conditional areas: conditionally approved and conditionally restricted. Any growing area in the conditionally approved classification must meet the criteria for the approved classification when it is in the open status of this classification. When the growing area is in the closed status of this classification, it may be used for relaying or depuration if it meets the requirements for the restricted classification and if this use is specified in its management plan, or it may be closed to any use. Any growing area in the conditionally restricted classification must meet the criteria for the restricted classification when it is in the open status of this classification and no harvesting is permitted when it is in its closed status.

Growing areas that are subject to intermittent microbiological pollution from predictable pollution events may be placed in the conditionally approved or conditionally restricted classification under the NSSP. Examples of predictable pollution events include the failure of wastewater treatment facilities to maintain a performance standard needed to maintain an established effluent quality; changes in seasonal populations affecting growing area water quality; and nonpoint source pollution events such as caused by as certain rainfall intensities. Conditional classifications may also be used to manage growing areas affected by toxigenic micro-algae that produce marine biotoxins.

An example of a common situation where use of the conditionally approved classification might be appropriate is when water quality is dependent upon the operation and performance of a sewage treatment plant. In this example, the growing area would meet the criteria for the approved classification when the sewage treatment plant is performing satisfactorily. If there is some interruption in sewage treatment, the likely result will be degradation of water quality in the growing area. This degradation would ~~require the establishment of a prohibited area large enough to dilute and assimilate the effluent discharged during the interruption in treatment~~ present a threat to consumers of shellfish harvested from the impacted area if it is left in the open status, so the impacted area cannot be classified as approved. ~~If an interruption can be predicted and is at a manageable frequency necessitating repeated closings (e.g., one (1) or two (2) times a year), the Authority may consider a conditionally approved classification for a portion of the growing area classified as prohibited.~~ Interruptions could include a disinfection failure or certain climatic conditions which are known to affect the wastewater treatment facility's performance. Although many Authorities are burdened by administrative procedures, the use of a management plan for the conditional classification allows the Authority to act quickly to implement closures~~,~~ and avoid unnecessary delays in returning the conditionally approved portion of the growing area to the open status. The added administrative burden might be offset by the shellfish resource gained by upgrading a ~~prohibited~~ portion of a growing area where harvest for direct market is not allowed to the conditionally approved classification.

The concept of the conditionally approved classification is also applicable to other situations in which there may be a rapid or seasonal change in water quality. Examples include:

* The water quality in a growing area adjacent to a resort community may vary according to seasons of the year. During the summer months, when the community experiences a significant population increase, water quality may be

adversely affected. However, during the winter when there are few people in the community, water quality might improve sufficiently to allow the growing area to be placed in the open status. In some ~~S~~states, this is known as a seasonal closure.

* The water quality in a protected harbor in a sparsely settled area, which provides anchorage for a fishing fleet several months a year, might vary. When the fishing fleet is in the harbor, the water might be of poor sanitary quality. The area would be closed for shellstock harvesting when the fishing fleet is using the harbor. During the remainder of the year, however, the quality of the harbor water might meet the criteria for the approved classification and be opened to shellstock harvesting.

* The water quality in an area may fluctuate with the discharge of a major river, or rainfall in the area may cause runoff of pollutants from adjacent land surfaces (non-point pollution) into the growing area. During periods of low runoff or river discharge, the area might meet the criteria for the approved classification.

The use of the conditional classification option offers the Authority the ability to increase the availability of water for shellfishing that would otherwise be closed. The management plan dictates the circumstances and procedures for immediate response to situations requiring closure. The administrative procedures included in the management plan allows the Authority to reopen the area to harvesting as soon as the pollution condition is over and the water quality and shellstock have returned to acceptable quality.

Suitability of the Conditional Classification

The first step is to determine, through a thorough investigation conducted as part of the sanitary survey, if the growing area is suitable for conditional management by evaluating the impacts of actual and potential sources of pollution in terms of their actual or potential effect on water quality. Information must be gathered to ~~support~~ evaluate that ~~the supposition that the growing area will be in the open status of its conditional classification for a reasonable period of time; that~~ the pollution events which determine ~~this~~ the open-status period of time are known, and predictable. ~~and are not so complex that the factors of these events cannot be reasonably managed; and that the~~ If management is based on predicted changes in microbiological water quality, analyses must show that the ~~bacteriological~~microbiological water quality can be correlated with the factors affecting ~~the distribution of pollutants~~ microbiological indicator levels in the growing area. The investigation may also consider relevant resource management, legal statutes and regulations, patrol and enforcement considerations, and jurisdictional boundaries ~~social, economic, or political factors~~ that may influence the open and closed periods, and the establishment of boundaries, for the conditional classification of a growing area. The management plan for each growing area placed in a conditional classification is based on the information gathered during the investigation. Some potential sources of pollution which could be managed under a conditional classification management plan include: bypasses and overflows within a sewage collection and treatment system, intermittent discharges from boats~~, seasonally related pollution occurrences,~~ seasonal variation in microbiological indicator levels, seasonal changes in population (human), animals, land runoff, and freshwater flows.

The second step in determining the suitability of conditional classifications is to determine whether the Authority has sufficient resources available to survey, manage, monitor, control harvesting, close and reopen the area as required. ~~Use of these classifications imposes additional manpower and resource burdens on the Authority. For example sources of pollution must be routinely monitored; coordination between State, local and industry officials must be timely; performance standards must be monitored; and closures must be immediate and effective. Any Authority that has elected to use the conditionally approved or conditionally restricted classifications has found the resource investment to be substantial and this investment must be balanced against the benefit of the additional shellfish resource available.~~

The third step is to evaluate each source of pollution ~~in terms of the pollution load and~~ to determine if performance standards can be formulated for each ~~pollution~~ source having a significant effect on the sanitary quality of the growing area. The conditional classification management plan ~~must~~ establishes ~~a strict set of~~ criteria which must be met for the growing area to remain in the open status. The following are examples of different types of performance standards that could be used:

* Performance standards might stipulate the bacteriological quality of effluent from sewage treatment plants. The microbiological quality can be monitored in terms of disinfection residual or dosage for ultraviolet light disinfection. An example of a performance standard for an effluent discharge is:

  "The median fecal coliform MPN, in any one (1) month, shall not exceed 200 per 100 ml, based on not less than sixteen (16) samples per month, and not more than ten (10) percent of the samples shall have an MPN in excess of 1,000 per 100 ml. This fecal coliform limit shall be presumed to be met if the chlorine residual in the effluent is at least 1.0 ppm and the chlorine residual in the effluent is continuously recorded on a chart by chlorine residual analyzer or is measured hourly and recorded in the daily monitoring records as required for the plant's NPDES permit."

* For disinfection by ultraviolet (UV) light, the disinfection is based on dosage. An example of a performance standard is, "A minimum UV dose of 37 mW-Sec/cm^2 is to be maintained. The calculation of intensity of the UV light is to include factors for effluent quality, including turbidity, suspended solids, and transmittance. The effluent factors contributing to the dose, including turbidity, suspended solids, transmittance, and flow will be continuously measured and recorded. An alarm will be activated if any of the factors are above design limits."

* Performance standards might be based upon the amount of vessel traffic in the area and the concomitant amount of sewage that can be expected.

* Performance standards might be based upon the amount of rainfall in the immediate area. An example is: "The growing area will be closed to harvesting for (number of days) when there has been two (2) inches or more rainfall registered at a rain gauge at (specified location) within a 24-hour period."

* Performance standards might be based upon the height of a river stage. An example could be: "When the river at (a specified area) reaches 3.66 meters (twelve [12] feet) or higher, the growing area will be closed."

The design of a waste treatment plant and the plant effluent specifications are critical to the use of the conditional classifications. Design criteria which may be useful in determining the quality of sewage which can be discharged into an area without exceeding the desired water quality standards include: population equivalent (fecal coliform) of sewage, predicted survival of fecal coliform in seawater, effectiveness of disinfection and the amount of clean dilution water in an area.

The mechanical equipment at critical sewage treatment or plant components should be such that interruptions will be minimized. Requirements which might be imposed depend upon the importance of the unit's relationship to maintenance of water quality in the growing area. Important design features, which should be considered in the design of the sewage collection system, include:

* Exclusion of storm water runoff from sewer collection systems and use of devices such as flow equalization tanks to control effects of storm water infiltration on treatment plant performance;

* Provision of standby power at critical sewage pumping stations through the use of on-site emergency generators, or other alternate power sources;

* Use of gauges, charts and other recording devices to monitor flows and performance standards; and

* Use of alarms, telemetering or other devices to report immediately failure of any critical components at the WWSD and in the collection system at sewage pumping stations.

A detailed discussion of ways to increase the reliability of sewage treatment plants can be found in *Protection of Shellfish Waters* (USEPA, 1974) and *Design Criteria for Mechanical, Electric and Fluid System Component Reliability*

(USEPA, 1974).

The fourth step is to determine the water quality, which will occur in the growing area when the performance standards are not met, and what portion of the growing area will be affected. Once these determinations are made, the Authority can select the appropriate management strategy for the portion of the growing area that will be placed in the closed status when performance standards are not met and can select the boundaries for the closed status. The boundaries of that portion of the growing area to be placed in the closed status would depend upon such items as the distance and travel time from the pollution source to the area, the concentration of pollutants in the discharge during the breakdown condition, amount of effluent and hydrographic factors including dilution available in the receiving water.

The use of the conditional classification where a sewage treatment plant is the pollution source being managed requires a fifth step. An area in the prohibited classification ~~(closed safety zone)~~ must be established between the sewage treatment plant and the growing area placed in the conditionally approved or conditionally restricted classification. The size of the prohibited area should be based on the level of sewage treatment; the total time it would take for the person responsible for the operation of the sewage treatment facility to detect a failure and notify the Authority; and the time it would take the Authority to issue a notice to stop shellstock harvesting. The size of the area in the prohibited classification should allow for an effluent travel time through the prohibited area that is at least twice that required for the notification process to become effective. Due consideration should be given to the possibility that emergency actions might be necessary on holidays or at night. A minimum effluent dilution is to be determined at the prohibited boundary and can be the controlling factor in situations where there is efficient detection and notification of breakdowns.

The length of time that a growing area should be in the closed status of its conditional classification will depend upon several factors. These factors include the degree of pollution in the growing area and flushing capacity of the estuary, the species of shellfish, water temperature, shellstock activity and cleansing rates, and presence of silt or other chemicals that might interfere with the physiological activity of the shellstock. Additional information on the natural cleansing of shellstock is provided in the NSSP Guidance Document, *Shellstock Relay*.

Minimum Requirements for a Conditional Area Management Plan

The management plan for a growing area in the conditionally approved or conditionally restricted classification must meet certain minimum requirements to ensure that the safety of the shellfish for human consumption is maintained. The use and success of the conditional classification depends upon a thorough and accurate management plan. Therefore, it is important that all aspects of the management plan be fully considered and implemented. The minimum requirements to be addressed are:

A. An understanding of and an agreement to the conditions of the management plan by the one (1) or more Authorities involved, other local, ~~S~~state, and Federal agencies which may be involved, the affected shellfish industry, and the persons responsible for the operation of any treatment plants or other discharges that may be involved. Signatures verifying this agreement are not required to be included in the plan but are a recognized and effective way to demonstrate that all parties to the plan are in agreement.
B. A written management plan for the growing area being placed in the conditional classification, which includes a general description of the growing area with a map showing the area's boundaries, and which addresses all items in C. through H.
C. A sanitary survey that shows the growing area meets the requirements for its classification ~~will be in the open status of its conditional classification for reasonable periods of time~~. The survey must provide a description of the factors determining the growing area's suitability for being classified conditionally approved or conditionally restricted, and the supporting information and data.
D. A description of the predictable pollution event or events that are being managed and the performance standards established for each pollution source contributing to the pollution event including:
    (1) For a wastewater treatment facility, the performance standard should be based on:
        * Peak effluent flow

* Bacteriological quality of the effluent
* Physical and chemical quality of the effluent
* Bypasses from the treatment plant or its collection system
* Design, construction, and maintenance to minimize mechanical failure or overloading (i.e., the reliability of the treatment system and collection system components)
* Provisions for verifying and monitoring efficiency of the wastewater treatment plant and the feedback system for addressing inadequate treatment.
* Identification of conditions that lead to WWSD failure and closure of the conditionally approved area.

(2) For meteorological or hydrological events, the performance standard should be based on:
* Identification of the specific meteorological and/or hydrologic event that will cause the growing area to be placed in the closed status;
* Discussion and data analyses concluding that effects on water quality from these specific meteorological and/or hydrologic events are predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for the growing area placed in the conditional classification; and
* The predicted number of times, based on historical findings, that the pollution event will occur within one (1) year.

(3) For seasonal events, such as marina operation, seasonal rainfall, and waterfowl migration, the performance standard should be based on:
* Identification of the seasonal event that will cause the growing area to be placed in the closed status, including its estimated duration; and
* Discussion and data concluding that the seasonal event is predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for a growing area placed in the conditional classification;

E. A description of the plan for monitoring water quality including numbers and frequency;

F. A description of how the closed status for the conditional classification will be implemented, which must include:

(1) A clear statement that when the performance standards are not met, the growing area will immediately be placed in the closed status;

(2) A requirement to notify the Authority or Authorities that the management plan performance standards have not been met, including:
* The name of the agency or other party responsible for notifying the Authority;
* The anticipated response time between the performance standards not being met and notification of the Authority; and
* The procedures for prompt notification including contingencies such as night, weekend, and absences of key personnel (It is recommended to include back-up contacts in addition to primary contact information);

(3) A description of the implementation and enforcement, including:
* The response time between the notification to the Authority of the failure to meet performance standards and activation of the legal closure of the growing area by the Authority;
* The procedures and methods to be used to notify the shellfish industry; and
* The procedures and methods to be used to notify the patrol agency (enforcement agency) including:
  - The name of the responsible patrol agency;
  - The anticipated response time between the Authority's legal closure of the growing area and notification of closure to the patrol agency; and
  - A description of the patrol agencies anticipated activities to enforce the closed status.

G. A description of the criteria that must be met prior to reopening a growing area in the closed status, including the need to determine that:

(1) The performance standards established in the management plan are again fully met;

(2) The flushing time for pollution dissipation is adequate;

(3) A time interval has elapsed which is sufficient to permit reduction of human pathogens as measured by the coliform indicator group in the shellstock;
(4) Where necessary, the bacteriological quality of the water must be verified; and
(5) Shellstock feeding activity is sufficient to achieve reduction of pathogens to levels present prior to the pollution event.

H. A commitment to a reevaluation of the management plan at least annually using, at a minimum, the reevaluation requirements in the NSSP Model Ordinance.

References

1. Casper, Victor L. 1982. *Memorandum, Quarterly Report - Shellfish, October 1, 1982 Through December 31, 1982; Oyster Related Gastroenteritis Outbreaks*. U.S. Food and Drug Administration, Washington, D.C.
2. Interstate Shellfish Sanitation Conference. Sanitary Survey and the Classification of Growing Waters. *In* ISSC (ed.), NSSP Guide for the Control of Molluscan Shellfish. Interstate Shellfish Sanitation Conference, Columbia, SC.
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4. U.S. Environmental Protection Agency. 1974. *Design Criteria for Mechanical, Electric, and Fluid System and Component Reliability*. EPA 430/9-74-001.
5. U.S. Environmental Protection Agency. 1974. *Protection of Shellfish Waters*. Office of Water Program Operations. EPA 430/9-74-010.

.02 Protocol for Reviewing Classification of Areas Implicated by Pathogens in Shellfish Meat Samples

NOTE: Please click on the link below to view, print or download these documents:

Protocol for Reviewing Classification of Areas Implicated by Pathogens in Shellfish Meat Samples

https://issc.org/sites/default/files/uploads/-2023/2023-guide-docs/011-2017-guidance-chapter-ii-16-protocol-classification-areas-implicated-by-pathogens.pdf

.03 Classification of Shellfish Growing Waters Adjacent to Waste ~~W~~water Treatment Plants

Note: NSSP Model Ordinance excerpts are listed in italics.

I. Introduction

~~One of the goals of the NSSP is to control the safety of shellfish for human consumption by preventing its harvest from contaminated growing areas. The positive relationship between sewage pollution of shellfish growing areas and disease has been demonstrated many times. Shellfish-borne infectious diseases are generally transmitted via a fecal-oral route. The pathway can become quite circuitous. The cycle usually begins with fecal contamination of the growing waters.~~

~~The primary responsibility of the Authority is to ensure the public health safety of the shellfish growing areas through compliance with the NSSP Model Ordinance. The Authority must perform a sanitary survey that collects and evaluates information concerning actual and potential pollution sources that may adversely affect the water quality in each growing area. Based on the sanitary survey information, the authority determines what use can be made of the shellstock from the growing area and assigns growing areas one (1) of five (5) classifications.~~

~~The shoreline survey (also known as the pollution source survey) is the sanitary survey component in which the actual and potential pollution sources that may adversely affect the growing area are identified. These sources may introduce infectious disease agents or poisonous and deleterious substances to the growing waters where they may be taken up and concentrated by shellfish. Detailed and accurate information concerning the pollution sources is necessary for a proper growing area classification.~~

~~The key to the accurate classification of shellfish growing areas is the sanitary survey. The principal components of a sanitary survey include: (1) an evaluation of the pollution sources that may affect the areas; (2) an evaluation of the meteorological factors; (3) a review of hydrographic factors that may affect distribution of pollutants throughout the area; and (4) an assessment of water quality.~~

~~A pollution source survey must be conducted of the shoreline area and watershed to locate direct discharges (e.g., municipal and industrial waste discharges and package treatment units) and non-point sources of pollution (e.g., septic tanks, storm water runoff and agricultural and wildlife area runoff). Municipal and industrial wastewater treatment facilities should be evaluated in terms of design capacity versus actual loading, type and concentration of pollutants discharged, and the type and effectiveness of pollution control devices.~~

~~Water samples are collected to determine if the water quality meets the water quality standards for this growing area classification. The NSSP recognizes two (2) water quality monitoring strategies: adverse pollution condition and systematic random sampling. Presence of point sources of pollution requires the use of the adverse pollution condition monitoring system to collect data for the application of the water quality standard. In growing areas not affected by point sources, the Authority may elect to use either system. The presence or absence of point sources of pollution and the monitoring system used dictate the frequency of samples that must be collected for application of the water quality standards.~~

The original NSSP principles have historically proved effective in controlling bacterial illness associated with shellfish harvested from polluted waters. These principles, namely a robust sanitary survey, regular water and shellfish monitoring using bacterial indicators, controlled harvest times and labelling the origin of shellstock remain applicable as the primary preventative food safety control measures for growing areas.

However, there is now ample scientific evidence to show that the current bacterial indicators are inadequate to predict the risk of viral illness for the following reasons:

(1) Enteric viruses are resistant to treatment and disinfection processes in a ~~Waste Water~~Wastewater Treatment Plant (WWTP) and are frequently detected in the WWTP's final effluent under normal operating conditions (Baggi et al. 2001; Burkhardt et al. 2000; Pouillot et al. 2015).
(2) Shellfish can bioaccumulate enteric viruses up to 100-fold from surrounding water (Seraichekas et al. 1968; Maalouf et al. 2011).
(3) Certain enteric viruses are retained by molluscan shellfish to a greater extent and for longer than the indicator bacteria currently used to classify shellfish growing areas (Sobsey et al. 1987; Dore & Lees 1995; Love et al. 2010). It has been well documented that enteric virus detection is not indexed by levels of conventional indicator bacteria.

For several decades now viral illnesses, in particular norovirus (NoV) and hepatitis A (HAV), have been identified as common food safety problems associated with the consumption of bivalve molluscan shellfish (Woods 2010; Iwamoto et al 2010; Scallan et al. 2011; Batz et al. 2012; Hall et al. 2012). NoV genogroups I, II and IV and HAV are typically associated with ill-individuals and transferred by the fecal-oral route. Because WWTPs do not completely remove infectious enteric viruses, emphasis should be placed on the importance of ensuring there is adequate dilution between a sewage source and a shellfish growing area.

In addition to the risk of enteric viruses present in wastewater, WWTP effluents may also contain chemicals and other deleterious substances including pharmaceuticals, nanoparticles, and other contaminants of emerging concern. Establishment of appropriate classification based upon virus removal efficacy and proximity and source strength of

WWTP discharges is an effective strategy to reduce the risk posed by both enteric viruses and other contaminants found in WWTP effluents. NSSP requires that shellfish growing waters be classified into one (1) of five (5) classifications. They include:

(1) Prohibited - A classification used to identify a growing area where the harvest of shellstock for any purpose, except depletion or gathering of seed or nursery culture for aquaculture or resource enhancement, is not permitted.
(2) Restricted - A classification used to identify a growing area where harvesting shall be by special license and the shellstock, following harvest, is subjected to a suitable and effective treatment process through relaying or depuration.
(3) Conditionally Restricted - A classification used to identify a growing area that meets the criteria for the restricted classification except under certain conditions described in a management plan.
(4) Conditionally Approved - A classification used to identify a growing area which meets the criteria for the approved classification except under certain conditions described in a management plan.
(5) Approved - A classification used to identify a growing area where harvest for direct marketing is allowed.

This guidance document provides information on the five (5) shellfish harvest classifications and the appropriate use of these classifications impacted by WWTP effluents. ~~A sanitary survey report is required prior to the establishment of the classifications listed above with the exception of areas classified as prohibited.~~

~~II. General Requirements for Growing Area Classification~~

~~A. Chapter IV. Shellstock Growing Areas~~

~~***@.01 Sanitary Survey***~~

~~*A. General.*~~

~~*(1) The sanitary survey is the written evaluation report of all environmental factors, including actual and potential pollution sources, which have a bearing on water quality in a shellfish growing area. The sanitary survey shall include the data and results of:*~~

~~*(a) A shoreline survey;*~~

~~*(b) A survey of the microbiological quality of the water. In growing areas adjacent to WWSDs the Authority may utilize MSC results from analysis of shellfish meat samples and the analysis of the data will be included in the sanitary survey report;*~~

~~*(c) An evaluation of the effect of any meteorological, hydrodynamic, and geographic characteristics on the growing area; and*~~

~~*(d) A determination of the appropriate growing area classification.*~~

~~*(2) The sanitary survey shall be periodically updated through the triennial reevaluation and the annual review in accordance with Section C. to assure that data is current and that conditions are unchanged.*~~

~~*(3) The documentation supporting each sanitary survey shall be maintained by the Authority. For each growing area, the central file shall include all data, results, and analyses from:*~~

~~*(a) The sanitary survey reviewed and signed by the Sanitary Survey Officer;*~~

~~*(b) The triennial reevaluation; and*~~

~~*(c) The annual review.*~~

~~*(4) The Authority shall maintain a current comprehensive, itemized list of all growing areas, including maps showing the boundaries and classification of each shellstock growing area.*~~

~~*B. Sanitary Survey Required.*~~

~~*(1) A sanitary survey shall not be required to classify growing areas as prohibited. The findings of a sanitary survey, however, may result in a growing area being classified as prohibited.*~~

~~*(2) A sanitary survey, including the triennial reevaluation, when available, of each growing area shall be required prior to:*~~

~~*(a) The harvest of shellstock for human consumption; and*~~

~~(b) The classification of a growing area as approved, conditionally approved, restricted, or conditionally restricted.~~

~~C. Sanitary Survey Performance.~~

~~(1) A sanitary survey of each growing area shall be performed at least once every twelve (12) years and shall include the components in Section A. (1).~~

~~(2) When a written sanitary survey report is not completed, the area shall be placed in the closed status.~~

~~(3) The growing area classification and the supporting data from the sanitary survey shall be reviewed at least every three (3) years.~~

~~(a) This triennial reevaluation shall include:~~

~~(i) A review in accordance with Section C. (5) and (6) of the water quality samples;~~

~~(ii) Documentation of any new pollution sources and an evaluation of their effect on the growing area;~~

~~(iii) Reevaluation of all pollution sources, including the sources previously identified in the sanitary survey, as necessary to fully evaluate any changes in the sanitary conditions of the growing area. The reevaluation may or may not include a site visit;~~

~~(iv) A comprehensive report which analyzes the sanitary survey data and makes a determination that the existing growing area classification is correct or needs to be revised; and~~

~~(v) If the triennial reevaluation determines that conditions have changed based on the information and data collected during the triennial review and that the growing area classification is incorrect, immediate action shall be initiated to reclassify the area.~~

~~(b) When a written triennial reevaluation report is not completed, the Authority shall place the growing area in the closed status.~~

~~(4) The triennial reevaluation may include:~~

~~(a) Inspection of WWSD or collection of additional effluent samples to determine their impact on the growing area;~~

~~(b) Hydrodynamic studies;~~

~~(c) Additional field work to determine the actual impact of pollution sources; and~~

~~(d) Collection of additional water samples.~~

~~(5) On an annual basis, the sanitary survey shall be updated to reflect changes in the conditions in the growing area. The annual reevaluation shall include:~~

~~(a) A field observation of the pollution sources which may include:~~

~~(i) A drive-through survey;~~

~~(ii) Observations made during sample collection; and~~

~~(iii) Information from other sources.~~

~~(b) Review, at a minimum, of the past year's water quality sample results by adding the year's sample results to the data base collected in accordance with the requirements for the bacteriological standards and sample collection required in Section @.02;~~

~~(c) Review of available inspection reports and effluent samples collected from pollution sources;~~

~~(d) Review of available performance standards for various types of discharges that impact the growing area; and~~

~~(e) A brief report which documents the findings of the annual reevaluation.~~

~~(f) The Authority may use MSC meat sampling data and/or MSC waste water~~wastewater~~ sampling data in the annual revaluation of (5) (b), (c), and (d) above to evaluate the viral contributions of the performance standards of WWSD impacts on shellfish growing areas. If MSC meat and/or water data is being used, the Authority shall conduct annual sample collection and analysis in determining performance standards.~~

(6) If the annual reevaluation determines that conditions have changed based on the information and data collected during the annual review and that the growing area classification is incorrect, immediate action shall be initiated to reclassify the area.

D. Shoreline Survey

Requirements.

(1) In the shoreline survey for each growing area, the Authority shall:
(a) Identify and evaluate all actual and potential sources of pollution which may affect the growing area;
(b) Determine the distance from the pollution sources to the growing area and the impact of each source on the growing area;
(c) Assess the reliability and effectiveness of sewage or other waste treatment systems;
(d) Determine if poisonous or deleterious substances adversely affect the growing area; and
(e) Consider the presence of domestic, wild animal or resident and migrating bird populations for possible adverse effects on growing areas.
(f) Conduct an assessment of pollution sources which may include:
(i) A drive-through survey;
(ii) Observations made during sample collection;
(iii)Other in-field assessments; and/or
(iv) Information from other sources.

(2) The Authority shall assure that the shoreline survey meets the following minimum requirements:

(a) The boundaries, based on the area topography, of each shoreline survey area are determined by an investigation which identifies the properties with the potential to impact the shellfish waters;
(b) Each shoreline survey area is identified by a unique designation which results in identification of all data associated with each shoreline survey by the unique designation;
(c) Each shoreline survey area is investigated and pollution sources evaluated by qualified, trained personnel; and
(d) Documentation for each pollution source identified by the Authority as affecting a growing area includes:
(i) The location of the site on a comprehensive map of the survey area; and
(ii) The determination that the pollution source has a direct or indirect impact on shellfish waters: and
(e) A written summary of the survey findings.

III. Guidance for Growing Area Classification

As a result of the information gathered during the sanitary survey, the Authority is responsible for distinguishing those growing areas suitable for harvest of shellstock for direct human consumption, those growing areas where the shellfish will require treatment prior to consumption, and those growing areas unsuitable to harvest for human consumption. The probable presence or absence of pathogenic microorganisms, marine biotoxin or other poisonous or deleterious substances in growing area waters is important to the Authority in deciding how the shellfish obtained from the growing area should be used. The Authority's decision, based on the sanitary survey information, will place all actual and potential growing areas in one (1) of the five (5) possible NSSP growing area classifications.

The five (5) growing area classifications are approved, conditionally approved, restricted, conditionally restricted and prohibited. Except for an emergency situation such as conditions following a hurricane when a growing area in the approved classification may be placed temporarily in the closed status, a growing area in the approved classification is

~~always in the open status. The remaining four (4) growing area classifications all place some type of restriction on shellstock harvesting. For more information concerning the enforcement of these restrictions, see the NSSP Guidance Document, *Growing Area Patrol and Enforcement of Growing Area Restrictions* .~~

~~IV. Prohibited Classification~~

~~A. Definition~~

~~A classification used to identify a growing area where the harvest of shellstock for any purpose, except depletion or gathering of seed or nursery culture for aquaculture or resource enhancement, is not permitted.~~

~~B. Requirements for a Prohibited Area Adjacent to a WWTP under NSSP MO Chapter IV. @.03 E.(5)~~

~~(1) Model Ordinance Chapter IV. Shellstock Growing Areas~~

~~***@.03 Growing Area Classification.***~~

~~*A. General. Each growing area shall be correctly classified as approved, conditionally approved, restricted, conditionally restricted, or prohibited, as provided by this Ordinance.*~~

~~*(1) Emergency Conditions…*~~

~~*(2) Classification of Growing Areas. Each growing area which:*~~

~~*(a) Is not subjected to a sanitary survey every twelve (12) years shall be classified as prohibited or, if unclassified, shall be treated as prohibited for NSSP purposes; or*~~

~~*(b) Have a sewage treatment plant outfall or other point source outfall of public health significance within or adjacent to the growing area shall have an area in the prohibited classification established adjacent to the outfall in accordance with Section E. Prohibited Classification; and*~~

~~*(c) Is subjected to…*~~

~~*(3) Boundaries…*~~

~~*(4) Revision of Classifications…*~~

~~*(5) Status of Growing Areas…*~~

Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification

E. Prohibited Classification.

~~*(1) Exception…*~~

~~*(2) General…*~~

~~*(3) Sanitary Survey. A growing area shall be classified as prohibited if:*~~

~~*(a) No current sanitary survey exists;*~~

~~*(b) A sanitary survey determines:*~~

~~*(i) The growing area is adjacent to a sewage treatment plant outfall or other point source outfall with public health significance;*~~

~~*(ii) Pollution sources may unpredictably contaminate the growing area;*~~

~~*(iii) The growing area is contaminated with fecal waste so that the shellfish may be vectors of disease microorganisms;*~~

~~*(iv) The concentration of…*~~

~~*(v) The area is contaminated with poisonous or deleterious substances causing the shellfish to be adulterated.*~~

~~*(4) Risk Assessment. A growing area shall be classified as prohibited if a risk assessment performed in accordance with Chapter II. Risk Assessment and Risk Management indicates the shellstock are not safe for human consumption.*~~

(1) ~~*(5)*~~ *Wastewater Discharges.*

(a) An area classified as prohibited shall be established adjacent to each sewage treatment plant outfall or any other point source outfall of public health significance.

(b) The determination of the size of the area to be classified as prohibited adjacent to each outfall shall include the following minimum criteria:

(i) The volume flow rate, location of discharge, performance of the wastewater treatment plant and the microbiological quality of the effluent. The Authority

may utilize MSC ~~waste water~~wastewater sample data in the determination of the performance of the sewage treatment plant;

(ii) The decay rate of the contaminants of public health significance in the wastewater discharged;

(iii) The wastewater's dispersion and dilution, and the time of waste transport to the area where shellstock may be harvested; and

(iv) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries.

~~**C. Allowable Uses of Shellfish from a Prohibited Growing Area**~~

~~(1) Depletion~~

~~Depletion means the removal, under the direct control of the Authority, of shellstock from a growing area classified as prohibited.~~

~~(2) Seed~~

~~Seed means shellstock which is less than market size and complies with the criteria in NSSP Model Ordinance Chapter VI. Shellfish Aquaculture @.02 Seed Shellstock where necessary.~~

~~**D. Model Ordinance Requirements for Depletion and Gathering of Seed**~~

~~(1) Chapter IV. Shellstock Growing Areas~~

~~***@.03 Growing Area Classification***~~

~~*E. Prohibited Classification*~~

~~*(1) Exception…*~~

~~*(2) General. The Authority shall:*~~

~~*(a) Not permit the harvest of shellstock from any area classified as prohibited, except for the harvest of shellstock for the gathering of seed or nursery culture for aquaculture or resource enhancement or the depletion of the areas classified as prohibited; and*~~

~~*(b) Ensure that shellstock removed from any growing area classified as prohibited is effectively excluded from human consumption unless it is seed to be cultured as outlined in the NSSP Model Ordinance Chapter VI. Shellfish Aquaculture @.02 Seed Shellstock.*~~

~~*(3) Sanitary Survey…*~~

~~*(4) Risk Assessment…*~~

~~*(5) Wastewater Discharges…*~~

~~(2) Chapter VI. Shellfish Aquaculture~~

~~*Requirements for the Harvester/Dealer*~~

~~***.03 Seed Production in Water Classified as Prohibited or Unclassified***~~

~~*Seed may come from any growing area, or from any growing area in any classification, provided that:*~~

~~*A. The source of the seed, if from waters classified as prohibited or unclassified, is sanctioned by the Authority; and*~~

~~*B. Operational Plan. Each aquaculture site that cultures seed in waters classified as prohibited or unclassified shall have a written operational plan. The plan shall be approved by the Authority prior to its implementation and shall include:*~~

~~*(1) A description of the design and activities of the culture facility;*~~

~~*(2) The specific site and boundaries in which shellfish aquaculture activities will be conducted;*~~

~~*(3) The types and locations of any structures, including rafts, pens, cages, nets, or floats which will be placed in the waters;*~~

~~*(4) The species of shellfish to be cultured and harvested;*~~

~~*(5) Procedures to assure that no poisonous or deleterious substances are introduced from the seed production activities; and*~~

~~*(6) Corrective actions for addressing seed exceeding the maximum seed size as defined by the Authority.*~~

II. ~~E.~~ Guidance for Determining the Size of Each Prohibited Area Adjacent to a WWTP

The NSSP M~~odel O~~rdinance ~~also~~ requires that an area classified as prohibited ~~must~~ be established between any sewage treatment plants or other waste discharge of public health significance and any growing area placed in the approved, conditionally approved, restricted, or conditionally restricted classification. The size of the prohibited area should be based on the effectiveness and level of sewage treatment; the location of the shellstock resource that would be affected; the classification of adjacent waters, the total time it would take for the person responsible for the operation of the sewage treatment facility to detect a failure and notify the Authority; the time it would take the Authority to issue a notice to stop shellstock harvesting, and the degree of effluent dilution. The size of the area in the prohibited classification should allow for an effluent travel time through the prohibited area that is at least twice that required for the notification process to become effective. Due consideration should be given to the possibility that emergency actions might be necessary on holidays or at night. A minimum effluent dilution is to be determined at the prohibited boundary and can be the controlling factor in situations where there are efficient detection and notification of breakdowns.

There are several important considerations for the shellfish authority ~~to consider~~ when establishing the size of each prohibited area adjacent to a WWTP discharge:

(1) The area is large enough to ensure that there is adequate dilution for the type of classification that will be used adjacent to the prohibited area. If a conditional classification (either conditionally restricted or conditionally approved) based on WWTP function is established adjacent to the prohibited area, adequate dilution should be determined when the WWTP is operating as normal. "Normal" means that the WWTP is operating fully within the plant's design specifications, including design flows; treatment stages; disinfection; as well as compliance with all permit conditions that relate to the WWTPs effectiveness in reducing enteric viruses in discharged wastewater.

Should a restricted area for the purposes of relaying or depuration be established adjacent to the prohibited area, establishing the size of the prohibited area should be based on worst case plant operating conditions. This same consideration would apply for an approved area adjacent to the prohibited area.

Below are several scenarios that could occur and are critical for Authorities on evaluating each WWTP when determining appropriate classifications:

(a) Bypassing stage of treatment
A treatment plant should be considered operating outside of normal operation if a treatment stage such as primary or secondary treatment is bypassed which may result in an increased load of solids in the disinfection step and reduce the effectiveness of disinfection. An additional example would be when a WWTP experiences a loss in disinfection and thus the ability to effectively treat the final effluent. ~~SCAs~~ The Authority should determine the significance of these types of events and determine appropriate classification for the growing waters adjacent to the prohibited area.

(b) Operating outside design specifications/other types of failures or events
It is not uncommon for a WWTP to periodically experience mechanical failures of equipment that could alter the treatment of sewage. Additionally, a WWTP may also need to periodically ~~perform~~undergo routine maintenance to the various stages of treatment and may need to temporarily take a portion of a treatment stage off-line for cleaning. Other unexpected maintenance may need to occur. For example, cleaning of filters or membranes that have become bio-fouled.

(c) Operating above design flow
Some WWTPs may operate above ~~its~~their design flows and not necessarily bypass any particular stage of treatment. During these events it is typical for WWTP operators to adjust the operation of

the WWTP which may include reducing the treatment time in the aeration stage and/or solids separation/settling stage of treatment. Under some circumstances this could lead to a significant reduction in the effectiveness of disinfection. ~~SCAs~~ The Authority may consider assessing the efficiency of WWTPs to determine the significance of these type of events.

(d) WWTP permit violations

If a WWTP is exceeding the permitted bacterial indicator levels in the final effluent this indicates that effectiveness of the disinfection step has been reduced. Other measured parameters in the effluent (e.g. Total Suspended Solids (TSS), Biochemical Oxygen Demand (BOD)) may also indicate a reduction in treatment efficiency has occurred. ~~SCAs~~ The Authority may consider assessing the efficiency of WWTPs to determine the significance of these types of events.

Compliance ~~of~~with WWTP operation permit limits~~compliance~~ does not necessarily eliminate the potential transmission of pathogens present in wastewater effluent to contaminating shellfish in the impacted area.

There could be situations in which a particular WWTP could be in compliance with a permit~~,~~ and could still pose a risk to the shellfish harvest area. For example, a WWTP may have permit conditions to allow for flow blending during high flow periods where a portion of the sewage may receive full treatment, but a portion of the sewage may only be partially treated and "blended" in the final disinfection step. Although this may be an acceptable practice under a permit it could result in conditions in which the efficiency of the WWTP to remove enteric viruses is considerably reduced. ~~SCAs~~ The Authority may consider assessing the efficiency of WWTPs to determine the significance of these events.

(2) The integrity of the collection system. Collection system malfunctions, bypasses or other factors can lead to significant leakages of untreated sewage to the marine environment.

(3) That there is adequate detection and response time when a malfunction occurs to ensure that all harvesting ceases and closures are enforced, so that contaminated product does not reach the market.

III. ~~F.~~ **Guidance for the Use of MSC in Shellfish Meats in determining the size of the prohibited area impacted by WWTP discharge.**

MSC has been demonstrated to accurately assess enteric virus dynamics through contaminant mitigation strategies such as relay. MSC levels in shellstock from growing areas adjacent to WWTP discharge are a function of WWTP performance, seasonal persistence of viruses in the environment and the shellfish, species-specific anomalies, and distance from the outfall. The regulatory level of 50 PFU/100 g is a conservative value used for re-opening approved growing areas (after 7 days) after a sewage spill and end point target values for viral relay. Before using MSC for these purposes, the Authority should perform preliminary studies to familiarize themselves with the seasonal viral persistence patterns as well as regional and species-specific anomalies.

Seasonal persistence of MSC in shellfish meats can vary greatly from warm summer months to the cooler fall, winter, and spring months. MSC levels can be 2 to 3 logs (100 to 1000 times) higher in the late fall, winter, and early spring months demonstrated by multiple studies ~~from~~ conducted in northern temperate latitudes using ~~both~~ MSC. ~~and m~~Molecular enumeration using PCR for enteric viruses also demonstrates this trend. This dramatic tendency to accumulate and retain virus particles by levels 2 to 3 logs higher during ~~over~~ the winter months than those accumulated and retained during the summer months has species-specific implications for warm-water adapted species such as American oysters and northern quahogs, which tend to reduce or stop pumping ~~shut down~~ as cooling water temperatures approach 10°C. Viruses and bacteria bio-accumulated in shellfish behave very differently; fecal coliforms (FC) are~~is~~ prone to die-off in a week or two over colder months while viruses can persist at high levels under these cold water conditions for months. Cold-water adapted species such as soft-shelled clams, Pacific oysters, European oysters, and mussels all demonstrate the tendency to increase accumulation values by 2 to 3 log values over the colder months.

If the Authority is interested in using MSC in shellfish meats, it is recommended that monthly samples be taken over the course of a year in multiple growing areas inside the 1000:1 dilution line to understand these seasonal, spatial, and species-specific variations. Thesesis data can be very useful toin assessing the feasibility of using the conditionally restricted classification for the purpose of relay.

IV. ~~G.~~ **Use of MSC in Evaluating WWTP Efficiency**

At a minimum, MSC may be used in conjunction with conventional bacterial indicators to conduct a comprehensive assessment of WWTP microbiological performance. The differences between influent, pre-disinfection effluent, and final effluent samples taken under normal and challenged conditions can be used to assess the viral deactivation efficiency of a specific ~~waste water~~wastewater treatment process. The analysis is somewhat similar to the determination of WWTP efficacy using bacterial indicators such as *E. coli*, which is currently used to comply with EPA's National Pollution Discharge Elimination System (NPDES) permit requirements for municipal wastewater treatment plant discharge. ~~Many~~ Studies have shown that deactivation of bacterial and viral indicators (and pathogens) can be significantly different in different treatment processes and under challenged conditions. There are several case studies showing that under certain conditions, differential bacterial indicators may indicate highly effective treatment of wastewater while differential MSC samples show little deactivation efficiency.

By collecting differential wastewater samples including influent, pre-disinfection effluent, and final effluent and evaluating those samples for MSC, the viral performance of the wastewater treatment process can be determined. If a comprehensive sampling program includes sufficient samples to assess the WWTP under typical operating conditions as well as challenged condition such as high flow, the viral elimination efficiency of the WWTP can be determined. A comprehensive assessment of WWTP microbiological performance using MSC as well as the conventional bacterial indicators can inform the Authority on the risk associated with a growing area adjacent to a WWTP outfall. An assessment of a WWTP must demonstrate the range of effluent quality during routine operation through an appropriate sampling study and the ability to accurately predict those times when effluent microbiological quality is detrimentally impacted by challenged conditions.

~~**H. Public Health Significance**~~

~~The positive relationship between disease and consuming contaminated shellfish has been clearly established. Prevention of consumption of contaminated shellfish is the primary objective of the NSSP. The prohibited area classification is the most restrictive growing area classification and is used for areas subject to gross pollution. The use of this classification is also required for all growing areas immediately adjacent to a wastewater treatment plant and where the shellfish authority has not performed a sanitary survey. The harvesting of shellstock is not allowed for any human food use except for the gathering of seed or nursery culture for aquaculture or resource enhancement. For additional information concerning the classification of growing waters and the sanitary survey, see the NSSP Model Ordinance. Depletion and Gathering of Seed (Chapter IV @.03 E. Prohibited Classification (2) (a) & (b) and Chapter VI .03 Seed Shellstock A. & B.)~~

~~A growing area is placed in the prohibited classification when the sanitary survey or marine biotoxin surveillance program indicates that fecal material, pathogenic microorganisms, poisonous or deleterious substances, marine biotoxin, or radionuclides may reach the harvest area in excessive concentrations. The NSSP Model Ordinance also requires that a growing area for which there is no sanitary survey be placed in the prohibited classification as a precautionary measure. Taking shellstock from a prohibited area for any human food purpose is not allowed.~~

~~The NSSP Model Ordinance also requires that an area classified as prohibited must be established between any sewage treatment plants or other waste discharge of public health significance and any growing area placed in the approved, conditionally approved, restricted, or conditionally restricted classification. The size of the prohibited area should be based on the effectiveness and level of sewage treatment; the location of the shellstock resource that would be affected; the classification of adjacent waters, the total time it would take for the person responsible for the operation of the sewage treatment~~

~~facility to detect a failure and notify the Authority; the time it would take the Authority to issue a notice to stop shellstock harvesting, and the degree of effluent dilution.~~

V. ~~I.~~ **Establishment of Boundaries for the Prohibited Area**

The establishment of the boundary for the prohibited area is dependent upon ~~other~~ classification ~~which may be~~ of the area(s) adjacent to the prohibited area. Examples could include water bodies in which the Authority chose to use all five (5) classifications or a situation where the Authority only uses prohibited and approved. The decision of adjacent classifications is often based on shellfish uses for the water body or environmental control and protection efforts by ~~S~~state law enforcement ~~Water Control~~ agencies. The requirements of the classification adjacent to the prohibited area and the allowable uses in the areas will often dictate the distance the boundary line for the prohibited area is from the outfall.

Guidance for Dilution Ratios

To determine the impact of a WWTP on adjacent waters, it is imperative that the Shellfish Control Authority assess the ~~waste water~~wastewater dispersal and dilution and the time of transport to the area where shellstock may be harvested. In determining the appropriate dilution for establishing the size of the prohibited area, the Shellfish Control Authority must determine the classification which will be adjacent to the prohibited area. The dilutions below outlines recommended dilution for the boundary line between prohibited and other possible classifications based on dilutions of WWTP effluent, based on initial FC values of 1.4 x 10^6 FC/100 ml(. Each of these dilutions will be discussed in more detail in the context of each classification.

(1) Prohibited to Restricted Boundary

Minimum dilution – The ~~SCA~~Authority should determine the effluent quality based on a worst case scenario and should establish a dilution ratio that would accomplish a dilution equivalent to a MPN of 88 (or 163) which is the upper limit restricted standard for depuration and relaying without a contaminant reduction study.

(2) Prohibited to Conditionally Restricted Boundary

Minimum dilution of 320:1 based on "Critical Dilution for Toxics to Ambient (Background)" from the Clean Water Act and EPA's Regulatory Mixing Zone (RMZ).

(3) Prohibited to Conditionally Approved Boundary

Minimum dilution 1000:1 or justified by other data.

(4) Prohibited to Approved Boundary

Minimum dilution >100,000:1 ~~dilution~~ based on worst case scenario or justified by other data.

~~V. Restricted Classification.~~

~~**A. Definition**~~

~~A classification used to identify a growing area where harvesting shall be by special license and the shellstock, following harvest, is subjected to a suitable and effective treatment process through relaying or depuration.~~

~~**B. Requirements for Use of the Restricted Classification**~~

~~(1) Chapter IV. Shellstock Growing Areas~~

~~***@.03 Growing Area Classification***~~

~~*A. General…*~~

~~*B. Approved Classification…*~~

~~*C. Conditional Classifications…*~~

~~*D. Restricted Classification.*~~

~~*(1) General*~~

~~*(a) A growing area may be classified as restricted when:*~~

~~*(i) A sanitary survey indicates a limited degree of pollution; and*~~

~~*(ii) Levels of fecal pollution, human pathogens, or poisonous or deleterious substances are at such levels that shellstock can be made safe for human consumption by either relaying, depuration or low acid canned*~~

food processing.

(b) The Authority shall have effective controls to assure that shellfish are harvested from restricted areas only:

(i) By special license; and

(ii) Under the supervision of the Authority.

(2) Water Quality. Water quality in the growing area shall meet the bacteriological standards in Section @.02 for a growing area in the restricted classification if the growing area is used for depuration. (These standards are included later in this section.)

(3) Shellstock Quality Criteria. The Authority shall establish shellstock quality criteria for use in placing an area in the restricted classification. Depending on the treatment process to be applied to the shellstock, the criteria shall be established in accordance with:

(a) Chapter V. Shellstock Relaying; or

(b) Chapter XV. Depuration

E. Prohibited Classification…

C. Allowable Uses of Shellfish from a Restricted Growing Area

(1) Relay with a Contaminant Reduction Study

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(2) Relay without a Contaminant Reduction Study

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(3) Depuration

Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(4) Seed

Seed means shellstock which is less than market size.

D. Model Ordinance Requirements for Relaying with a Contaminate Reduction Study

(1) Chapter V. Shellstock Relaying

@.01 General

The Authority shall assure that:

A. The shellstock used in relaying activities is harvested from growing areas classified as conditionally approved, restricted, or conditionally restricted;

B. The level of contamination in the shellstock can be reduced to levels safe for human consumption;

C. The contaminated shellstock are held in growing areas classified as approved or conditionally approved for a sufficient time under adequate environmental conditions so as to allow reduction of pathogens as measured by total coliform or fecal coliform or poisonous or deleterious substances that may be present in shellstock. For shellstock harvested from areas impacted by WWSD, MSC may be used as a measure for viral reduction.

D. If shellstock are relayed in containers:

(1) The containers are:

~~(a) Designed and constructed so that they allow free flow of water to the shellstock; and~~

~~(b) Located so as to assure the contaminant reduction required in Section C.; and~~

~~(2) The shellstock are washed and culled prior to placement in the containers.~~

~~***@.02 Contaminant Reduction.***~~

~~A. The Authority shall establish species-specific critical values for water temperature, salinity, and other environmental factors which may affect the natural treatment process in the growing area to which shellstock will be relayed. The growing area to be used for the treatment process shall be monitored with sufficient frequency to identify when limiting critical values may be approached.~~

~~B. The effectiveness of species-specific contaminant reduction shall be determined based on a study. The study report shall demonstrate that, after the completion of the relay activity:~~

~~(1) The microbiological quality of each shellfish species is the same microbiological quality as that of the same species already present in the approved or conditionally approved area; or~~

~~(2) Contaminant levels of poisonous or deleterious substances in shellstock do not exceed FDA action levels, tolerances and/or guidance levels and/or levels that are deemed safe through risk evaluations; or~~

~~(3) When the source growing area is impacted by WWSD, the viral quality of each shellfish species meets the MSC standard of 50 PFU/100 g or predetermined levels established by the Authority based on studies conducted on regional species under regional conditions.~~

~~C. The authority may waive the requirements for a contaminant reduction study if:~~

~~(1) Only microbial contaminants need to be reduced; and~~

~~(2) The shellstock are relayed from a conditionally approved, restricted, or conditionally restricted area meeting the bacteriological water quality for restricted areas used for shellstock depuration per Chapter IV. @.02 G. and Chapter IV. @.02 H.; and~~

~~(3) The treatment period exceeds sixty (60) days.~~

~~D. The time period shall be at least fourteen (14) consecutive days when environmental conditions are suitable for shellfish feeding and cleansing unless shorter time periods are demonstrated to be adequate.~~

~~E. When container relaying is used and the Authority allows a treatment time of less than fourteen (14) days, the Authority shall require more intensive sampling including:~~

~~(1) Product sampling before and after relay; and~~

~~(2) Monitoring of critical environmental parameters such as temperature and salinity; and~~

~~(3) For Authorities using MSC, monitoring before and after relay for shellstock relayed from areas impacted by WWSD.~~

~~F. The Authority shall establish the time period during the year when relaying may be conducted.~~

~~In addition to the requirements of Chapter IV. @.02 G. & H., restricted growing waters used for relaying without a contaminant reduction study must meet the requirements of Chapter IV. @.03 D.~~

VI. ~~E.~~ Guidance for Restricted Classification for Relaying with a Contaminant Reduction Study

Refer to NSSP MO Chapter IV. @.03 D. for requirements for use of the restricted classification and to NSSP MO Chapter V. for requirements for shellstock relaying.

~~Model Ordinance~~ NSSP MO Chapters IV. and V. do not include microbial standards for classifying growing areas as restricted that are the source of shellstock for relaying when a contaminant reduction study is required. In establishing of the boundary between prohibited and restricted classifications, the Authority must ensure that levels of fecal pollution, human pathogens, or poisonous or deleterious substances are at such levels that shellstock can be made safe for human consumption by either relaying, depuration or low acid-canned food processing.

~~In determining an appropriate boundary, the Authority shall consider the following factors associated with the wastewater discharge:~~

~~(1) The volume flow rate, location of discharge, performance of the wastewater treatment plant and the microbiological quality of the effluent. The Authority may utilize MSC waste water~~wastewater~~ sample data in the determination of the performance of the sewage treatment plant;~~

~~(2) The decay rate of the contaminants of public health significance in the wastewater discharged;~~

~~(3) The wastewater's dispersion and dilution, and the time of waste transport to the area where shellstock may be harvested; and~~

~~(4) The location of the shellfish resources, classification of adjacent waters and identifiable landmarks or boundaries.~~

A growing area may be placed in the restricted classification instead of the prohibited classification when the sanitary survey indicates a limited degree of pollution. This option may be used when the sanitary survey for the growing area indicates that the microbiological quality or poisonous or deleterious substances in the growing area are such that additional treatment through relay can render the shellstock safe for human consumption. The Authority should use the restricted classification only when sufficient relay studies have been conducted to establish raw product quality requirements at the harvest level; and when the Authority has sufficient administrative and technical resources to properly administer this classification. These resources include monitoring of pollution sources; providing coordination between ~~S~~state, local and industry officials; issuing special harvesting permits; and supervising the harvesting and transport of shellstock to relay sites. For a complete discussion of the supervision requirements at the harvest level, see the NSSP Guidance Document, *Shellstock Relay*.

~~Use of the restricted classification for relaying with a contaminant reduction study requires the Authority to develop the controls necessary to assure that the shellfish are relayed prior to consumption. The criteria may vary according to the use to be made of the shellstock and the effectiveness of the relay process used to cleanse the shellstock. Process effectiveness is determined through a study, which establishes the levels of microbiological quality indicators in shellstock at the time of harvest, and the density that can be achieved at the completion of the process. Effectiveness of the process is likely to vary between growing areas used for natural cleansing treatment in relay operations. The species of shellstock may also affect the effectiveness of the relay. For a complete discussion of relay, see the NSSP Guidance Document, *Shellstock Relay* .~~

~~F. Guidance for Conducting a Contaminant Reduction Study for Relay~~

~~The use of the restricted classification for the purpose of relaying with a contaminant reduction study does not require the authority to demonstrate that the growing area meets a microbiological water quality standard. However, in determining the boundary between the prohibited area and the restricted area for relaying with a contaminant reduction study, the authority shall give consideration to the types of contamination that may be in the growing area prior to allowing the area to be in the source of shellfish for relaying. The contaminants may include:~~

- ~~Pathogenic Organisms~~
- ~~Poisonous or Deleterious Substances~~
- ~~Marine Biotoxins~~
- ~~Physical and Chemical Contaminants~~

~~Contaminant Reduction is a specified activity defined in Chapter V. Shellstock Relaying @.02. The authority shall establish species-specific critical values for water temperature, salinity and other environmental factors such as dissolved oxygen and turbidity which may affect the natural treatment process (e.g. relay process). These critical values must be monitored and the Authority shall establish the time of year when relay may be conducted. The relay process requires that shellstock are held in the receiving growing area for a sufficient time under adequate environmental conditions to allow reduction of pathogens as measured by total coliform or fecal coliform. To verify the effectiveness of a relay process, contaminant reduction studies are required. The only exception to this requirement is when water quality in the restricted growing area meets Chapter IV.@.02-G-H, only microbial contaminants need to be reduced, and the treatment period exceeds sixty (60) days. For all other relay operations, the Authority shall establish species-specific critical values for water temperature, salinity, and other environmental factors which may affect the natural treatment process in the receiving growing area. The receiving waters shall be monitored with sufficient frequency to identify when limiting critical values may be approached. The effectiveness of species-specific contaminant reduction shall be determined based on a study. The study shall demonstrates that after the completion of the relay activity, the microbiological quality of each shellfish species is the same microbiological quality as that of the same species already present in the approved or conditionally approved area or contaminants levels of poisonous or deleterious substances in shellstock do not exceed NSSP tolerance levels. Based on the study, the Authority shall establish the time period during the year when relaying may be conducted. Shellstock shall be relayed for at least fourteen (14) consecutive days when environmental conditions are suitable for shellfish feeding and cleansing unless shorter time periods are demonstrated with the contaminant reduction study to be adequate. If the shellstock are container relayed and the treatment times are less than fourteen (14) days, intensive sampling is required. This intensive sampling includes lot sampling before and after relay as well as monitoring of critical environmental parameters such as seawater temperature and salinity.~~

~~Although minimum requirements for contaminant reduction studies have not been specified in the Model Ordinance, there are certain principles of process verification studies that should be considering including; study design, sampling replicates, and data analysis providing statistical reliability. Shellstock and water samples collected during a contaminant reduction study must be analyzed in NSSP-conforming laboratories using NSSP-approved methods. Shellfish samples should be collected at regular intervals from both source and receiving growing areas over the time period of the relay and the natural cleansing process that is proposed. It is important to produce a sufficiently robust database to demonstrate the process is consistently working and the variables affecting the cleansing process are understood. All shellfish samples of ten (10) to twelve (12) animals should be collected in triplicate so that the mean as well as standard deviation or standard error can be calculated. Water temperature and salinity should be measured at both source and receiving waters at the time of shellstock collection. Fecal coliform levels of shellstock already present in the receiving growing area should be collected in triplicate and evaluated for comparison to relayed shellstock microbial levels. Contaminant reduction studies are specific to species, source growing area, and receiving growing area. In States with extensive experience with relay practices, the Authority may approach contaminant reduction studies on a more regionally basis covering multiple source and receiving growing areas.~~

~~When the source growing area is adjacent to a WWTP outfall, the Authority may utilize MSC in conducting the contaminant reduction study. Should the Authority utilize MSC sampling, the MSC levels in each shellfish species after the relay process must be assessed. The MSC standard of 50 PFU/100 g or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions are both approved for these assessments. Relay dynamics for bacterial and viral pathogens can be very different and assessing both offers more insight into the potential health risk. Seasonal persistence of bio-accumulated viral particles in shellfish can range 1,000 times higher in the winter months verses the summer months. Depuration rates can vary from 1 log in 44 hours at receiving water temperature above 18 °C to 1 log in 25 days when receiving water temperature fall below 10 °C. Understanding these dynamics for each species and region is paramount to successful relay from restricted or conditionally restricted growing areas adjacent to WWTP outfall. When container relaying is considered and treatment times of less than 14 days are planned, an intensive MSC sampling program based on before and after relay samples can be utilized to assure relayed~~

shellstock are less than the 50 PFU/100 g standard or pre-determined levels established by the authority based on studies conducted on regional species under regional conditions.

G. Guidance for the use of MSC in Contaminant Reduction Studies and Process Control for Shellstock Relay

MSC has been shown to be an appropriate modeling organism for contaminant reduction studies and process verification for shellstock from growing areas impacted by a WWTP outfall. The ability of MSC to model enteric viral dynamics in relay and depuration has been demonstrated in several studies using different species in different parts of the northern temperate zone. The MSC standard of 50 PFU/100 g used in process end-point samples was shown to be conservative with respect to public health outcomes.

The conditionally restricted classification recommended for relay adjacent to WWTP outfalls where contaminant studies will be used, should have limits such as zero-hour maximum MSC limits in the shellstock from the source growing areas, seasonal limits, and receiving water temperature and salinity limits as determined by comprehensive contaminant reduction studies. This is in addition to controls to assure the continued operation of the adjacent WWTP under the management plan to keep the source growing area in the restricted status. MSC data from sampling shellstock from the source growing area may help determine those times when viral loading and/or viral persistence in the shellstock are low and viral mitigation strategies are feasible. In both viral depuration and viral relay pilot studies using soft-shelled clams in Maine, periods of time were identified using bi-weekly MSC assays of the target species to understand those times when bio-accumulated MSC levels in the shellstock were at a seasonal low (low viral persistence). Receiving waters temperatures were correspondingly high in those summer months resulting in significantly higher depuration rates, especially when water temperature exceeded 64.4 °F (18 °C). Studies showed the depuration rate approached a single log reduction in forty-four (44) hours when water temperatures were above 64.4 °F (18 °C). In contrast, those studies also determined that as water temperature approached 41 °F (5 °C), it would take approximately twenty (20) days to see a comparable single log reduction in MSC levels. The combination of seasonally low MSC levels in the soft-shelled clams and higher summertime depuration rates resulted in successful depuration consistently meeting a shellfish end-point of 50 PFU/100 g.

Species-specific and regional anomalies in persistence and relay and depuration dynamics require that comprehensive contaminant reduction studies be performed for each growing area for each relay or depuration process being considered. In planning a comprehensive contaminant reduction study, sufficient quantities of target specie(s) from the source area should be collected on a regular basis and evaluated for fecal coliform and MSC (triplicate samples of 10-12 animals), during that period of time when the restricted harvest is being considered. Background levels of MSC are not known in a new species or region, the Authority might consider collecting samples year round in the first year to understand the range of viral persistence throughout the year to understand those times when viral mitigation strategies are feasible.

Trial lots of shellstock should be evaluated monthly during the period of time when the relay is being considered. One (1) to two (2) bushels are adequate for relay trials. Triplicate shellfish samples of ten (10) to twelve (12) animals from the approved relay site should be collected at appropriate intervals and analyzed for fecal coliform and MSC. Contaminant reduction studies should use triplicate samples so that variation as well as mean value can be assessed yielding improved statistical reliability for the contaminant reduction studies. If little is known about the depuration rates of the target species, it may be necessary to conduct a separate study using shellfish that are highly contaminated with MSC to assess the viral depuration rate in that region. The goal of contaminant reduction studies is to show those periods of time and the conditions when relay is effective.

The Authority may permit an end-point value other than 50 PFU/100 g based if pre-determined levels established by the Authority based on studies conducted on regional species in regional conditions are known.

H. Model Ordinance Requirements for Relaying without a Contaminant Reduction Study

~~(1) Chapter V. Shellstock Relaying~~

~~***@.01 General***~~

~~*The Authority shall assure that:*~~

~~*A. The shellstock used in relaying activities is harvested from growing areas classified as conditionally approved, restricted, or conditionally restricted.*~~

~~*B. The level of contamination in the shellstock can be reduced to levels safe for human consumption;*~~

~~*C. The contaminated shellstock are held in growing areas classified as approved or conditionally approved for a sufficient time under adequate environmental conditions so as to allow reduction of pathogens as measured by total coliform or fecal coliform. For shellstock harvested from areas impacted by WWSD, MSC may be used as a measure for viral reduction, or poisonous or deleterious substances that may be present in shellstock.*~~

~~*D. If shellstock are relayed in containers:*~~

~~*(1) The containers are:*~~

~~*(a) Designed and constructed so that they allow free flow of water to the shellstock; and*~~

~~*(b) Located so as to assure the contaminant reduction required in Section C.; and*~~

~~*(2) The shellstock are washed and culled prior to placement in the containers.*~~

~~(2) Chapter V. Shellstock Relaying~~

~~***@.02 Contaminant Reduction***~~

~~*C. The Authority may waive the requirements for a contaminant reduction study if:*~~

~~*(1) Only microbial contaminants need to be reduced; and*~~

~~*(2) The shellstock are relayed from a conditionally approved, restricted, or conditionally restricted area meeting the bacteriological water quality for restricted areas used for shellstock depuration per Chapter IV. @.02 G. and Chapter IV. @.02 H.; and*~~

~~*(3) The treatment period exceeds sixty (60) days*~~

~~(3) Chapter IV. Shellstock Growing Areas~~

~~***@.02 Microbiological Standards***~~

~~*G. Standard for the Restricted Classification of Growing Areas when Evaluated for Adverse Pollution Conditions and Used as a Shellstock Source for Shellstock Depuration.*~~

~~*(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section G. (2).*~~

~~*(2) Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed an MPN or MF (mTEC) of:*~~

~~*(a) 300 MPN per 100 ml for a three-tube decimal dilution test;*~~

~~*(b) 173 MPN per 100 ml for a twelve-tube single dilution test; or*~~

~~*(c) 163 CFU per 100 ml for a MF (mTEC) test.*~~

~~*(3) Required Sample Collection. Samples shall be collected in accordance with Section E. (3).*~~

~~*H. Standard for the Restricted Classification of Growing Areas when Evaluated for Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration.*~~

~~*(1) Exception. If the tidal stage increases the fecal coliform concentration, the Authority shall use samples collected under that tidal stage to classify the area.*~~

~~*(2) Pollution Sources. Growing areas shall meet the requirements in Section F. (2).*~~

~~*(3) Water Quality. The bacteriological quality of every sample station in the growing area shall meet the fecal coliform standard in Section G. (2) or Section H. (4).*~~

~~*(4) Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed a MPN or MF (mTEC) of:*~~

~~*(a) 260 MPN per 100 ml for a five-tube decimal dilution test;*~~

~~*(b) 300 MPN per 100 ml for a three-tube decimal dilution test; or*~~

~~*(c) 163 CFU per 100 ml for a MF (mTEC) test.*~~

~~*(5) Estimated 90th Percentile. The estimated 90th percentile shall be calculated by the same method described in Section F. (5).*~~

~~*(6) Required Sample Collection.*~~

~~*(a) Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity and frequency as described in Section E. (3) for application of the standard under Section G. (2).*~~

~~*(b) Systematic Random Sampling Standard. The Authority shall collect samples in the same intensity and frequency, and shall apply the sample results in the manner described in Section F. (6) for the application of the standard under Section H. (4).*~~

~~In addition to the requirements of Chapter IV @.02 G & H., restricted growing waters used for relaying without a contaminant study must meet the requirements of Chapter IV @.03 D. (Page 12)~~

VII. Guidance for Restricted Classification for Relay Without a Contaminant Reduction Study

The NSSP Model Ordinance provides Authorities the option to allow relaying from a restricted area affected by a point source without a contaminant reduction study. The requirement for establishing the restricted classification for this use is different than the requirements for relay with a contaminant reduction study. The Authority must assure that the bacteriological quality of every station meets Chapter IV, @.02 G, (2). Additionally, the treatment period must exceed sixty (60) days. Should the Authority have viral concerns, the use of MSC sampling of the shellfish would be appropriate. The Authority could use the 50 PFU/100 g level or predetermined levels established by the Authority based on studies conducted in the area.

~~**J. Model Ordinance Requirements for Depuration**~~

~~(1) Chapter IV. Shellstock Growing Areas~~

~~***@.02 Microbiological Standards.***~~

~~*G. Standard for the Restricted Classification of Growing Areas when Evaluated for Adverse Pollution Conditions and Used as a Shellstock Source for Shellstock Depuration.*~~

~~*(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section G. (2).*~~

~~*(2) Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed an MPN or MF (mTEC) of:*~~

~~*(a) 300 MPN per 100 ml for a three-tube decimal dilution test;*~~

~~*(b) 173 MPN per 100 ml for a twelve-tube single dilution test; or*~~

~~*(c) 163 CFU per 100 ml for a MF (mTEC) test.*~~

~~*(3) Required Sample Collection. Samples shall be collected in accordance with Section E. (3).*~~

~~*H. Standard for the Restricted Classification of Growing Areas when Evaluated for Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration.*~~

~~*(1) Exception. If the tidal stage increases the fecal coliform concentration, the Authority shall use samples collected under that tidal stage to classify the area.*~~

~~*(2) Pollution Sources. Growing areas shall meet the requirements in Section F. (2).*~~

~~*(3) Water Quality. The bacteriological quality of every sample station in the growing area shall meet the fecal coliform standard in Section G. (2) or Section H. (4).*~~

~~*(4) Fecal Coliform Standard for Systematic Random Sampling. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed 88 per 100 ml and the estimated 90th percentile shall not exceed a MPN or MF (mTEC) of:*~~

~~*(a) 260 MPN per 100 ml for a five-tube decimal dilution test;*~~

~~*(b) 300 MPN per 100 ml for a three-tube decimal dilution test; or*~~

~~*(c) 163 CFU per 100 ml for a MF (mTEC) test.*~~

~~*(5) Estimated 90th Percentile. The estimated 90th percentile shall be calculated by the same method described in Section F. (5).*~~

~~*(6) Required Sample Collection.*~~

~~*(a) Adverse Pollution Condition Standard. The Authority shall collect samples in the same intensity and frequency as described in Section E. (3) for application of the standard under Section G. (2).*~~

~~(b) Systematic Random Sampling Standard. The Authority shall collect samples in the same intensity and frequency, and shall apply the sample results in the manner described in Section F. (6) for the application of the standard under Section H. (4).~~

~~(2) Chapter XV. Depuration~~

~~***.01 Critical Control Points.***~~

~~*A. Receiving Critical Control Point - Critical Limits.*~~

~~*(1) The dealer shall…*~~

~~*(2) The dealer shall…*~~

~~*(3) Should a dealer…*~~

~~*(4) The dealer shall receive and depurate only shellstock obtained from a special licensed harvester who has:*~~

~~*(a) Harvested or supervised the harvest of shellstock from a Restricted or Conditionally Restricted area in the open status.*~~

~~*(b) Identified the shellstock…*~~

VII. ~~K.~~ Guidance for Restricted Classification for Depuration

Use of the restricted classification for depuration requires the Authority to conduct a sanitary survey of the growing area as required in Chapter IV. @ .01 and establish a monitoring program to ensure the water quality requirements of Chapter IV. @-.02 G. & H. and @.03 D.

~~Depuration process verification described in Chapter XV. @.03 Section J. is based on conditional and approved protocols. The protocol is conditional when statistical analysis of the database containing the 10 most recent FC end point samples fails to meet prescribed species-specific indices. The intent of which is to ensure an appropriate level of testing and quality assurance, including release criteria, during those periods of time when the depuration process is being challenged. These process verification protocols are based on fecal coliform assays of shellfish meats.~~ The requirement for adverse case sampling of the restricted growing area is to assure that water quality in the restricted harvest growing areas does not exceed a median FC score of 88/100 ml (or 163 FC.100 ml) and P90 requirements.

Water quality requirements for the restricted growing area used for depuration were put in place to prevent grossly contaminated shellfish from being processed. It was not the inability to depurate high FC levels from contaminated shellstock, but rather that viruses associated with grossly contaminated shellstock were thought to not effectively depurate viruses in forty-four (44) hours. In contrast, restricted growing areas adjacent to WWTP discharges used for relay with contamination reduction studies are considered effective for viral reductions and do not require a water quality sampling program based on fourteen (14) consecutive days of

relay. The inability to detect viruses using fecal coliform based process verification and the lack of any suitable viral indicator assays was the original rationale behind restricted growing areas for depuration requiring water quality limits.

~~**L. Model Ordinance Requirements for Use of a Restricted Area as the Source of Seed**~~

~~(1) Chapter VI. Shellfish Aquaculture~~

~~***.03 Seed Production in Water Classified as Prohibited or Unclassified***~~

~~*Seed may come from any growing area, or from any growing area in any classification, provided that:*~~

~~*A. The source of the seed, if from waters classified as prohibited or unclassified, is sanctioned by the Authority; and*~~

~~*B. Operational Plan. Each aquaculture site that cultures seed in waters classified as prohibited or unclassified shall have a written operational plan. The plan shall be approved by the Authority prior to its implementation and shall include:*~~

~~*(1) A description of the design and activities of the culture facility;*~~

~~*(2) The specific site and boundaries in which shellfish aquaculture activities will be conducted;*~~

~~*(3) The types and locations of any structures, including rafts, pens, cages, nets, or floats which will be placed in the waters;*~~

~~*(4) The species of shellfish to be cultured and harvested;*~~

~~*(5) Procedures to assure that no poisonous or deleterious substances are introduced from the seed production activities; and*~~

~~*(6) Corrective actions for addressing seed exceeding the maximum seed size as defined by the Authority.*~~

~~**M. Seed**~~

~~If a restricted growing area is used as a source for seed and the Authority requires that the shellfish must be cultured in the approved growing area for a minimum of six (6) months, the classification requirements for relay and depuration are adequate for this use.~~

VIII. ~~N.~~ Determination of the Boundaries Between Prohibited and Restricted Areas

The establishment of boundaries separating prohibited and restricted growing areas is dependent upon the uses to be allowed within the restricted growing area. MO Chapters IV and V address the classification requirements for allowable shellfish uses in the restricted classification. These uses include the following:

(1) Relay with a contaminant reduction study
(2) Relay without a contaminant reduction study
(3) Depuration

~~If harvesting for relay with a contaminant reduction study, the boundary line should be based on an acceptable dilution ratio. If harvesting for relay without a contaminant reduction study or depuration, the boundary line must be based on a fecal coliform sampling program. The SCA~~Authority has the option to utilize MSC.

Guidance for Dilution Ratios

Restricted areas that are the source for shellstock relaying with a contaminant reduction study are not required to meet a microbiological standard. Shellstock from restricted areas used for relaying without a contaminant reduction study or for depuration do have to meet a microbiological standard. In the absence of a microbiological standard, dilution ratios become very important to protect public health. An Authority ~~Shellfish Control Authority~~ should not allow relay with a contaminant reduction study from any portion of a restricted area that does not meet a minimum dilution. The Authority~~SCA~~ should determine the effluent quality based on a worst case scenario and should establish a dilution ratio that would accomplish a dilution equivalent to a MPN of 88 ~~(or 163)~~ which is the upper limit restricted standard for relaying without a contaminant reduction study and for depuration. This dilution is 16,000:1. Should the ~~Shellfish Control~~ Authority choose to classify waters not meeting a dilution ratio equivalent to the upper limit MPN standard of 88 ~~(or 163)~~, the classification should be supported by fecal or MSC sampling of WWTP effluent to demonstrate a wastewater quality level less than

1.4 X 10^6 or the results of the contaminate reduction studies conducted over worst-case scenarios at the upstream WWTP discharge.

IX. ~~VI.~~ Establishment of Conditional Classifications

~~The basic concept of the NSSP is to control the safety of shellfish by preventing their harvest from contaminated growing areas. In reviewing growing area classifications and sanitary surveys conducted by Shellfish Control Authorities, it appears that a common misinterpretation is the classification of an area as approved when in fact the area should have been classified as conditional. Critical investigations usually reveal that the area is subject to intermittent pollution events. Careful consideration of an intermittent pollution event, development and application of a management plan, and cooperation and compliance by all parties may also allow upgrading of an area to a conditionally approved or conditionally restricted classification instead of requiring the area to be restricted or prohibited at all times.~~

~~Intermittent pollution to shellfish growing waters has been a significant cause of shellfish borne infectious disease outbreaks worldwide. In 1978, at least 20,000 persons were involved in an outbreak of oyster associated gastroenteritis attributed to Norwalk virus. The investigation of the outbreak indicated that a combination of meteorological and hydrographic events had caused inadequately treated and diluted sewage from a nearby municipal facility to reach the area. In an incident in 1982, at least 471 persons developed gastroenteritis after consumption of sewage contaminated oysters when a combination of raw sewage bypasses, high rainfall, strong winds, and abnormally low tides caused contamination of an area that was classified as approved. In both of these instances, application of the conditionally approved area concept probably could have prevented the outbreaks.~~

~~A common situation where this classification might be appropriate is when water quality is, to some degree, dependent upon the operation of a WWTP. For example, the boundaries of an approved shellfish area might be improperly determined during a period when a WWTPSD is operating at a satisfactory level. If there is some interruption in treatment, it follows that there will be some degradation of water quality in the growing area which may require a relocation of the boundaries. The degree of relocation would depend upon such items as the distance between the pollution source and the growing area, hydrography, the amount of water, and the amount of pollution.~~

~~The first step in determining whether an area should be classified as conditionally approved or conditionally restricted is to determine whether sufficient State resources are available to manage, survey, monitor, control harvesting, affect closures, and reopen the area as required. It should be noted that sources of pollution must be routinely monitored; coordination between State, local and industry officials must be timely; performance standards must be monitored; and closures must be immediate and effective. States electing to classify areas as conditionally approved have found the public resource investment to be substantial.~~

~~The second step in determining whether an area should be placed in the conditionally approved or conditionally restricted classification is to evaluate the potential sources of pollution in terms of their effect on water quality in the area. Potential sources of pollution involving a WWTP include: bypasses and overflows within a sewage collection and treatment system.~~

~~The third step in establishing a conditionally approved or conditionally restricted area is to evaluate the source of pollution in terms of the water quality standards to be maintained, and to formulate performance standards for each pollution source having a significant effect on the sanitary quality of the area. The following is an example of performance standards that might be developed:~~

~~Performance standards or closure criteria may be based upon the bacteriological quality of effluent from sewage treatment plants. This might be stated in terms of chlorine residual if the bacteriological quality of the effluent can be positively related to chlorine residual. The following is an example of a performance standard for an effluent discharge: "The median coliform MPN, in any one (1) month, shall not exceed 500 per 100 ml, based on not less than sixteen (16)~~

composite samples per month, and not more than ten (10) percent of the samples shall have an MPN in excess of 10,000 per 100 ml. Determinations of the chlorine residual of the effluent should be made hourly and recorded in the permanent plant records."

A performance standard may be based upon total quality of sewage, which can be discharged from any given unit, or from a combination of units, without causing the basic water quality standards to be exceeded.

The design of a waste treatment plant and the plant effluent specifications may be critical to the designation of an area classified as conditionally approved or conditionally restricted. Design criteria which may be useful in determining the quality of sewage which can be discharged into an area without exceeding the desired water quality standards include: population equivalent (coliform) of sewage, predicted survival of coliform in seawater, effectiveness of chlorination and the total quality of clean dilution water in an area. Results of many studies on the survival of bacteria in seawater have been published.

The mechanical equipment at critical sewage treatment or pumping units should be such that interruptions will be minimized. Wherever possible, operations should be automatically recorded on charts. Requirements that might be imposed depend upon the importance of the unit's relationship to water quality. Important design features of a sanitary waste collection system that should be considered include:

Storm water should be excluded from the sanitary system. There should be stand-by equipment to ensure that treatment or pumping will not be interrupted. It should be taken into account that interruptions may occur because of damage to a single unit or a power failure.

The pumps and critical units should be fitted with meters or gauges so the regulatory agency can monitor performance standards.

Installation of recording scales to indicate rate of chlorine use is helpful. Chlorine flow meters are available that integrate hydraulic flow with chlorine demand.

Liquid level recording gauges fitted with alarms and located in overflow channels of sewage treatment plants and wet wells of lift stations are useful. They can be set to indicate when overflow takes place. It is good operating procedure to date recording charts. Gauges should be calibrated and maintained so that indicated discharge rates are accurate.

Automatic devices to warn of failure or malfunctioning at self-operated pumping stations or treatment plants can be an important control.

Another factor to consider in developing a conditionally approved or conditionally restricted area is that a prohibited area must be interposed between the conditionally approved or restricted area and the source of pollution. The size of such area should be based on the total time it would take for the operating agency to detect a failure, notify the Authority, and for the latter agency to issue a notice to stop shellfish harvesting. It is recommended that the area be of such size that the flow time through the safety area is at least twice that required for the notification process to become effective. Due consideration should be given to the possibility that closure actions might be necessary on holidays or at night.

The length of time a conditionally approved or conditionally restricted area should be closed following a temporary closure will depend upon several factors including the species of shellfish, water temperature, shellfish activity and cleansing rates, presence of silt or other chemicals that might interfere with the physiological activity of the shellfish, and the degree of pollution of the area.

The conditional classifications are designed to address growing areas that are subject to intermittent microbiological pollution. These optional classifications offer the Authority an alternative to placing the area in the restricted or prohibited classification year round when during certain times of the year or under certain conditions, the shellstock from the growing area may be safely harvested. Public health protection and the control of shellfish safety in the use

of the conditional classifications are afforded through the use of a management plan. The management plan for each growing area placed in a conditional classification is based on the information gathered during the sanitary survey. The plan establishes a strict set of criteria that must be met for the growing area to remain in the open status. Failure to meet the criteria automatically places the growing area in the closed status, with immediate notice to the public, the affected industry, and the plan's participants. Two (2) of the most important components of the management plan are: the acceptance of and the agreement to the conditions of the management plan by the one (1) or more Authorities involved, other local, State and Federal agencies which may be involved, the affected shellfish industry, and the persons responsible for the operation of any treatment plants or other discharges that may be involved; and the annual reevaluation of compliance with the plan to assure public health protection. Use of the conditional classification requires more intense monitoring and more frequent reevaluation because of the intermittent nature of the pollution event.

When the Authority has sufficient resources to manage a conditional classification, the use of the conditional classification could allow the safe use of growing areas that might otherwise not be available to the shellfish industry. For a complete discussion of the conditional classification, see the NSSP Model Ordinance Guidance Documents: *Management Plans for Growing Areas in the Conditional Classification* . For additional information concerning the classification of growing waters and the sanitary survey, see the NSSP Model Ordinance Guidance Documents: *Sanitary Survey and the Classification of Growing Waters* .

A. Requirements for Conditional Area Adjacent to a WWTP

(1) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification.

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(1) Survey Required. The sanitary survey meets the following criteria:

(a) The factors determining the period the growing area is in open status are known and predictable and are not so complex as to preclude a reasonable management approach as determined by the Authority;

(b) Each potential source of pollution that may adversely affect the growing area is evaluated;

(c) When conditional management is based at least in part on predicted changes in microbiological water quality, microbiological water quality correlates with environmental conditions or other factors affecting the distribution of pollutants into the growing area; and

(d) For Authorities utilizing MSC meat sample data, when conditional management is based at least in part on predicted changes in MSC levels, those data correlate with environmental conditions or other factors affecting the distribution and persistence of viral contaminants into the growing area.

(2) Management Plan Required. For each growing area, a written management plan shall be developed and shall include:

(a) For management plans based on wastewater treatment plant function, performance standards that include:

(i) Peak effluent flow, average flow, and infiltration flow;

(ii) Microbiological quality of the effluent;

(iii) Physical and chemical quality of the effluent;

(iv) Conditions which cause plant failure;

(v) Plant or collection system bypasses;

(vi) Design, construction, and maintenance to minimize mechanical failure, or overloading;

(vii) Provisions for monitoring and inspecting the waste water<u>wastewater</u> *treatment plant; and*

(viii) Establishment of an area in the prohibited classification adjacent to a

~~wastewater treatment plant outfall in accordance with Section E. Prohibited Classification;~~

~~(b) For management plans based on pollution sources other than waterwastewater treatment plants:~~

~~(i) Performance standards that reliably predict when criteria for conditional classification are met; and~~

~~(ii)Discussion and data supporting the performance standards.~~

~~(c) For management plans based on WWSD function or pollution sources other than WWSD criteria that reliably predict when an area that was placed in the closed status because of failure to comply with its conditional management plan can be returned to the open status. The minimum criteria are:~~

~~(i) Performance standards of the plan are fully met;~~

~~(ii)Sufficient time has elapsed to allow the water quality in the growing area to return to acceptable levels;~~

~~(iii) Sufficient time has elapsed to allow the shellstock to reduce pathogens that might be present to acceptable levels. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of coliform levels in the shellstock to pre-closure levels. The study may establish criteria for reopening based on coliform levels in the water. If the conditional management plan is based on effects of non-point sources of pollution such as rain events and/or storm water runoff, an area can be reopened 48 hours after the water quality has met acceptable classification criteria as long as shellstock are actively feeding. The Authority may utilize MSC in growing areas adjacent to WWSD. Studies establishing sufficient elapsed time shall document the interval necessary for reduction of viral levels in the shellstock. Analytical sample results shall not exceed a level of 50 MSC per 100 grams or pre-determined levels established by the Authority based on studies conducted on regional species under regional conditions. These studies may establish criteria for reopening based on viral levels in the shellfish meats or the area must be in the closed status until the event is over and twenty-one (21) days have passed; and~~

~~(iv) Shellstock feeding activity is sufficient to achieve microbial reduction.~~

~~(d) For management plans based on a risk assessment made in accordance with Chapter II. Risk Assessment and Risk Management, criteria that reliably determine when the growing area may be placed in the open status and shellfish may be harvested;~~

~~(f) Procedures for immediate notification to the Authority when performance standards or criteria are not met;~~

~~(g) Provisions for patrol to prevent illegal harvest; and~~

~~(h) Procedures to immediately place the growing area in the closed status in 24 hours or less when the criteria established in the management plan are not met.~~

~~(3) Reevaluation of Conditional Classification.~~

~~(a) The classification shall be reevaluated at least once each year. The reevaluation shall include:~~

~~(i) Evaluation of compliance with the management plan;~~

~~(ii)Determination of adequacy of reporting of failure to meet performance standards;~~

~~(iii) Review of the cooperation of the persons involved;~~

~~(iv) Evaluation of water quality in the growing area with respect to the bacteriological standards for its classification;~~

~~(v) Field inspection of critical pollution sources, where necessary; and~~

~~(vi) Written findings, evaluations and recommendations.~~

~~(b) Water Sample Collection.~~

~~(i) When the conditional management plan is based on the absence of pollution from marinas for certain times of the year, monthly water samples are not required when the growing area is in the open status of its conditional classification provided that at least three (3) of the water samples collected to satisfy the bacteriological standard for the open status are collected when the growing area is in the open status.~~

~~(ii) When the conditional management plan is based on the operation and performance of a WWSD(s); combined sewer overflow(s); or other point sources of pollution, monthly water samples are required when the growing area is in the open status of its conditional classification except when.~~

~~(a) Hydrographic or dilution analysis has been completed to determine the impact of a WWSD performance failure and communication requirements are documented and the WWSD operator provides immediate notification to the Shellfish Authority during a performance failure; or~~

~~(b) Mooring assessment determines the mooring area is not a pollution source.~~

~~(iii) If a monthly sample cannot be collected due to environmental constraints, the monthly sampling requirement will be satisfied if an additional water sampling run is conducted the following month.~~

~~(iv) When the conditional management plan is based on the effects of non-point sources of pollution, such as rainfall events, storm water runoff, and seasonal variations, a minimum of five (5) sets of water samples (when the Adverse Pollution Condition sampling regimen is used) or six (6) sets of water samples (when the Systematic Random Sampling regimen is used) are required. The samples shall be collected when the growing area is in the open status.~~

~~(v) When the conditional management plan is based on the effects of non-point sources of pollution, such as rainfall events or storm water runoff, and the area is in the open status for less than six (6) months a minimum of five (5) sets of water samples are required (Adverse Pollution Condition and Systematic Random Sampling). At least one (1) sample shall be collected each month the area is placed in the open status. This sample shall be collected while the area is open. If closed status samples are used to meet the minimum sample requirements only two (2) sets of samples may be utilized and they must have been taken within five (5) days of when the Authority anticipates that the area will be placed in the open status. For growing areas in the open status less than two (2) months, at least one (1) sample must be collected while the area is in the open status. Samples collected during the closed status to meet the minimum five (5) sets of water samples shall be applied to annual and triennial reevaluations of the area.~~

~~(vi) When the conditional management plan is based on the seasonal opening and closing of the area, and the area is in the open status for a predetermined period of less than six (6) months, a minimum of five (5) sets of water samples are required (Adverse Pollution Condition and Systematic Random Sampling). All samples shall be collected while the area is in the open status unless the Authority has historical water quality data to demonstrate that the area meets open status criteria while in the closed status. If closed status samples are used to meet the minimum sample requirements they must be collected within thirty (30) days prior to the area being placed in the open status.~~

(4) Understanding of and Agreement With the Purpose of the Conditional Classification and Conditions of Its Management Plan by All Parties Involved.

(a) The management plan shall be developed by the Authority in coordination with:

(i) The local shellfish industry;

(ii) The individuals responsible for the operation of any WWSDs involved; and

(iii) Any local or State agencies; and

***(b)** Failure of any one party to agree shall constitute sufficient justification to deny the application of the conditional classification to a growing area.*

(5) Conditional Area Types. There are two (2) types of conditional areas:

(a) Conditionally approved; and

(b) Conditionally restricted

B. Guidance for a Conditional Area Management Plan

The management plan for a growing area in the conditionally approved or conditionally restricted classification must meet certain minimum requirements to ensure that the safety of the shellfish for human consumption is maintained. The use and success of the conditional classification depends upon a thorough and accurate management plan. Therefore, it is important that all aspects of the management plan be fully considered and implemented. The minimum requirements to be addressed are:

(1) An understanding of and an agreement to the conditions of the management plan by the one (1) or more Authorities involved, other local, State and Federal agencies which may be involved, the affected shellfish industry, and the persons responsible for the operation of any treatment plants or other discharges that may be involved;

(2) A written management plan for the growing area being placed in the conditional classification, which includes a general description of the growing area with a map showing the area's boundaries, and which addresses all items in C. through H..

(3) A sanitary survey that shows the growing area will be in the open status of its conditional classification for reasonable periods of time. The survey must provide a description of the factors determining the growing area's suitability for being classified conditionally approved or conditionally restricted, and the supporting information and data.

(4) A description of the predictable pollution event or events that are being managed and the performance standards established for each pollution source contributing to the pollution event including:

(a) For a wastewater treatment facility, the performance standard should be based on:

(i) Peak effluent flow

(ii) Bacteriological quality of the effluent

(iii) Physical and chemical quality of the effluent

(iv) Bypasses from the treatment plant or its collection system

(v) Design, construction, and maintenance to minimize mechanical failure or overloading (i.e., the reliability of the treatment system and collection system components)

(vi) Provisions for verifying and monitoring efficiency of the wastewater treatment plant and the feedback system for addressing inadequate treatment.

(vii) Identification of conditions that lead to WWTP failure and closure of the conditionally approved area.

(b) For meteorological or hydrological events, the performance standard should be based on:

(i) Identification of the specific meteorological and/or hydrologic event that will cause the growing area to be placed in the closed status;

(ii) Discussion and data analyses concluding that effects on water quality from these specific meteorological and/or hydrologic events are predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for

the growing area placed in the conditional classification; and

(iii) The predicted number of times, based on historical findings, that the pollution event will occur within one (1) year.

(c) For seasonal events, such as marina operation, seasonal rainfall, and waterfowl migration, the performance standard should be based on:

i. Identification of the seasonal event that will cause the growing area to be placed in the closed status, including its estimated duration; and

(ii) Discussion and data concluding that the seasonal event is predictable, and that the data are sufficient to establish meaningful performance standards or criteria for the establishment and implementation of a management plan for a growing area placed in the conditional classification;

(5) A description of the plan for monitoring water quality including numbers and frequency;

(6) A description of how the closed status for the conditional classification will be implemented, which must include:

(a) A clear statement that when the performance standards are not met, the growing area will immediately be placed in the closed status;

(b) A requirement to notify the Authority or Authorities that the management plan performance standards have not been met, including:

(i) The name of the agency or other party responsible for notifying the Authority;

(ii) The anticipated response time between the performance standards not being met and notification of the Authority; and

(iii) The procedures for prompt notification including contingencies such as night, weekend and absences of key personnel;

(c) A description of the implementation and enforcement, including:

(i) The response time between the notification to the Authority of the failure to meet performance standards and activation of the legal closure of the growing area by the Authority;

(ii) The procedures and methods to be used to notify the shellfish industry; and

(iii) The procedures and methods to be used to notify the patrol agency (enforcement agency) including:

- The name of the responsible patrol agency;
- The anticipated response time between the Authority's legal closure of the growing area and notification of closure to the patrol agency; and
- A description of the patrol agencies anticipated activities to enforce the closed status.

(7) A description of the criteria that must be met prior to reopening a growing area in the closed status, including the need to determine that:

(a) The performance standards established in the management plan are again fully met;

(b) The flushing time for pollution dissipation is adequate;

(c) A time interval has elapsed which is sufficient to permit reduction of human pathogens as measured by the coliform indicator group in the shellstock;

(d) Where necessary, the bacteriological quality of the water must be verified; and

(e) Shellstock feeding activity is sufficient to achieve reduction of pathogens to levels present prior to the pollution event.

(8) A commitment to a reevaluation of the management plan at least annually using, at a minimum, the reevaluation requirements in the NSSP Model Ordinance.

VII. Conditionally Restricted

A. Definition

A classification used to identify a growing area that meets the criteria for the restricted classification except under certain conditions described in a management plan.

B. Requirements for Conditionally Restricted Area Adjacent to a WWTP

(1) Model Ordinance Chapter IV. Shellstock Growing Areas

@.03 Growing Area Classification.

C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:

(7) Conditionally Restricted Classification. Any growing area in the conditionally restricted classification shall:

(a) Meet the requirements for:

(i) A restricted classification when the conditionally restricted classification is in the open status; and

(ii) A prohibited classification when the conditionally restricted classification is in the closed status; and

(b) Designate in its management plan whether the harvested shellstock are to be relayed or depurated.

(2) Use of the conditionally restricted classifications by the Authority is optional. The conditionally restricted classification is designed to address growing areas that are subject to intermittent microbiological pollution. These classifications offer the Authority an alternative to placing the area in the prohibited classification year round when, under certain conditions, the shellstock from the growing area may be safely harvested for restricted purposes. The concept also applies to situations where conditions are acceptable for harvest when wastewater treatment plant operation is satisfactory, but not when a malfunction occurs. A management plan is required that describes the controls to provide public health protection in the use of the conditionally restricted classification. For a full explanation of the conditional classifications and their use, see the NSSP Guidance Document, *Management Plans for Growing Areas in the Conditional Classifications* .

Authorities that allow relaying or depuration may utilize the conditionally restricted classification adjacent to prohibited areas established as a result of a WWTP outfall. The use of the conditionally restricted classification is dependent upon the predictable factors associated with the WWTP discharge. These factors may include volume, treatment efficient, seasonality or other factors which affect the quality of the WWTP effluent. The quality concerns are bacterial, viral, toxic chemical and poisonous deleterious substances. Portions of the prohibited area that are less impacted by the WWTP outfall during predictable time periods can be classified conditional and used as a source of shellfish for relaying and depuration.

The conditionally restricted classification management plan must establish a strict set of criteria, which must be met for the growing area to remain in the restricted status. The following are examples of different types of performance standards that could be used:

(a) Performance standards might stipulate the bacteriological quality of effluent from sewage treatment plants. The microbiological quality can be monitored in terms of disinfection residual or dosage for ultraviolet light disinfection. An example of a performance standard for an effluent discharge is:

"The median fecal coliform MPN, in any one (1) month, shall not exceed 200 per 100 ml, based on not less than sixteen (16) samples per month, and not more than ten (10) percent of the samples shall have an MPN in excess of 1,000 per 100 ml. This fecal coliform limit shall be presumed to be met if the chlorine residual in the effluent is at least 1.0 ppm and the chlorine residual in the effluent is continuously recorded on a chart by chlorine residual analyzer or is measured hourly and recorded in the daily monitoring records as required for the plant's NPDES permit."

(b) For disinfection by ultraviolet (UV) light, the disinfection is based on dosage. An example of a performance standard is, "A minimum UV dose of 37 mW-Sec/cm^2 is to be maintained. The calculation of intensity of the UV light is to include factors for effluent quality, including turbidity, suspended solids, and transmittance. The effluent factors contributing to the dose, including turbidity, suspended solids, transmittance, and flow will be continuously measured and recorded. An alarm will be activated if any of the factors are above design limits."

A detailed discussion of ways to increase the reliability of sewage treatment plants can be found in *Protection of Shellfish Waters* (USEPA, 1974) and *Design Criteria for Mechanical, Electric and Fluid System Component Reliability* (USEPA, 1974).

The fourth step is to determine the water quality, which will occur in the growing area when the performance standards are not met, and what portion of the growing area will be affected. Once these determinations are made, the Authority can select the appropriate management strategy for the portion of the growing area that will be placed in the closed status when performance standards are not met, and can select the boundaries for the closed status. The boundaries of that portion of the growing area to be placed in the closed status would depend upon such items as the distance and travel time from the pollution source to the area, the concentration of pollutants in the discharge during the breakdown condition, amount of effluent and hydrographic factors including dilution available in the receiving water.

The use of the conditional classification where a sewage treatment plant is the pollution source being managed requires a fifth step. An area in the prohibited classification must be established between the sewage treatment plant and the growing area placed in the conditionally approved or conditionally restricted classification. The size of the prohibited area should be based on the level of sewage treatment; the total time it would take for the person responsible for the operation of the sewage treatment facility to detect a failure and notify the Authority; and the time it would take the Authority to issue a notice to stop shellstock harvesting. The size of the area in the prohibited classification should allow for an effluent travel time through the prohibited area that is at least twice that required for the notification process to become effective. Due consideration should be given to the possibility that emergency actions might be necessary on holidays or at night. A minimum effluent dilution is to be determined at the prohibited boundary and can be the controlling factor in situations where there is efficient detection and notification of breakdowns.

The length of time that a growing area should be in the closed status of its conditional classification will depend upon several factors. These factors include the degree of pollution in the growing area and flushing capacity of the estuary, the species of shellfish, water temperature, shellstock activity and cleansing rates, and presence of silt or other chemicals that might interfere with the physiological activity of the shellstock. Additional information on the natural cleansing of shellstock is provided in the NSSP Guidance Document, *Shellstock Relay* .

C. Allowable Uses of Shellfish from a Conditionally Restricted Growing Area

(1) Allowable Uses When Area is in Restricted Status

(a) Relay without a Contaminant Reduction Study

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(b) Relay with a Contaminant Reduction Study

~~Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.~~

~~(c) Depuration~~

~~Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.~~

~~(d) Seed~~

~~Seed means shellstock which is less than market size.~~

~~(2) Allowable Uses When Area is in Prohibited Status~~

~~(a) Seed~~

~~Seed means shellstock which is less than market size.~~

~~**D. Model Ordinance Requirements for Relay with a Contaminant Study**~~

~~The Requirements for Relay with a Contaminant Study are defined in Section V. D.~~

~~**E. Model Ordinance Requirements for Relay without a Contaminant Study**~~

~~The Requirements for Relay without a Contaminant Study are defined in Section V. H.~~

~~**F. Model Ordinance Requirements for Depuration**~~

~~The Requirements for Depuration are defined in Section V. J.~~

~~**G. Model Ordinance Requirements for Seed**~~

~~The Requirements for Seed are defined in Section V. L.~~

X. Determining Boundaries for Conditionally Restricted Growing Areas

Should the Authority utilize the conditionally restricted classification to allow relay or depuration, the area classified as conditionally restricted would be established within the portion of the prohibited area established adjacent to the WWTP. Shellfish uses allowed in the restricted classification would be allowed in the conditionally restricted area when the plant is operating within the satisfactory conditions outlined in the conditionally restricted management plan. (Chapter IV@ .03 C (2). Use of the conditionally restricted classification for relay without contaminant reductions studies and depuration requires the Authority to determine whether the growing area is impacted by additional point and non-point sources of pollution in addition to the management plan which is intended to address all potential problems with the adjacent WWTP. The bacteriological quality of every sample station in the growing area shall meet the fecal coliform standard in Chapter IV.@.02 Section G. (2) or Section H. (3) depending upon whether there is an additional point source or just non-point sources of contamination impacting the conditionally restricted growing area. Sufficient water quality samples shall be collected in accordance with Chapter IV.@.02 Section E. (3) at representative water quality sampling stations throughout the impacted restricted growing area.

The establishment of boundaries separating prohibited and conditionally restricted growing areas is dependent upon the uses to be allowed within the restricted growing area. MO Chapters IV and V address the classification requirements for allowable shellfish uses in the restricted classification. These uses include the following:

(1) Relay with a contaminant reduction study
(2) Relay without a contaminant reduction study
(3) Depuration

If harvesting for relay with a contaminant reduction study, the boundary line should be based on an acceptable dilution ratio. If harvesting for relay without a contaminant reduction study or depuration, the boundary line must be based on a fecal coliform sampling program. The Authority has the option to utilize MSC.

The use of the conditionally restricted classification should not affect other adjacent classifications such as restricted, conditionally approved or approved. The area will be considered in the prohibited status when the management plan criteria are not met.

Guidance for Dilution Ratios

For Authorities that choose to establish conditionally restricted areas, the operating efficiency of the plant must be a primary consideration. A portion of what might be the standard prohibited area could be classified as conditionally restricted when the WWTP is operating efficiently. An explanation for operating efficiency is included in Section VI paged 26 of this document. Conditionally restricted areas, when meeting the NSSP requirement for the restricted classification, can be used for a source for shellstock relaying with a contaminant reduction study. These areas are not required to meet a microbiological standard. Shellstock from restricted areas used for relaying without a contaminant reduction study or for depuration do have to meet a microbiological standard. In the absence of a microbiological standard, dilution ratios become very important to protect public health.

An Authority should not consider any portion of a growing area that does not meet a 320:1 dilution ratio as a source for relaying with a contaminant reduction study. The concept of a 320:1 dilution ratio was first documented in a technical paper written by Virgil Carr of FDA. The technical paper was based on studies conducted at WWTP utilizing UV for disinfection.

This study proposed that the prohibited area, could approach the size requirements for Critical Dilution for Toxics to Ambient (Background) from the Clean Water Act. Similarly, the EPA's Regulatory Mixing Zone (RMZ) is 300:1, which is approximately the transition line from near field dilution zone to far field dilution zone where most mixing has already occurred. The 320:1 dilution ratio is needed to assure that poisonous and deleterious substances are not present in high enough concentrations to present a public health concern.

From a pragmatic point of view, dilution from the outfall to the 320:1 line is a dilution factor of 320 while dilution from 320:1 to 1000:1 is a dilution factor of 3.1. This roughly equates to 100 times more dilution of the originate effluent occurring within the 320:1 dilution line than occurs from the 320:1 dilution line to the 1000:1 dilution line. This is an important factor to consider when one is attempting to understand the viral density in growing waters overlying growing areas adjacent to WWTP discharge and the associated risk.

~~**VIII. Conditionally Approved**~~

~~**A. Definition**~~

~~A classification used to identify a growing area which meets the criteria for the approved classification except under certain conditions described in a management plan.~~

~~**B. Requirements for Conditionally Approved Area Adjacent to a WWTP**~~

~~(1) Model Ordinance Chapter IV. Shellstock Growing Areas~~

~~***@.03 Growing Area Classification.***~~

~~*C. Conditional Classifications. Growing areas may be classified as conditional when the following criteria are met:*~~

~~*(6) Conditionally Approved Classification. Any growing area in the conditionally approved classification shall:*~~

~~*(a) Meet the requirements for:*~~

~~*(i) An approved area classification when the conditionally approved classification is in the open status; and*~~

~~*(ii) A restricted or prohibited classification when the conditionally approved*~~

classification is in the closed status; and

(b) If the closed status meets the criteria for the restricted classification, designate in its management plan whether the shellstock may be harvested for relaying or depuration.

Growing areas are placed in the approved classification when the sanitary survey information and marine biotoxin surveillance data indicate that fecal material, pathogenic microorganisms, poisonous, or deleterious substances are not present in the growing area in unacceptable concentrations. Shellstock harvested from these growing areas may be sold directly to the public for consumption raw or cooked.

C. Allowable Uses of Shellfish in a Conditionally Approved Growing Area

(1) Allowable Uses when the Conditionally Approved Area is in the Open Status

(a) Direct Marketing

Direct Marketing means the sale for human consumption of shellfish which:

(i) Does not require depuration or relaying prior to sale; or

(ii) Has been subjected to depuration or relaying activities

(b) Relay

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(c) Depuration

Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(d) Seed

Seed means shellstock which is less than market size.

(e) Post Harvest Processing

Post Harvest Processing means any process which has been validated using NSSP validation procedures which reduces the levels of pathogenic hazards to below the appropriate FDA action level or in the absence of such a level, below the appropriate level as determined by the ISSC.

(2) Allowable Uses when the Conditionally Approved Area is in the Closed Status

(a) Relay

Relay means to transfer shellstock from a growing area classified as restricted or conditionally restricted to a growing area classified as approved or conditionally approved for the purpose of reducing pathogens as measured by the coliform indicator group or poisonous or deleterious substances that may be present in the shellstock by using the ambient environment as the treatment process.

(b) Depuration

Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.

(c) Seed

Seed means shellstock which is less than market size.

D. Model Ordinance Requirements for Direct Marketing

There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for direct market.

E. Model Ordinance Requirements for Relay

The Requirements for Relay are defined in Section V. H.

~~There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for relay.~~

~~**F. Model Ordinance Requirements for Depuration**~~

~~There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for depuration.~~

~~(1) Model Ordinance Chapter XV. Depuration~~

~~.01 Critical Control Points.~~

~~*A. Receiving Critical Control Point - Critical Limits.*~~

~~*(1) The dealer shall receive and depurate only shellstock which is obtained from a licensed harvester who has:*~~

~~*(a) Harvested the shellstock from an Approved or Conditionally Approved area in the open status as indicated by the tag; [C] and*~~

~~*(b) Identified the shellstock with a tag on each container or transaction record on each bulk shipment; [C] and*~~

~~*(c) Harvested the shellstock in compliance with the time/temperature requirements of Chapter VIII. @.02 A. (1), (2) or (3) as determined from records supplied by the harvester described in Chapter VIII. .02 G. (2) [C].*~~

~~*(2) The dealer shall…*~~

~~*(3) Should a dealer…*~~

~~*(4) The dealer shall…*~~

~~The Requirements for Depuration of shellfish harvested from conditionally approved areas in the closed status are defined in Section V.J.~~

~~**G. Model Ordinance Requirements for Seed**~~

~~The Requirements for Seed are defined in Section V.L.~~

~~There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for seed.~~

~~**H. Model Ordinance Requirements for Post-Harvest Processing**~~

~~There are no classification restrictions on shellfish harvested from conditionally approved areas in the open status for PHP.~~

~~**I. Model Ordinance Requirements for Relay with a Contaminant Reduction Study**~~

~~The Requirements for Relay with a Contaminant Reduction Study are defined in Section V.D.~~

~~**J. Model Ordinance Requirements for Relay without a Contaminant Reduction Study**~~

~~The Requirements for Relay without a Contaminant Reduction Study are defined in Section V.H.~~

XI. ~~K.~~ Determining Boundaries for Conditionally Approved Growing Areas

Should the Authority utilize the conditionally approved classification to allow harvest for direct marketing, the area classified as conditionally approved would be established within the portion of the prohibited or restricted area established adjacent to the WWTP. Shellfish uses allowed in the approved classification would be allowed in the prohibited or restricted area when the plant is operating within the satisfactory conditions outlined in the conditionally approved management plan. (Chapter IV. @ .03 C (2)).

In addition to meeting the satisfactory conditions outline in the conditionally approved management plan, the area must also conduct a sanitary survey of the growing area as required in Chapter IV. @ .01 and establish a monitoring program to ensure the water quality requirements of Chapter IV. @ .02 E. The area will be considered in the prohibited or restricted status when the management plan criteria are not met.

Guidance for Dilution Ratios

For Shellfish Control Authorities that choose to establish conditionally approved areas for harvest uses allowable within the approved classification, the operating efficiency of the plant must be a primary consideration. A portion of the prohibited or restricted area could be classified as conditionally approved when the WWTP is operating efficiently. An explanation for operating efficiency is included in Section VI page 26 of this document. The minimum dilution of 1000:1 is recommended for establishing a conditionally approved area adjacent to a WWTP. The rationale for the 1000:1 dilution rate was included in Section IV: Guidance Document Chapter II 19., which was adopted by the ISSC in 2015. Conditionally approved areas, when not in the approved status, can be used for a source for shellstock relaying with a contaminant reduction study, shellstock relaying without a contaminant reduction study and depuration. To utilize shellfish for these purposes, these areas are required to meet the Model Ordinance requirements associated with those uses (e.g. restricted water quality standard).

~~**IX. Approved Classification**~~

~~**A. Definition**~~

~~A classification used to identify a growing area where harvest for direct marketing is allowed.~~

~~**B. Requirements for Use of the Approved Classification**~~

~~(1) Model Ordinance Chapter IV. Shellstock Growing Areas~~

~~***@.03 Growing Area Classification.***~~

~~*B. Approved Classification. Growing areas shall be classified as approved when the following criteria are met.*~~

~~*(1) Survey Required. A sanitary survey finds that the area is:*~~

~~*(a) Safe for the direct marketing of shellfish;*~~

~~*(b) Not subject to contamination from human or animal fecal matter at levels that, in the judgment of the Authority, presents an actual or potential public health hazard; and*~~

~~*(c) Not contaminated with:*~~

~~*(i) Pathogenic organisms;*~~

~~*(ii) Poisonous or deleterious substances;*~~

~~*(iii) Marine biotoxins; or*~~

~~*(iv) Bacteria concentrations exceeding the bacteriological standards for a growing area in this classification.*~~

~~*(2) Water Quality. The water quality in the growing area shall meet the bacteriological standards for an approved classification in Section @.02.*~~

~~***@.02 Microbiological Standards***~~

~~*E. Standard for the Approved Classification of Growing Areas when Evaluated for Adverse Pollution Conditions.*~~

~~*(1) Water Quality. The bacteriological quality of every station in the growing area shall meet the fecal coliform standard in Section E. (2).*~~

~~*(2) Fecal Coliform Standard for Adverse Pollution Conditions. The fecal coliform median or geometric mean MPN or MF (mTEC) of the water sample results shall not exceed fourteen (14) per 100 ml, and not more than ten (10) percent of the samples shall exceed an MPN or MF (mTEC) of:*~~

~~*(a) 43 MPN per 100 ml for a five-tube decimal dilution test;*~~

~~*(b) 49 MPN per 100 ml for a three-tube decimal dilution test;*~~

~~*(c) 28 MPN per 100 ml for a twelve-tube single dilution test; or*~~

~~*(d) 31 CFU per 100 ml for a MF (mTEC) test.*~~

~~*(3) Required Sample Collection.*~~

~~*(a) A minimum of five (5) samples shall be collected annually under adverse pollution conditions from each sample station in the growing area.*~~

~~*(b) A minimum of the most recent fifteen (15) samples collected under adverse pollution conditions from each sample station shall be used to calculate the median or geometric mean and percentage to determine compliance with this standard.*~~

~~*(c) Sample station locations shall be adjacent to actual or potential sources of pollution.*~~

~~**C. Allowable Uses of Shellfish in an Approved Growing Area**~~

~~(1) Direct Marketing~~

~~Direct Marketing means the sale for human consumption of shellfish which:~~

~~(a) Does not require depuration or relaying prior to sale; or~~

~~(b) Has been subjected to depuration or relaying activities~~

~~(2) Depuration~~

~~Depuration means the process of reducing the pathogenic organisms that may be present in shellstock by using a controlled aquatic environment as the treatment process.~~

~~(3) Seed~~

~~Seed means shellstock which is less than market size.~~

~~(4) Post Harvest Processing~~

~~Post-Harvest Processing means any process which has been validated using NSSP validation procedures which reduces the levels of pathogenic hazards to below the appropriate FDA action level or in the absence of such a level, below the appropriate level as determined by the ISSC.~~

~~**D. Model Ordinance Requirements for Direct Marketing**~~

~~There are no classification restrictions on shellfish harvested from approved areas for direct market.~~

~~**E. Model Ordinance Requirements for Depuration**~~

~~The Requirements for Depuration are defined in Section XIII.F.~~

~~There are no classification restrictions on shellfish harvested from approved areas for depuration.~~

~~**F. Model Ordinance Requirements for Seed**~~

~~The Requirements for Seed are defined in Section V.L.~~

~~There are no classification restrictions on shellfish harvested from approved areas for seed.~~

~~**G. Model Ordinance Requirements for Post-Harvest Processing**~~

~~There are no classification restrictions on shellfish harvested from approved areas for PHP.~~

XII. Determining Boundaries for ~~Conditionally~~ Approved Growing Areas

In establishing boundaries between approved areas and other classifications adjacent to a WWTP, the Authority should consider dilution ratios and the approved area must meet the microbiological standards for approved growing areas.

Guidance for Dilution Ratios

When determining if a WWTP or collection system discharge within the watershed or catchment area draining to a shellfish estuary potentially impacts a shellfish growing area, the NSSP recommends that a worst case raw sewage discharge be assumed. In this circumstance, if a level of 1.4×10^6 FC/100 ml is assumed for a raw sewage release, a 100,000:1 dilution would be required to dilute the sewage sufficient to meet the approved area standard of 14 FC/100 ml. If dilution analysis determines that the location of the discharge is such that the dilution of effluent would be greater than 100,000:1 then the WWTP could be considered located outside the zone of influence to the shellfish growing area. Different dilution ratios may be applied depending on the known

concentration of sewage, a performance history of the treatment and collection system and a database of influent and effluent quality, provided that the water quality objective of the downstream harvest area is met.

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.04 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood

Because shellfish are filter feeders, they can readily accumulate substances from the water column. The types of poisonous or deleterious substances that have been recovered from shellfish include heavy metals, pesticides, petroleum products, polychlorinated biphenyls, and naturally occurring marine biotoxins. The source of these contaminants may be industrial, agricultural, mining, spillage, sewage, dredging operations, sludge dumps, and naturally occurring toxigenic marine organisms.

The FDA has established action levels, tolerances, and guidance levels for poisonous or deleterious substances to control the levels of contaminants in human food, including seafood (FDA Federal Register, 1977; FDA, 2002). Action levels are established and revised according to criteria specified in the ***Code of Federal Regulations*** (21 CFR 109 and 509), and are revoked when a regulation establishing a tolerance for the same substance and use becomes effective. Action levels and tolerances represent limits at or above which FDA will take legal action to remove adulterated products, including shellfish, from the market. Action levels and tolerances are established based on the unavoidability of the poisonous or deleterious substance and do not represent permissible levels of contamination where it is avoidable.
Guidance levels are used to assess the public health impact of the specified contaminant.

Table 1 lists action levels, tolerances and guidance levels established by the FDA for poisonous or deleterious substances in seafood, including shellfish. Notices are published in the ***Federal Register*** as new action levels are established or as existing action levels are revised or revoked. Should any of these notices affect Table 1, FDA will issue an interpretation advising NSSP participants of this revision or addition.

Table 1
Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood

Class of Substance	Substance	Level	Food Commodity	Reference
Deleterious Substance	Aldrin/Dieldrin[c]	0.3 ppm	All Fish	CPG sec 575.100b
	Chlordane	0.3 ppm	All Fish	CPG sec 575.100b
	Chlordecone[d]	0.3 ppm	All Fish	CPG sec 575.100b
	DDT, DDE, TDE[e]	5.0 ppm	All Fish	CPG sec 575.100b
	Diquat[g]	2.0 ppm	All Fish	40 CFR 180.226
	Diquat[g]	20.0 ppm	Shellfish	40 CFR 180.226
	Glyphosate[g]	0.25 ppm	Fin Fish	40 CFR 180.364
	Glyphosate[g]	3.0 ppm	Shellfish	40 CFR 180.364
	Carbaryl	0.25 ppm	Oysters	40 CFR 180.169
	Endothall and its Monomethyl ester	0.1 ppm	All Fish	40 CFR 180.293
	Methyl Mercury	1.0 ppm	All Fish	CPG sec 540.600
	Heptachlor / Heptachlor Epoxide[f]	0.3 ppm	All Fish	CPG sec 575.100
	Mirex	0.1 ppm	All Fish	CPG sec 575.100
	Polychlorinated Biphenyls (PCBs)[g]	2.0 ppm	All Fish	21 CFR 109.30
	2,4-D[g]	0.1 ppm	Fish	40 CFR 180.142
	2,4-D[g]	1.0 ppm	Shellfish	40 CFR 180.142
Chemotherapeutics	Chloramphenicol	No Residue	All Fish	21 CFR 530.41
	Clenbuterol	No Residue	All Fish	21 CFR 530.41
	Diethylstilbestrol (DES)	No Residue	All Fish	21 CFR 530.41
	Demetridazole	No Residue	All Fish	21 CFR 530.41
	Ipronidazole and other nitroimidazoles	No Residue	All Fish	21 CFR 530.41
	Furazolidone and other nitrofurans	No Residue	All Fish	21 CFR 530.41
	Fluoroquinolones	No Residue	All Fish	21 CFR 530.41
	Glycopeptides	No Residue	All Fish	21 CFR 530.41
Natural Toxins	Paralytic Shellfish Poisoning (PSP) toxins	80 µg/100g	All Fish	CPG sec 540.250
	Neurotoxic Shellfish Poisoning (NSP) toxins	20 MU/100g	Clams, mussels, oysters, fresh frozen or	NSSP MO
	Azaspiracid Shellfish Poisoning (AZP) toxins	0.16 mg/kg	Clams, mussels, oysters, fresh frozen or	NSSP MO
	Diarrhetic Shellfish Poisoning (DSP) toxins	0.16 mg/kg	Clams, mussels, oysters, fresh frozen or	NSSP MO
	Amnesic Shellfish Poisoning (ASP) toxins	20 mg/kg	All Fish (except in the viscera of Dungeness crab where 30 mg/kg is permitted)	Compliance Program 7303.842

Note: the term "fish" refers to fresh or saltwater fin fish, crustaceans, other forms of aquatic animal life other than birds or mammals and all mollusks as defined in *21 CFR 123.3(d)*.

Footnotes for Table 1

a) Unless otherwise specified, the action levels, tolerances and other values listed apply to both the raw and processed food commodity. Procedures for sample collection and analyses are specified in Sections 420 and 450 of the ***FDA Investigations Operation Manual***; ***FDA Pesticide Analytical Manual*** **(PAM)** Volume I or II; ***AOAC Official Methods of Analysis***; ***APHA Recommended Procedures for the Examination of Sea Water and Shellfish***, Fourth Edition, 1970; or, peer reviewed literature for Domoic Acid (ASP) methodologies.

b) References designated as CPG represent the FDA Compliance Policy Guides and all associated numbers as they appear in appropriate sections of FDA's Compliance Policy Guides Manual.

c) The action level for aldrin and dieldrin are for residues of the pesticides individually or in combination. However, in adding amounts of aldrin and dieldrin do not count aldrin or dieldrin found at the level below 0.1 ppm for fish.

d) Previously listed as Kepone, the tradename for chlordecone.

e) The action level for DDT, TDE, and DDE are for residues of the pesticides individually or in combination. However, in adding amounts of DDT, TDE, and DDE do not count any of the three (3) found below 0.2 ppm for fish.

f) The action level for heptachlor and heptachlor epoxide are for the pesticides individually or in combination. However, do not count heptachlor or heptachlor epoxide found below 0.1 ppm.

g) The levels published in 21 CFR and 40 CFR represent tolerances rather than guidance levels or action levels.

References

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@.04 Marine Biotoxin Control

.01 Marine Biotoxin Control Guidance

NSSP guidance documents provide Authorities with information and best practices on how to implement the components of the Model Ordinance. NSSP Model Ordinance requirements apply only to interstate commerce although most ~~S~~states apply the requirements intrastate. For the most up to date and detailed listing of requirements, the reader should consult the most recent edition of the Model Ordinance. An overview of marine biotoxins including associated biological vectors, diseases, historic outbreaks, and emerging trends can be found in Section III Public Health Reasons and Explanations Chapter IV. @.04 Marine Biotoxin Control.

In accordance with the NSSP, FDA and receiver ~~S~~states should have assurance that shellfish producing ~~S~~states or countries with shellfish sanitation arrangements are taking and can take adequate measures to prevent harvesting, shipping, and consumption of toxic shellfish. To provide this assurance, the NSSP requires the Authority to develop and adopt either a marine biotoxin contingency plan and/or a marine biotoxin management plan for a specific list of biotoxins that covers each marine and estuarine shellfish growing area. Single plans can be developed for a whole state or can cover particular growing areas or toxins. An Authority may have an area with a contingency plan for some biotoxins and a management plan for others, a contingency plan for all biotoxins, or a management plan for all biotoxins. A contingency plan is developed by an Authority that has no history or reason to expect toxin-producing phytoplankton in their growing areas. A marine biotoxin management plan is developed by an Authority that has historic occurrence of toxin-producing phytoplankton and toxicity in shellfish from their growing areas.

The Marine Biotoxin Contingency Plan
Section II. ~~MO~~ Ch IV. Shellstock Growing Areas @.04 Marine Biotoxin Control (A)

Purpose

The purpose of a contingency plan is for the Authority to be prepared to mitigate risk and protect public health if an unanticipated biotoxin event occurs in a classified shellfish growing area. Examples of an unanticipated biotoxin event include an illness outbreak or an emergence of a toxin-producing phytoplankton in a growing area where it has not historically occurred.

The Model Ordinance requires that a contingency plan:

1. Address the toxins that cause each of the following illnesses (except those addressed in a biotoxin management plan): PSP, ASP, NSP, DSP, and AZP.
   a. Even if the toxin has never been known to occur in the area or it is biologically unlikely to occur in the area, it still must be addressed.

2. Define the administrative procedures and resources necessary to: initiate an emergency shellfish sampling program; close growing areas and embargo shellfish; prevent harvesting of contaminated species; provide for product recall; disseminate information; coordinate control actions; and establish reopening criteria.
   a. It is important to note that the Model Ordinance does not require an Authority to take any actions following the development of a contingency plan, unless the Authority elects to include specific actions in their plan such as phytoplankton or biotoxin sampling protocols. Instead, this plan should define the procedures an Authority would follow in the event of a bloom or illness outbreak, as well as how the Authority would go about acquiring the resources needed to implement those procedures.

Contingency Plan Content Guidance

Element	Recommended Plan Contents
Emergency Sampling Program	• Identify area(s), phytoplankton, and/or shellfish for sampling • A procedure to promptly expand this sampling program, including increasing sampling stations and sampling frequency, in the event of increased toxicity/cell counts at any indicator monitoring stations identified within the plan • Identify partner sampling agencies available • Identify laboratory support, including capacity, method(s), contract(s) o In some circumstances, the Authority may have the laboratory support available in-house, but in other circumstances may have to identify alternate NSSP labs to conduct the necessary methods o If there is no approved method available, the Authority should identify an appropriate method for analysis following the procedures described in MO Chapter III @.02 Methods • Describe training for samplers

	• Identify financial resources available, request processes, and necessary approvals • Though not required by the Model Ordinance, it may be appropriate for the Authority to implement an early warning system within the contingency plan, as described in the *Marine Biotoxin Management Plan* section below
Close Growing Areas and Prevent Harvest of Contaminated Species	• Identify the legal authority to close areas and restrict harvesting • Protocols to initiate closures, taking into consideration public health, economic, and conservation concerns. The rapidity with which toxin levels can increase, inherent delays in sample collection and results, the number of samples required to initiate action, the size of the area to be closed (including a safety zone), and the type of harvesting restrictions to be invoked (all species or specific species) should all be considered • Describe the mechanism to quickly notify growers, harvesters, and dealers of closures • Describe protocols to notify patrol entities of emergency closures, as well as patrol procedures necessary to prevent harvest from closed areas
Embargo Shellfish	• Identify the legal authority to embargo, detain, quarantine, or otherwise prevent the movement of shellfish in commerce and to withdraw interstate shipping permits • Describe procedures for embargoing shellfish, including the identification of affected lots and distribution networks, as well as any associated forms, tags, or other administrative tools used • Describe the mechanism for destruction of embargoed product if it is found to be adulterated, or the mechanism to release embargoed product if it is found to be free of contamination
Coordinate Control Actions	• Describe the mechanism to notify partner state, local, federal, and/or tribal agencies to avoid duplicative efforts and streamline response
Product Recall	• Identify the legal authority to recall product that may already be in commerce • Identify agency protocols for implementing a product recall • It may be helpful to develop templates and forms for recall, if they are not already in place
Disseminate Information to Partners	• Establish relationships and procedures to notify: o ISSC and FDA Shellfish Specialist o Adjacent states or states that may have received adulterated product o Local, tribal, and state public health and safety partners

	○ Poison control partners • Describe efforts to inform and educate the public about risks associated with biotoxins • It may be useful to develop information sheets or fillable templates for notifications, press releases, partner agency communications, or other routinely used documents • Develop a model communications plan or agree in advance on lead communications points of contact
Reopening Criteria	• Establish how the authority will determine when an area can be reopened based on data ○ Reopening criteria should integrate public health, conservation, and economic considerations ○ To establish a detoxification curve or other environmental indices, such as phytoplankton concentration trends, more than one (1) sample collected at different times is needed. For example, some states collect two (2) samples over seven (7) days and others collect three (3) samples over fourteen (14) days. These samples should show the absence of biotoxins or levels below the closure guidance level ○ If species-specific sampling regimes are employed, then each species that exceeded the quarantine threshold must be tested independently to reopen • Identify laboratory support, including capacity, methods, and contracts ○ In some circumstances, the Authority may have the laboratory support available in-house, but in other circumstances, may have to identify alternate NSSP labs to conduct the lab methods approved for reopening • If there is no approved method available, the Authority should identify an appropriate method for analysis following the procedures described in MO Chapter III @.02 Methods

Additional Considerations:

a. If an Authority has a management plan and/or protocols such as patrol manuals or existing MOUs that are relevant and appropriate, the Authority may reference those documents within its contingency plan.
b. Relationships with academia, government, non-government, and industry partners can be extremely helpful in identifying the presence of previously unseen phytoplankton or biotoxins. It can be helpful to develop and maintain a general list of contact people or organizations that can collaborate on phytoplankton and biotoxin monitoring efforts.
c. The Model Ordinance also requires that certain records be maintained during and following an event. It is recommended that the contingency plan include details on record maintenance.
    i. Appropriate records of illnesses should be compiled and maintained by the Authority. These records should include data on the incidence of illness and appropriate case history data. This information may be important in defining the severity of the problem, as well as for a retrospective evaluation of the adequacy of the entire control program.
    ii. Records of shellfish sample results from toxin testing should include analysis of trends, detoxification curves, phytoplankton and water sample analyses, and pertinent environmental observations.
    iii. Whenever possible, the Authority should archive shellfish or shellfish homogenates for additional analysis.

The Marine Biotoxin Management Plan
Section II. ~~MO~~ Ch IV. Shellstock Growing Areas @.04 Marine Biotoxin Control (B)

Purpose
The marine biotoxin management plan is required for proactive management of marine biotoxins in growing areas with a history of toxin- producing phytoplankton, toxins in shellfish at or above the guidance level in their growing areas, and/or a previous illness outbreak. Similar to a contingency plan, the Model Ordinance requires that a management plan define the administrative procedures and resources necessary to: close growing areas and embargo shellfish; prevent harvesting of contaminated species; provide for product recall; disseminate information; coordinate control actions; and establish reopening criteria. Please refer to the Contingency Plan Content Guidance above for recommendations on how to develop these portions of the management plan/

Additionally, the Model Ordinance requires that:

1. For any areas covered by a management plan, the Authority must maintain a toxin-producing phytoplankton and/or shellfish sampling program.
2. The management plan includes procedures to ensure that all shellfish harvested from growing areas or portion(s) of growing areas placed in the controlled access status will meet all conditions of harvest restrictions prior to being placed in distribution.

Early Warning System

It is recommended that any Authority with a management plan should have an early warning system in place (https://www.fao.org/3/cc4794en/cc4794en.pdf). Early warning systems may include additional phytoplankton and/or shellfish monitoring efforts conducted by the Authority and/or by use of a network of observers and partnerships as well as communications with other organizations to identify environmental or biological warning signs.

- Establish relationships and communication procedures with resource agencies to rapidly report to the Authority any abnormal environmental phenomena that might be associated with shellfish growing areas, such as bird or fish kills, water discoloration or abnormal behavior of shellfish or marine scavengers.

- Establish relationships and communication procedures for health agencies to report any toxin-like illnesses.
- Primary sampling stations should be located at sites where experience has shown toxins or blooms are most likely to appear first.
- If monitoring shellfish, samples should be collected of species which are most likely to reveal the early presence of toxin and which are most likely to show the highest toxin levels. For example, in some circumstances, mussels have been found to be useful for early detection.
- Sampling design should always consider what species are present in the growing area and commercially harvested.

Marine Biotoxin Management Strategies

It is necessary to recognize that different marine biotoxin management strategies are essential to address specific risks as well as geographic and logistical conditions. Marine biotoxin management strategies must include an appropriate number of samples to adequately address the specific risks. The Authority initiating biotoxin management plans should employ sampling in accordance with the strategies below until a baseline dataset of at least 36 samples per growing area or hydrographically linked waterbodies is developed (i.e., 36 phytoplankton samples for a phytoplankton strategy or 36 shellfish samples for a shellfish-related strategy). These samples should cover representative environmental conditions and a time span of at least three (3) years. Once this baseline dataset is developed and trends are established, the Authority may consider reducing sample numbers, frequency, and lot testing and/or increasing harvest days allowed in the marine biotoxin management plan in accordance with the strategies below.

All marine biotoxin management plans must establish, at a minimum, the below criteria:
- screening levels,
- methods,
- laboratory(s)/analyst(s),
- a representative sampling plan,
- representative sample locations (stations),
- representative sampling frequency; and
- a dataset that supports management decisions.

A. Phytoplankton monitoring: this strategy involves a routine program for sampling growing area waters for the presence of phytoplankton documented or suspected to produce marine biotoxins. This complementary management strategy that enhances predictive capabilities of anticipating toxicity in shellfish must be used in combination with other management strategies.

The level of monitoring required will vary based on the historical database available to inform the sampling strategy (i.e., growing areas with a long history of defined temporal and spatial patterns of toxin-producing phytoplankton may have a more targeted approach to sampling, requiring less monitoring than for growing areas where temporal and spatial patterns have not been determined).

Phytoplankton monitoring can be applied to all growing areas where collecting, transporting and processing water samples is logistically feasible, taking into consideration effects of zooplankton grazing and durability of various cell types to temperature and transport. This management strategy may be applied to aquaculture or wild harvest. Appropriate venues for this management strategy include, but are not limited to, easily accessible wild harvest areas and aquaculture sites in state waters or aquaculture sites in federal waters.

The phytoplankton monitoring strategy shall be used together with one (1) or more of the other biotoxin

management strategies. If it were used as the sole management strategy, phytoplankton monitoring would likely misrepresent the actual risk of marine biotoxins. Cell counts, as measured per liter of water, are often used to trigger additional testing of shellfish in biotoxin monitoring programs. These cell count criteria can only be established with a robust dataset; therefore, new monitoring programs should employ low cell count criteria to trigger shellfish toxicity samples to establish or refine the cell concentrations responsible for toxins accumulating in shellfish.

When an early warning system such as phytoplankton monitoring detects increased toxicity/cell counts or other information suggests that toxin levels are increasing, it is important that the Authority have procedures to promptly expand sampling to additional stations and/or increase the frequency of sampling for marine biotoxins. The procedures should include plans for obtaining the additional resources necessary to implement the expanded sampling and laboratory analysis program. If a plan consists of water sampling for phytoplankton cell counts as surveillance, the Authority should identify its plan to be able to initiate shellfish sampling.

Considerations should be made for how sampling is conducted such as phytoplankton net tows, filtered surface water, or whole water samples. The depth of water sampled should also be considered and evaluated for all species of phytoplankton being targeted. Some species of phytoplankton are known to display diurnal, vertical migration patterns within the water column, while other species are known to occur in dense patches.

Laboratory and field methods may include, but are not limited to light microscopy, flowcytometry, DNA fingerprinting, rapid toxin detection tests, and PCR assays. Analysts should be trained in each method employed and consideration should be given to complimentary methods of analysis such as light microscopy with phytoplankton identification confirmed by a rapid test at least in the initial phases of the monitoring program.

An appropriate sampling plan, station location, and sampling frequency should all factor in the location and type of the resource being monitored, the species of phytoplankton anticipated or observed, and the environmental conditions that might result in a rapid bloom or trigger the production of toxicity in an existing population. Primary sampling stations (also referred to as indicator or sentinel stations) should be located at sites where toxic phytoplankton are most likely to first appear, based either on experience or knowledge of site conditions. The geographic distribution for collection of samples should take into consideration the randomness of toxic algal blooms. Establishing the frequency and period for collection of samples to identify an event as early as possible is an important consideration.

Historical occurrences and fluctuations in coastal phytoplankton populations due to the influence of meteorological and hydrographic events are also significant. For example, a large rain storm may cause nutrient loading in coastal waters and trigger a toxic phytoplankton bloom, or a hurricane may drive an offshore phytoplankton bloom onshore. To facilitate knowledge transfer, it is advisable that the authority describe its rationale in selecting sampling sites.

B. Routine shellfish toxicity monitoring: this strategy involves a routine program for sampling and testing shellfish meats for the presence of marine biotoxins. Unless species-specific shellfish testing is conducted, the highest risk species (e.g., species that metabolizes toxin most quickly) occurring in the growing area shall be used. Many biotoxin monitoring programs have found mussels to be the best sentinel species. This strategy may be used alone or in combination with other management strategies.

The level of monitoring required will vary based on the historical database available to inform the sampling strategy (i.e., growing areas with a long history of defined temporal and spatial patterns of shellfish toxicity may have a more targeted approach to sampling, requiring less monitoring than for growing areas where temporal and spatial patterns have not been determined). Until the Authority is

confident they understand the risk posed by marine biotoxins in the growing area, sampling should be as robust as possible, and managers should consider that harmful algal blooms can change dramatically from year to year.

This management strategy can be applied to all growing areas where collecting, transporting and processing shellfish samples is feasible. This management strategy can be applied to aquaculture or wild harvest. Appropriate venues for this management strategy include but are not limited to, easily accessible wild harvest areas and aquaculture sites in state waters or wild harvest areas and aquaculture sites in federal waters.

The routine shellfish toxicity monitoring strategy may be used independently or together with one (1) or more of the other biotoxin management strategies. If used as the sole management strategy, predicting future toxicity levels in shellfish and the appropriate sampling frequency can be difficult. Long-term databases can provide valuable historic information on the timing of toxicity occurring in shellfish as well as toxicity elimination from shellfish. Shellfish toxin levels that are below the regulatory levels may trigger emergency or expanded testing, or precautionary closures. Growing areas should be placed in the closed status at a level that provides an adequate margin of safety, since in many instances, toxicity levels will change rapidly and the time between sampling and results should be considered. Precautionary closures can be made to prevent the harvest of potentially toxic shellfish while sample results are being collected and processed.

Consideration should be given to the different species of shellfish present in a growing area, the intensity and duration of harmful algal blooms and the uptake and elimination rates of specific toxins from all species of shellfish harvested from the growing areas (e.g., sea scallops).

Methods shall be used in accordance with Section IV. Guidance Documents Chapter II Growing Areas.14 or Section II. Chapter III. @.02 C. Additionally, the Authority should identify laboratories that can perform approved methods for marine biotoxins and identify laboratory capacity.

An appropriate sampling plan, station location and sampling frequency should factor in the location and type of the resource being monitored, the species of shellfish harvested in the growing area and environmental conditions that might affect toxin uptake, such as water temperatures.
Primary sampling stations (also referred to as indicator or sentinel stations) should be located at sites where toxin is most likely to first appear, based either on past experience or knowledge of site conditions. The geographic distribution for collection of samples should take into consideration the randomness of toxic algal blooms. Establishing the frequency and period for collection of samples to identify an event as early as possible is an important consideration.

Sample collection, sample transportation, and sample analysis procedures should be developed, and predictable timeframes established between collection and results. The Authority should ensure that in an emergency, such as a suspected biotoxin illness, the normal timeframe can be compressed, and sample results known as quickly as possible. It is important to consider emergency coverage schedules for staff and lab availability outside of normal office hours during harmful algal bloom events.

When an early warning system detects increased toxicity/cell counts or other information suggests that toxin levels are increasing, it is important that the Authority have procedures to promptly expand sampling to additional stations and/or increase the frequency of sampling for marine biotoxins. The procedures should include plans for obtaining the additional resources necessary to implement the expanded sampling and laboratory analysis program.

C. Pre-harvest shellfish toxicity testing: this strategy involves sampling and testing shellfish meats for the presence of marine biotoxins in the intended harvest area specifically in advance of harvesting. This

strategy, if used independent of any other strategy, shall permit harvest in specific geographic locations and for short durations. This strategy may also be used in combination with other management strategies and should be considered as a complementary strategy while developing datasets for alternative management strategies (e.g., pre-harvest shellfish toxicity testing in combination with phytoplankton monitoring which can evolve into a robust shellfish toxicity monitoring strategy).

This strategy requires representative samples that cover the spatial distribution of the area to be harvested. The duration of permitted harvest following sampling will vary based on the species being tested and the historical database available to inform the sampling strategy. Without at least 36 samples per harvest area over the span of at least three (3) years, the short duration of permitted harvest shall not exceed three (3) days from the time of shellfish collection for toxicity testing to harvest. The dataset could then be used to modify the duration of permitted harvest.

This management strategy can be applied to harvest areas where collecting, transporting and processing shellfish samples is feasible. This management strategy can be applied to aquaculture or wild harvest. Appropriate venues for this management strategy include but are not limited to; easily accessible and remote wild harvest areas and aquaculture sites in state and federal waters. If toxicity in excess of the established threshold in Section II. Chapter IV. @.04 C. is detected, the growing area must be either be placed in the closed or controlled access status.

A marine biotoxin management plan that incorporates this strategy must also establish:

- a defined harvest area, and;
- appropriate duration for permitted harvesting subsequent to sampling.

This strategy is specifically for permitting harvest following shellfish testing. The duration of permitted harvesting will depend on the species being tested, the risk of increasing toxicity and the timing of additional sampling. Samples must be representative of the harvest area.
Methods shall be used in accordance with Section IV. Guidance Documents Chapter II Growing Areas .14 or Section II. Chapter III. @.02 C.

D. Shellfish lot testing: this strategy involves sampling and testing shellfish meats for the presence of marine biotoxins on a lot basis after harvest. This strategy may be combined with a pre-harvest shellfish toxicity testing strategy, the results of which permit harvest. Lot testing may also be used on a case-by-case basis to clear product harvested immediately prior to a biotoxin closure if the Authority determines it is necessary.

This strategy requires representative samples for each lot of harvested shellstock. Lot testing shall be permitted in growing areas in the Controlled Access Status and require Restricted Shellstock tags. The conditions for the area in Controlled Access Status shall be defined in the harvest permit and may include holding shellstock until lot tests are available.

This management strategy can be applied to all growing areas where harvest occurs. This management strategy can be applied to aquaculture or wild harvest. Appropriate venues for this management strategy include but are not limited to; easily accessible and remote wild harvest areas and aquaculture sites in state and federal waters.

A marine biotoxin management plan that incorporates this strategy must also establish:

- representative number of samples per lot.

Methods shall be used in accordance with Section IV. Guidance Documents Chapter II Growing Areas.14 or Section II. Chapter III. @.02 C.

E. Pre-harvest shellfish toxicity screening and lot testing: this strategy requires pre-harvest shellfish toxicity screening of the intended harvest area coupled with shellfish lot testing upon landing or receipt at the initial certified dealer.

This strategy shall permit harvest from intended harvest areas in the Controlled Access Status and require Restricted Shellstock tags. The conditions for the area in Controlled Access Status shall be defined in the harvest permit and may include holding shellstock until lot tests results are available. In the absence of an adequate dataset, the initial number and frequency of pre-harvest and lot samples must be sufficient to conduct an evaluation of risk in the intended harvest area. The initial number of samples must be adequate to address the size of the intended harvest area and the amount of shellfish harvested. Single samples are not adequate for evaluation of risk. Should initial samples indicate minimal toxin levels or the absence of toxins, sampling can be reduced but must be conducted at least monthly or as often as necessary to monitor risk.

This management strategy can be applied to all growing areas where harvest occurs. This management strategy can be applied to aquaculture or wild harvest. Appropriate venues for this management strategy include but are not limited to; easily accessible and remote wild harvest areas and aquaculture sites in state and federal waters.

A marine biotoxin management plan that incorporates this strategy must also establish:

- a defined harvest area, and;
- representative number of samples.

Heat Processing

In shellfish growing areas where low levels of biotoxins routinely occur, harvesting for thermal processing, referred to in the Model Ordinance as heat processing, purposes may be an alternative to consider. Thermal processing as defined by applicable FDA regulations (21 CFR 113) may reduce the biotoxin concentration of the shellfish via dilution, not destruction. While thermal processing has been demonstrated more for PSP toxins (Berenguer et al., 1993; Vieites et al., 1999; Dong et al., 2022), there are limited studies for the reduction of ASP and DSP toxins (McCarron et al., 2008, Vidal et al 2009). If thermal processing is practiced, the Authority must develop and implement procedures to control the harvesting and transportation of the affected shellfish to the processing plant, as well as end product testing of processed shellfish.

Shellfish Meat Analyses and Toxin Profiles
Section II. Chapter III. @.02 C
Section IV. Guidance Documents Chapter II Growing Areas.14

There are a wide range of methodologies developed for screening and confirmation of toxic phytoplankton and their toxins. Only methods adopted into the NSSP can be implemented for the purpose of confirming toxin concentration levels in shellfish and making decisions to reopen growing areas. Additionally, some screening methods have been evaluated by the ISSC and found fit for purpose for the NSSP, thereby providing confidence in their use for specific screening purposes.

Toxin analyses methods fall into two (2) categories in the NSSP:

1. Approved Methods for Marine Biotoxin Testing (Section IV. Guidance Documents Chapter II Growing Areas .14 Table 2.); and
2. Approved Limited Use Methods for Marine Biotoxin Testing (Section IV. Guidance Documents Chapter II Growing Areas .14 Table 4.).

The methods within these categories range from mouse bioassays to immunochromatography and other antibody-based platforms to chemical analytical methods such as high-performance liquid chromatography (HPLC). The mouse bioassay historically has been the most universally applied technique for examining shellfish toxins. Other bioassay procedures have been developed and are becoming more generally applied.

In recent years, considerable effort has been applied to development of chemical analyses to replace or provide alternatives to in-vivo (live animal) bioassays. For toxins that have no method adopted into the NSSP, best available science is employed and emergency use adoption may be considered following the requirements described in Model Ordinance Chapter III. Laboratory @
.02 Methods.

The following table provides information for each toxin covered by the NSSP.

Paralytic Shellfish Poisoning (PSP) Toxins	
Analogs	Water-soluble alkaloid neurotoxins that are collectively referred to as saxitoxins or paralytic shellfish toxins (PSTs). To date 57 analogs have been identified, although not all are always present, and they vary greatly in overall toxicity (Wiese et al., 2010). In addition to saxitoxin (the parent compound), monitoring laboratories typically analyze for approximately 12 other analogs that may contribute measurably to toxicity.
Guidance Level	0.8 ppm (80 µg saxitoxin equivalents /100 g tissue).
Origin	The regulatory limit was set in the 1930s (Wekell et al., 2004). The minimum concentration of PSP toxin that will cause intoxication in susceptible persons is not known. Epidemiological investigations of PSP in Canada, however, have indicated 200 to 600 micrograms of PSP toxin will produce symptoms in susceptible persons. A death has been attributed to the ingestion of a probable 480 micrograms of PSP toxin. Investigations indicate that concentrations of less than 200 ug of the toxin have no deleterious effects on humans.
Shellfish Lab Methods	The mouse bioassay is still the most widely accepted detection method for the saxitoxins around the world and has been shown to adequately protect the public's health. In 2009, the Interstate Shellfish Sanitation Conference approved a post-column oxidation HPLC-PCOX method, making it the newest regulatory method available for PSP toxins in the U.S. The receptor binding assay (RBA), a competition assay whereby radiolabeled saxitoxin competes with unlabeled saxitoxin for a finite number of available receptor sites, provides a measure of overall PSP toxicity in a sample (Van Dolah et al., 2009). The RBA was approved for mussels and approved limited use for clams and

	scallops in 2014.
General Molluscan Shellfish Associations	Mussels, clams, cockles, oysters, and scallops (excluding the scallop adductor muscle).

Neurotoxic Shellfish Poisoning (NSP) Toxins	
Analogs	Comprised of more than 10 lipid-soluble cyclic polyethers. Several analogs and metabolites have been identified. NSP-causing toxins in shellfish include intact algal brevetoxins and their metabolites (collectively known as neurotoxic shellfish toxins or NSTs) (Plakas and Dickey, 2010).
Guidance Level	0.8 ppm (20 mouse units/100 g tissue or 80 µg brevetoxin-2 equivalents /100 g tissue)
Origin	Uncooked clams from a batch eaten by a patient in Florida with NSP symptoms were found to contain 118 mouse units per 100 grams of shellfish meat. However, consumption of even a few contaminated shellfish may result in poisoning and the severity of the disease may be dependent on many factors, including dose, bodyweight, underlying medical conditions, and the age of the victim as well as possibly the toxin mixture of the particular bloom (Watkins, 2008).
Shellfish Lab Methods	The approved NSSP method for NSP toxins is the mouse bioassay. The MARBIONC ELISA is approved for limited use. Efforts are underway to validate *in vitro* methods for detection of brevetoxins in shellfish. The methods that follow may be used for screening purposes. For example, rapid, sensitive ELISA test kits already are commercially available for this purpose. Biomarkers of brevetoxin contamination in shellfish have been identified by using LC/MS. Structural confirmation of these metabolites and brevetoxins in shellfish can be made by LC/MS, a method that offers high sensitivity and specificity. A method for detection, identification, and quantification of brevetoxins is HPLC-MS. Radioimmunoassay (RIA) and Receptor Binding Assay (RBA) are also under current use (Watkins, 2008). Available detection methods are not equal in their ability to measure naturally produced brevetoxins, and most methods are hampered by the absence of specific reference standards for brevetoxin congeners (Watkins, 2008).

General Molluscan Shellfish Associations	Oysters and clams.

Amnesic Shellfish Poisoning (ASP) Toxin	
Analogs	The neurotoxin domoic acid is a water-soluble, non- protein, excitatory amino acid. Isomers of domoic acid have been reported but are less toxic than domoic acid itself.
Guidance Level	20 ppm (2mg domoic acid/100 g tissue)
Origin	In 1987 in eastern Canada, domoic acid poisonings sickened individuals, leading to Health Canada's establishment of the regulatory limit. (Wekell, 2004).
Shellfish Lab Methods	The NSSP approved method for detecting domoic acid in seafood is a reversed-phase HPLC method with ultraviolet (UV) detection. The Reveal 2.0 ASP is an approved limited use method. There is an AOAC approved ELISA for the detection of domoic acid which may be used for screening purposes.
General Molluscan Shellfish Associations	Mussels, clams, cockles, oysters, and scallops (excluding the scallop adductor muscle).
Diarrhetic Shellfish Poisoning (DSP) Toxins	
Analogs	A group of lipid-soluble polyether toxins that includes okadaic acid (OA), the dinophysistoxins (DTXs), and a series of fatty acid esters of okadaic acid and the dinophysistoxins (collectively known as DSTs) (Uchida, 2018).
Guidance Level	0.16 ppm (0.16 mg total okadaic acid equivalents/kg tissue). Total okadaic acids equivalents equal combined free okadaic acid, dinophysistoxins, acyl- esters of okadaic acid and dinophysistoxins.
Origin	Established by FDA in 2011 for total (esterified plus nonesterified okadaic acid and the dinophysistoxins (Trainer, 2013).
Shellfish Lab Methods	Until recently, DSP was managed by mouse bioassay and/or monitoring shellfish growing waters for the presence of *Dinophysis* organisms. Unfortunately, the dose-survival times for the DSP toxins in the mouse assay vary considerably, and fatty acids interfere with the assay, giving false-positive results. A suckling mouse assay has been developed and used for control of DSP. This assay measures fluid accumulation after injection of the shellfish extract. In 2017 an LCMS/MS method for quantifying dinophysistoxins in clams was approved in the NSSP. For other

	species, the best available science is recommended.
General Molluscan Shellfish Associations	Mussels, clams, cockles, oysters, and scallops (excluding the scallop adductor muscle).

Azaspiracid Shellfish Poisoning (AZP) Toxins	
Analogs	The lipid-soluble toxin azaspiracid and several derivatives (AZAs). More than 30 AZA analogs have been identified, with three analogs routinely monitored in shellfish (AZA1, AZA2, and AZA3).
Guidance Level	0.16 ppm (160 µg azaspiracid-1 equivalents/kg tissue)
Origin	Estimation of consumption of a single portion of shellfish and through estimate of an Acute Reference Dose. Derived from epidemiological observations caused by a mixture of naturally occurring analogs (AZA 1, 2, and 3). Based on methods available in 2001.
Shellfish Lab Methods	AZAs are not routinely monitored in shellfish harvested in the U.S., but, in the EU, the mouse bioassay has been used. As for many of the lipophilic toxins, the mouse assay is not adequately sensitive or specific for public-health purposes. *In vitro* assays and analytical methods are now available to assess the toxicity of AZA-contaminated shellfish and to confirm the presence of AZA analogs in shellfish. These methods are in various stages of validation for regulatory use around the world. LC/MS is used as a confirmatory method for AZA, providing unambiguous structural confirmation of AZA analogs in shellfish samples. Currently, there is no NSSP method for AZP toxins.
General Molluscan Shellfish Associations	Detected in mussels, oysters, scallops (excluding the scallop adductor muscle), clams, and cockles.

Resources (click here for access)

1. 2012 FDA's Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins
2. FAO Paper 80: Marine Toxins. As follows:
    a. Paralytic Shellfish Poisoning
    b. Diarrhetic Shellfish Poisoning
    c. Neurotoxic Shellfish Poisoning
    d. Amnesic Shellfish Poisoning
    e. Azaspiracid Shellfish Poisoning
    f. References
3. FDA online course, Shellfish Growing Areas
4. Centers for Disease Control and Prevention, Morbidity and Mortality Weekly Report (MMWR)
5. NIH/PubMed: Various Shellfish-Associated Toxins

Model Ordinance, Public Health Reasons & Explanations, Guidance, and Appendices References

- Section I. Purposes & Definitions

- Section II. Model Ordinance –Chapter III. Laboratory @.02 MethoI(C) and (D)
- Section III. Public Health Reasons and Explanations– Chapter IV. Shellstock Growing Areas @.04 Marine Biotoxin Control (A)
- Section IV. Guidance Documents– Chapter II. Growing Areas @.03 Determining the Size of a Closed Area as a Result of Illnesses
- Section IV. Guidance Documents– Chapter II. Growing Areas @.04 Determining the Harvesting Periods Associated with Implicated Product for Identifying Shellfish to Be Included in the Recall
- Section IV. Guidance Documents - Chapter II. Growing Areas @.05 Determining the Scope of Implicated Product for Conducting a Recall
- Section IV. Guidance Documents – Chapter II. Growing Areas @.08 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood
- Section IV. Guidance Documents – Chapter II. Growing Areas @.12 Growing Area Patrol and Enforcement
- Section IV. Guidance Documents – Chapter II. Growing Areas @.13 Control of Shellfish Harvesting
- Section IV. Guidance Documents – Chapter II. Growing Areas @.14 Approved NSSP Laboratory Tests
- Chapter XVI. Recalls, Closures, and Special Events Checklist & Appendices

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@.05 Marinas

RESERVED

@.06 Mooring Areas

RESERVED

Proposal No. 27-137

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative

Submitter	Jonathan Deeds and Christopher Beekman
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Proposal Subject	Modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.
Specific NSSP Guide Reference	The intention is for this method to be an Approved Method for Biotoxin Testing for DSP toxins under the NSSP and that it should be added as a new listing in Section IV: Guidance Documents, Chapter III: Laboratory, @.02 Methods, .01 Approved NSSP Laboratory Tests, Table 2 Approved Methods for Marine Biotoxin Testing under the heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP) titled **Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) – Matrix Matched (Mussels only)**, and the applications should be (1) Growing Area Survey and Classification, (2) Dockside Testing Program, and (3) Controlled Relaying.
Text of Proposal/ Requested Action	See full SLV report and specific edits to existing method checklist.
Public Health Significance	Shellfish contaminated with natural toxins can cause consumer illnesses. The Food and Drug Administration (FDA) has established guidance levels for five groups of natural toxins in shellfish responsible for the following illnesses: amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). While management programs have existed in the United States for PSP, NSP, and ASP for many years, the need for management programs for lipophilic shellfish toxins, including DSP and AZP, are a relatively recent occurrence. The first shellfish harvesting closure due to DSP toxins (DSTs) in excess of the regulatory guidance level of 16 µg OA eq./100 g shellfish (0.16 ppm) occurred on the Texas Gulf Coast in 2008. The first harvesting closure, and illnesses, in the Puget Sound region occurred in 2011 and the first closure in the New England region occurred in 2015. In response to these events, a method for the detection and quantitation of DSTs based on liquid chromatography tandem mass spectrometry (LC-MS/MS) was validated and approved by the ISSC for use in clams in 2017 (Proposal #17-103). A checklist for the evaluation of this method in NSSP labs was approved in 2023 (Proposal #19-136). However, after subsequent failure of verification efforts in several NSSP labs, issues were noted with the method that were preventing its widespread use. This report describes the validation of a modified LC-MS/MS method for DSTs for use in the NSSP for the management of this toxin group in mussels. These modifications specifically address deficiencies in the original method for clams identified during method verification. If approved, this modified method will subsequently be extended to additional matrices, including clams and oysters.
Cost Information	**Capital equipment purchases:** 1. Ultra-high Performance Liquid chromatograph (UHPLC) or High-Performance Liquid Chromatograph (HPLC), capable of running in gradient mode [examples: Waters Corporation Acquity UPLC system (Manchester, UK) (approx. $60,000, new with government (GSA) discount), Shimadzu Corporation Nexera UHPLC system (Kyoto, Japan) (approx. $80,000, new with government (GSA) discount)]

	2. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities [example: Sciex QTrap 5500+ equipped with a Turbo V ionization source (Framingham, MA) (approx. $320,000, new with government (GSA) discount)] **Cost per sample (approx. for consumables):** $14/sample

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	Modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.
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Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Shellfish contaminated with natural toxins can cause consumer illnesses. The Food and Drug Administration (FDA) has established guidance levels for five groups of natural toxins in shellfish responsible for the following illnesses: amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). While management programs have existed in the United States for PSP, NSP, and ASP for many years, the need for management programs for lipophilic shellfish toxins, including DSP and AZP, are a relatively recent occurrence. A method for the detection and quantitation of DSP toxins based on liquid chromatography tandem mass spectrometry (LC-MS/MS) was validated and approved by the ISSC for use in clams in 2017 (Proposal #17-103). A checklist for the evaluation of this method in NSSP labs was approved in 2023 (Proposal #19-136). However, after subsequent failure of verification efforts in several NSSP labs, issues were noted with the method that were preventing its widespread use. This report describes the validation of a modified LC-MS/MS method for DSTs for use in the NSSP for the management of this toxin group in mussels. These modifications specifically address deficiencies in the original method for clams identified during method verification. If approved, this modified method will subsequently be extended to additional matrices, including clams and oysters.
2. What is the intended purpose of the method?	Y	The intention is for this method to be an Approved Method for Biotoxin testing for DSP toxins under the NSSP and that it should appear in Section IV. (Guidance Documents), Chapter II. (Growing Areas), Section .14

		(Approved Laboratory Tests), Table 2 (Approved Methods for Marine Biotoxin Testing) under the heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP) titled Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) – Matrix Matched (Mussels only), and the applications should be (1) Growing Area Survey and Classification (2) Dockside Testing Program, and (3) Controlled Relaying with the sample type of Shellfish for all.
3. Is there an acknowledged need for this method in the NSSP?	Y	In 2011, the European Union (EU) adopted LC-MS/MS as the reference method for lipophilic shellfish toxins, which include DSTs, and developed a harmonized SOP summarizing several similar methods that had been individually validated in various EU labs.[1] However, because the EU protocol is a summary of several distinct methods, measures a number of additional lipophilic toxins not regulated in the US, such as pectenotoxins and yessotoxins, and measures both free and esterified DSTs separately, it was necessary to pick a single method from the options in the EU SOP that measured total DSTs to validate for use in the NSSP. ISSC Proposal #17-103 was the first attempt at this effort, and although the method was found to be fit for purpose for the measurement of DSTs in clams in the developing laboratory, verification efforts by several NSSP labs failed due primarily to differences in the performance of the liquid chromatographs and mass spectrometers available in those laboratories. Specifically, the #17-103 method required the use of an ultra-performance liquid chromatograph (UPLC), which utilizes LC columns of smaller diameter and particle size running at elevated pressures and used a Sciex 5500 QTrap® triple quadrupole mass spectrometer. A combination of not having access to a UPLC system and differences in the performance of available mass spectrometers from other manufacturers, specifically increased susceptibility to matrix effects, led to the failure of participating NSSP labs from being able to verify the approved method. This new SLV proposal uses an HPLC column that can be used on both UPLC and standard HPLC systems and introduces the optional use of a matrix-matched standard curve to normalize responses between different brands and models of mass spectrometers. This new SLV was also validated using mussels while the original used clams. Once approved, this modified method will be extended to include additional matrices such as clams, oysters, etc.
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	Chemical Confirmatory Method: Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) specifically measures okadaic acid, dinophysistoxin 1, and dinophysistoxin 2. The inclusion of a required base hydrolysis step measures total DSTs (free plus esterified) in a single run.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	Modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Method Scope	Y	This method is fully validated for the determination of diarrhetic shellfish poisoning toxins in mussels.
References	Y	1. European Union Reference Laboratory for Marine Biotoxins. Harmonized Standard Operating Procedure for Determination of Lipophilic Marine Biotoxins in Molluscs by LC-MS/MS. Version 5, January 2015. 2. Codex Alimentarius. 1999. Recommended Methods of Analysis and Sampling, Codex Stan 234-1999, Adopted in 1999, Amendment: 2025. https://www.fao.org/fao-who-codexalimentarius/codex-texts/list-standards/en/ 3. Botana LM, Hess P, Munday R, Nathalie A, DeGrasse SL, Feeley M, et al. Derivation of toxicity equivalency factors for marine biotoxins associated with bivalve molluscs. Trends in Food Sci. Tech. (2017) 59; 15-24. 4. McNabb, P, Selwood AI, Holland PT. Multi-residue method for determination of algal toxins in shellfish: single-laboratory validation and inter-laboratory study. Journal of AOAC International, 88 (2005) 761. 5. US FDA. Guidelines for the Validation of Chemical Methods for the FDA Foods Program, 3rd Edition. 2019. https://www.fda.gov/food/laboratory-methods-food/foods-program-methods-validation-processes-and-guidelines
Principle	Y	Liquid chromatography tandem mass spectrometry (LC-MS/MS) is an analytical technique that provides both physical separation (liquid chromatography) and mass analysis (mass spectrometry) of sample components. In this work, a Shimadzu Nexera Ultra-High Performance Liquid Chromatography system was coupled with a Sciex 5500+ QTrap® triple quadrupole mass spectrometer by electrospray ionization for targeted quantitation of DSP toxins. Structurally informative transitions were specified for multiple reaction monitoring (MRM) for each toxin of interest. In MRM mode, an ion of interest (precursor ion) is preselected in the first quadrupole and fragmented in the second quadruple. Multiple product ions resulting from fragmentation of the precursor ion are then mass analyzed in the third quadrupole. Two analyte specific transitions, one for quantitation and one for confirmation, are specified for each analyte and monitored in both the calibration standard solutions and in the extracted shellfish matrices. Due to differing susceptibility to matrix enhancement between different manufacturers and models of mass spectrometers, this method includes the optional use of a matrix matched standard curve as well as a liquid/liquid partitioning sample clean-up step using hexane. A linear fit is applied to the peak area data for the quantitation ion collected for the calibration standards, and the equation for this line is utilized to calculate the concentration of each analyte in the spiked matrix samples. The enhanced resolving power afforded by LC and the selectivity gained by tandem mass spectrometry permitted the accurate detection and quantitation of DSP toxins in complex shellfish matrices. Total toxicity can be estimated as the sum of the molar

		concentrations of detected analogs multiplied by the relevant specific toxicity equivalency factors (TEFs)[3]. The currently accepted toxicity equivalency factors for DSP toxins are as follows: OA – 1, DTX1 – 1, and DTX2 – 0.6. To date, only OA and DTX1 have been found in shellfish responsible for illnesses or involved in harvesting closures the U.S.
Any Proprietary Aspects	Y	None
Equipment Required	Y	1. Instrumentation for sample preparation: knives for shucking, stainless steel laboratory spatulas, sieve for draining 2. 4 oz. plastic screw top specimen cups (if a sample of unextracted homogenate is to be saved) (e.g., Covidien #17099) 3. Balance, accuracy to the nearest 0.01 g 4. Blender, food processor, or laboratory homogenizer (e.g., Polytron Ultraturax equipped with a generator appropriate for shellfish (e.g. 20 mm saw toothed)) 5. Vortex mixer (either single or multi-tube) 6. Centrifuge, capable of 2000 x *g*, with adaptors for 50 mL and 15 mL centrifuge tubes 7. Heat block or water bath capable of maintaining 76 ± 2 °C 8. Volumetric flasks or graduated cylinders capable of accurately measuring 20, 100, 500, and 1000 mL 9. Disposable 50 mL centrifuge tubes 10. Disposable glass Pasteur pipettes with bulbs 11. 20 mL glass scintillation vials with coned polyethylene lined caps (if a sample of non-hydrolyzed methanolic extract is to be saved) (e.g., Wheaton #986560) 12. Disposable 16 × 100 mm glass screw cap tubes with phenolic PTFE lined caps (e.g., Fisher #14-959 35AA (tubes), Corning #9998-15 (caps)) 13. 0.01, 0.05, 0.1, 0.5, and 1 mL positive displacement microdispensers or syringes (e.g., Drummond #3-000-510, 3-000-575, 3-000-590 or Hamilton #80530, 80630, 81330, 81365) 14. 1-3 mL disposable syringes (e.g., Becton Dickinson #309602) 15. PTFE syringe tip membrane filters, 13 mm, pore size 0.2 µm (e.g., Pall #PN4542) 16. LC-MS autosampler vials with appropriate caps (e.g., Agilent #5182-0715) 17. Reversed Phase HPLC Column (e.g., Waters Xbridge Premier HPLC BEH C18 VanGuard Fit, 3.5 µm particle size, 2.1 × 100 mm, or Waters Acquity UPLC BEH C18, 1.7 µm particle size, 1.0 × 150 mm) 18. Standard High-Performance (HPLC) or Ultra-Performance (UPLC) Liquid chromatograph capable of running in gradient mode 19. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities
Reagents Required	Y	1. Certified Reference Standards -Okadaic acid: e.g. (NRC-CRM-OAd) NRC-CNRC Institute for Marine Biosciences, Canada -Dinophysistoxin-1: e.g. (NRC-CRM-DTX1c) NRC-CNRC Institute for Marine Biosciences, Canada -Dinophysistoxin-2: e.g. (NRC-CRM-DTX2c) NRC-

		CNRC Institute for Marine Biosciences, Canada 2. Chemicals Acetonitrile, HPLC Optima Grade Methanol, HPLC Optima Grade Water, HPLC Optima Grade Ammonium formate (≥98% purity) Formic Acid (≥98% purity) Hydrochloric acid (37%) Sodium hydroxide (≥98% purity) Hexanes (Certified ACS, ≥98.5%)
Sample Collection, Preservation and Storage Requirements	Y	Molluscan shellfish (e.g. clams, mussels, oysters, etc.) (10-12 animals per sample in most cases) should be collected fresh, shucked, drained of excess liquid, and homogenized thoroughly. Samples can either be extracted immediately or homogenates can be frozen at ≤-20°C. If samples cannot be homogenized immediately, they should be shucked, drained, and frozen at ≤-20°C. Samples should not be re-drained when thawed for homogenization and analysis if previously frozen. If a reserve sample is to be kept, all excess homogenates should be stored at ≤-20°C. Samples to be stored long term should be kept at ≤-80°C.
Safety Requirements	Y	Proper precautions should be taken to avoid inhalation of harmful reagents or contact with skin or eyes. Analyst should wear a lab coat, gloves and safety glasses when working with chemicals. Chemical reagents that are flammable and/or toxic should be used within a chemical fume hood to protect laboratory workers.
Clear and Easy to Follow Step-by-Step Procedure	Y	**1. Shellfish Preparation:** 1. Clean outside of the shellfish with fresh water. 2. Cut the adductor muscles to open and rinse the inside with fresh water to remove any debris. 3. Remove the meat from the shell and drain tissue using a sieve to remove salt water. 4. Combine at least 12 animals and homogenize thoroughly for 60 – 120 seconds using a blender/food processor or a laboratory homogenizer. **2. Extraction Procedure:** 1. Accurately weigh 2.00 g ± 0.05 g of tissue homogenate into a 50 mL disposable centrifuge tube. 2. Add 9.0 mL of methanol and mix thoroughly using a vortex mixer for 3 min. 3. Centrifuge at 2000 *g* for 10 min at approximately 20 °C. 4. Transfer the supernatant to a clean 20 mL graduated cylinder, volumetric flask, or glass scintillation vial. 5. Re-extract the residual tissue pellet with an additional 9.0 mL of methanol and mix thoroughly using a vortex mixer for 3 min. 6. Centrifuge at 2000 *g* for 10 min at approximately 20 °C. 7. Transfer the supernatant to a 20 mL volumetric flask or graduated cylinder and combine with the supernatant previously extracted. Adjust

		total extract volume to 20 mL with 100% methanol. 8. Transfer the 20 mL of total extract back into the 20 mL scintillation vial for storage (if desired). **3. Hydrolysis Procedure:** Total DSP toxins (i.e. free toxin plus toxin fatty acid esters) must be quantified when performing this method. To do this, all samples must go through an alkaline hydrolysis step prior to analysis. 1. Accurately transfer a 2 mL aliquot of the 20 mL methanolic extract to a 16 × 100 mm glass tube with a phenolic PTFE lined screw cap using a positive displacement microdispenser or syringe. 2. Add 250 µL of 2.5 M NaOH to the 2 mL extract aliquot. 3. Mix with a vortex mixer for 10 seconds and record the total weight of the tube. 4. Ensure caps are securely fastened and heat the mixture at 76 °C for 40 minutes. [Note: 76 °C is above the boiling point for methanol, therefore sample loss will occur if tube caps do not fit well and are not securely fastened.] 5. Dry the water from the outside of the tubes, allow the tubes to cool to room temperature (approx. 5-10 minutes), and then re-weigh each tube. If the weight has dropped by more than 0.1 g, replace lost weight using 100% MeOH. 6. Neutralize samples with 250 µL of 2.5 M HCL, mix by vortexing for 10 seconds. **4. Sample Clean-up Procedure:** Partitioning of the methanolic shellfish extracts with hexane is performed to remove nonpolar lipids such as triglycerides, which could contaminate the source region of the instrument and/or affect the responses of the DSTs[4]. 1. Add 7 mL of hexanes directly to the 2.5 mL hydrolyzed methanolic extract. 2. Mix by vortexing for 3 minutes. 3. Partition by centrifuging at 2,000 *g* for 10 min. 4. Using a disposable glass pasture pipette, collect the hexanes (top layer) and discard to an appropriate waste container. 5. Transfer approximately 1 mL of the methanolic extract (bottom layer) into a disposable syringe equipped with a 0.2 µm PTFE syringe tip filter. 6. Filter directly into an LC-MS certified glass vial and cap. **5. LC-MS/MS Procedure:** **A. Instrumentation Used for Validation:** Sciex QTrap 5500+ equipped with a Turbo V ionization source (Framingham, MA) and a Shimadzu Corporation Nexera UHPLC system (Kyoto, Japan). **B. LC Parameters:** HPLC column used for validation: Waters Xbridge Premier BEH C18 VanGuard FIT, 3.5 µm particle size,

2.1 × 100mm
Column Temperature: 40 °C
Autosampler Temperature: 10 °C
Injection Volume: 5µL

Note: Waters BEH C18, 1.7 µm, 1.0 mm × 150 mm [flow rate 0.120 mL/min; column temperature 40 °C; 5 µL injection] that was used in proposal #17-103 (UHPLC only) was also tested during this study (see ruggedness section) and was found to provide equivalent results.

C. LC Gradient:

Mobile phase A: 2mM ammonium formate and 50 mM formic acid in 100% water.

Preparation of 1000 mL: Dissolve 128 ± 10 mg ammonium formate in water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with water and add 1.9 mL of formic acid. Fill to mark with water.

Mobile phase B: 2mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water.

Preparation of 1000 mL: Dissolve 128 ± 10 mg ammonium formate in 48.1 mL water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with acetonitrile and add 1.9 mL of formic acid. Fill to mark with acetonitrile.

For the gradient in Table 2, LC flow is diverted to waste from time 0.0–4.5 min and from 7.75–15.0 min. This was approximately one minute before the first peak and one minute after the last peak to preserve instrument integrity from over spraying matrix into the source.

Table 2. LC Gradient

Time (min)	Flow Rate (mL/min)	%A	%B
0.0	0.300	50	50
2.0	0.300	50	50
6.0	0.300	30	70
8.0	0.300	1.0	99
10.0	0.300	1.0	99
10.5	0.300	50	50
15.0	0.300	50	50

D. MS Ion Source Parameters:
Turbo V ion source parameters were optimized in negative ionization mode for all analytes under the acidic chromatographic conditions listed above (Table 3). These parameters will vary between different instrument platforms or ionization sources.

Table 3. Turbo V ion source parameters

Source Temperature	550 °C
Ion Spray Voltage	−4500 V
Curtain Gas	25 au
Gas 1	40 au
Gas 2	40 au

		E. MRM Parameters: Manual or automatic compound optimization must be performed by every laboratory to determine the optimal settings for the MRM parameters as these may vary between different instrument platforms. Instruments must be correctly calibrated for negative ionization mode as OA, DTX1, DTX2 are optimally monitored at this polarity. The precursor and product ion mass-to-charge (m/z) is confirmed by acquiring full scan MS1 and MS/MS spectra for each toxin. At least two product ions must be monitored for each toxin, one for quantitation and one for confirmation. Dwell times for transitions are set such that at least 10 data points are acquired across each peak. Declustering Potential (DP), Entrance Potential (EP), Collision gas (CAD), Collison Energy (CE), and Collision Cell Exit Potential (CXP) must be optimized for each MRM transition monitored. Table 4 lists the compound-dependent parameters optimized for a Sciex QTRAP 5500+ detector. Note: To confirm the presence of each DST, two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio (S/N) is used for quantitation, and the transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation must be ≥ 3. For this study, the m/z 113.1 product ion was used for confirmation, but both the m/z 113.1 and 151.1 ions were monitored and either could be used. See SLV (Table 4) for full MRM Parameters used for Method Validation on a Sciex QTrap 5500+ Mass Spectrometer
Quality Control Steps Specific for this Method	Y	1. If available, a divert valve should be used to divert LC flow at the beginning and end of each chromatographic run. In preliminary studies, when a divert valve was not used, the ion source region and curtain plate needed to be cleaned between each batch (approximately every 24 hours) to maintain adequate sensitivity. [Divert valve was used in the SLV] 2. Use a new calibration curve each day of analyses or once every 24 hrs. for long analytical runs. Each calibration curve should be derived from at least six calibration points, and the linear regression should yield a correlation coefficient (R^2) greater than or equal to 0.99. If analyzing a large number of samples, the use of bracketing calibration curves is recommended. a. If using bracketing standard curves, the calibration curve should be split into two sets of vials (i.e., each vial only pierced once). b. The variation in the individual calibration curve slopes between the first and second set of calibration standards should not exceed 25%[1]. c. The variation in retention time of the standards in bracketing calibration curves should not exceed 3%[1]. d. The data from bracketing curves should be

		averaged and fitted with a line using least squares linear regression. The average calibration curves should yield an R^2 greater than or equal to 0.98[1]. 3. Reagent Blanks (methanol solvent) are analyzed after the high calibration standard and periodically (typically every 10 samples) after fortified samples to ensure that analyte carryover is not occurring. [Carryover defined as toxins detected > LOD]. 4. A Procedural Blank (methanol carried through sample preparation process at the same time as the samples) is performed with each batch of samples. 5. When bracketing standard curves are not used, a continuing calibration verification (CCV) standard is used; a separate mid-scale calibration standard is analyzed bracketing at least every 10 samples to assure that no retention time shifts (possibly due to column failure) or loss in signal intensity (due to fouling of the column or mass spectrometer) has occurred. a. If more than one CCV is used, sample should be split into separate vials such that each vial only pierced once. b. The variation in retention times of the CCV(s) does not exceed 3% compared to that of the standard curve. c. The peak areas of the CCV(s) do not vary by >15% compared to the corresponding standard in the calibration curve. 6. The retention time of analytes in all matrix solutions are within 3% that of the toxin standards. 7. Each chromatographic peak must be defined by at least 10 data points. 8. To confirm the presence of each DST, two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio (S/N) is used for quantitation, and the transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation must be ≥ 3. 9. Ion ratios between the quantitative and confirmation ion transitions should be within ±20% of the relative ion abundance of the neat toxin standards. 10. Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (R_s) of OA/DTX2 are calculated using the equation below, where RT is retention time and W is peak width. R_s must be ≥ 1 for correct identification[1]. $$R_s = \frac{2\times(RT_2 - RT_1)}{W_1 - W_2}$$

C. Validation Criteria		
1. Accuracy / Trueness	Y	Following the NSSP Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Accuracy/Trueness and Measurement Uncertainty (VII. #1), a total of 20 mussel samples were used, 10 samples from Maine and 10 from Washington. Each sample was first screened for DSTs using the described LC-MS/MS method to assure that each matrix sample did not contain any naturally accumulated toxins. Once these samples were established to be free of contamination, each was spiked at one of five concentrations ranging from 8 µg/100 g to 32 µg/100 g. Concentrations for spiking were selected to cover one half to two times the regulatory limit for DSTs in shellfish which is 16 µg of toxin per 100 g of shellfish tissue. Source locations were alternated (ME, WA, ME, WA, etc.) for all 20 samples to avoid any bias. All samples were quantified using both neat (MeOH) and matrix-matched standard curves, prepared as described above. The average accuracy for all samples combined was 100% for DTX1, 105% for DTX2, and 94% for OA when quantified using a MeOH standard curve. Accuracies were 91% for DTX1, 99% for DTX2, and 85% for OA when quantified using a matrix-matched standard curve. Full data provided in the SLV proposal report. FDA typically considers acceptable accuracy/trueness (as % recovery) to be 60-115% for concentrations in the range of 10 µg/kg (equivalent to 1 µg/100g) and 80-110% for concentrations in the range of 100 µg/kg (equivalent to 10 µg/100g)[5]. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends recoveries between 60-115% or 70-130% depending on the toxin and method for the DST group[2]. Based on this, the method was found to have acceptable accuracy when using both MeOH and matrix-matched standard curves.
2. Measurement Uncertainty	Y	Measurement uncertainties were determined to be 0.50, 0.50, and 0.54 µg/100g for DTX1, DTX2, and OA, respectively, when quantified using a MeOH standard curve, and 0.50, 0.50, and 0.47 µg/100g, respectively, when quantified using a matrix-matched standard curve. All data provided in the full SLV proposal.
3. Precision Characteristics (repeatability and reproducibility)	Y	Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Recovery and Precision (VII. #3), 10 samples (5 from Maine and 5 from Washington, alternating by source location) were each fortified at concentrations of 8, 16, and 32 µg/100 g, representing 1/2X, 1X, and 2X the regulatory level of 0.16 mg okadaic acid (OA) equivalents/kg (0.16 ppm). To assess method precision (as intermediate precision[5]), all samples were analyzed as replicate injections on separate days. As another measure of method precision (as repeatability[5]), percent relative standard deviation (%RSD) was calculated for five replicate extractions of two different naturally contaminated certified reference materials (CRMs) purchased from the National Research Council (NRC) Canada. All samples were quantified using both MeOH and matrix-matched standard curves. For analysis of intermediate precision data, following the

		SOP, a nested analysis of variance (F test) was performed, and significant differences were found for all treatments with the exception of DTX2 using a matrix matched standard curve only. This indicates that the precision of the method was not consistent across the range of concentrations tested, potentially due to additional sources of variation not accounted for with this test. It should be noted that variation in matrix effects was observed between individual samples in the initial matrix effects study, which led to the recommendation of pooling blank matrices for use in the matrix matched standard curves. As an alternative approach, Bartlett's test of homogeneity of variances was used, which differs from the F test in that it pools the data across replicates and samples and compares differences in the variance over the three spike levels. Using this approach, Bartlett's test was not significant for DTX1 using either standard curve but was still significant for DTX2 using the MeOH curve only ($p=0.0057$) and was weakly significant for OA using both the MeOH and matrix matched standard curves ($p=0.021$ and $p=0.034$, respectively). The SOP does not provide guidance for the calculation of precision if the initial F test fails. Therefore, instead of calculating a single precision score across the range of concentrations tested, as indicated in the SOP, the precision of the method, measured as the %RSD of replicate injections for 10 samples spiked at three concentrations, was calculated individually for each spike level. Using this approach, the %RSD ranged from 5.6-8.3 for DTX1, 4.4-8.4 for DTX2, and 9.4-15.8 for OA when using a MeOH standard curve; %RSD ranged from 4.6-7.1 for DTX1, 4.6-5.5 for DTX2, and 5.5-10.0 for OA when using a matrix-matched standard curve. To assess method repeatability, %RSDs for five replicate extractions of the two naturally contaminated CRMs ranged from 1.39-4.72 for all toxins when using a MeOH standard curve and 1.41-2.46 when using a matrix-matched standard curve Overall, the intermediate precision for all toxins and concentrations had <10 %RSD for both MeOH and matrix matched standard curves with the exception of OA and only at the lowest fortification level. Method repeatability for all toxins had <5 %RSD using both MeOH and matrix matched standard curves, FDA method validation guidelines recommend %RSDs of <22% being acceptable for concentrations in the range of 1 µg/100g and <11% in the range of 10 µg/100g. Therefore, the method was considered to be sufficiently precise for the intended purpose.
4. Recovery	Y	Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Recovery and Precision (VII. #3), 10 samples (5 from Maine and 5 from Washington, alternating by source location) were each fortified at concentrations of 8, 16, and 32 µg/100 g, representing 1/2X, 1X, and 2X the regulatory level of 0.16 mg okadaic acid (OA) equivalents/kg (0.16 ppm). Matrix fortifications and extractions were conducted as described above for accuracy/trueness. All samples were quantified using both MeOH and matrix-matched standard curves.

		Recoveries ranged from 102-103% for DTX1, 105-106% for DTX2, and 92-96% for OA when using a MeOH standard curve. Recoveries ranged from 89-95% for DTX1, 95-99% for DTX2, and 80-86% for OA when using a matrix-matched standard curve. One-way analysis of variance testing for significant differences between spike concentrations was not significant with the exception of DTX1 using the matrix-matched standard curve only ($p=0.042$). Tukey's test for honestly significant differences showed that the significant variance was between the low (8 µg/100 g) and the high (32 µg/100g) concentrations only. Full data provided in the SLV proposal report. FDA typically considers acceptable recoveries to be 60-115% for concentrations in the range of 10 µg/kg (equivalent to 1 µg/100g), and 80-110% for concentrations in the range of 100 µg/kg (equivalent to 10 µg/100g)[5]. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends recoveries between 60-115% or 70-130% depending on the toxin and method for the DST group[2]. In this study, recoveries using both MeOH and matrix matched standard curves were well within these guidelines. The significant difference in variance between the low and high fortification levels (for DTX1 using a matrix matched standard curve only) was not surprising considering most validation guidelines have a broader range of acceptable recoveries and precision at lower spike levels.
5. Specificity	Y	Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Specificity (VII. #4), all three DSTs were analyzed in the presence of the potentially co-occurring lipophilic toxins azaspiracids (AZA-1-3), pectenotoxin (PTX-2), and yessotoxin. Three aliquots of a single blank mussel tissue matrix were used. One aliquot served as a control blank, one sample contained an action level spike (16 µg/100 g) of each DST, and one contained the same concentration of DSTs plus a 16 µg/100 g spike each of AZA-1, AZA-2, AZA-3, PTX-2, and YTX. Five technical replicates of each aliquot, excluding the negative control blank, were analyzed. A one-way analysis of variance was used to test for significant differences from an SI of 1. The specificity index for all three DSTs was between 0.96-1.01 using both MeOH and matrix-matched standard curves. The one-way analysis of variance found that the SI was not significantly difference from 1 for DTX1 and OA but was significant for DTX2 ($P=0.020$). The average difference between samples containing the additional lipophilic shellfish toxins was only 0.60 ± 0.43 and 0.72 ± 0.53 µg/100g for MeOH and matrix-matched standard curves, respectively. In addition, the freeze-dried mussel CRM material analyzed as part of the comparability testing (5 replicates), which is reported to also contain domoic acid and multiple congeners of azaspiracids, yessotoxins, pectenotoxins, and the cyclic imines spirolides, pinnatoxins, and gymnodimines, was also used to assess method

		specificity. Despite the presence of these multiple potentially co-occurring and interfering compounds, the LC-MS/MS method produced results for DSTs that were between 99-105% and 93-108%, for MeOH and matrix-matched standard curves, respectively, compared to the informational values for total (post-hydrolysis) DSTs reported by NRC. Despite the significant finding for DTX2, the combined data shows that the method is specific for DST's and does not suffer from interference from potentially co-occurring lipophilic shellfish toxins. The significant finding for DTX2 is believed to be due to the high precision of the 5 technical replicates, as the average difference between samples with and without the potential interferants was very small (0.60 ± 0.43 and 0.72 ± 0.53 µg/100g for MeOH and matrix-matched standard curves, respectively).
6. Working and Linear Ranges	Y	Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity (VII. #5), 10 samples (5 from Maine and 5 from Washington, alternating by source location) were each fortified at concentrations spanning 50-150% of the range of interest (4, 8, 16, 32, and 48 µg/100 g). Matrix fortification and extraction were conducted as described above for accuracy/trueness. Coefficients of determination were greater than 0.98 for all three toxins showing linearity across the range of interest. Linearity data were also processed as suggested in SLV SOP #5 for each toxin by plotting the log of the response per unit toxin versus the log concentration of spiked toxin along with the 95% confidence intervals of the mean response across all concentrations. With the exception of a single data point (1/10 OA spikes at 4 µg/100g which was the lowest concentration tested), all other data points fell within the 5% brackets, indicating that between 4 µg/100 g and 48 µg/100 g the mass spectrometric response is linearly proportional to the concentration within the working range of the analysis for all three toxins. Full data provided in the SLV proposal report.
7. Limit of Detection	Y	Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity (VII. #5), 10 samples (5 from Maine and 5 from Washington, alternating by source location) were each fortified at concentrations spanning 50-150% of the range of interest (4, 8, 16, 32, and 48 µg/100 g). Matrix fortification and extraction were conducted as described above for accuracy/trueness. LODs for each toxin of interest were determined based on the standard deviation of the response and the slope from the linear range data. The equation was expressed as: LOD = 3.3σ/S, where σ is the standard deviation of the response from five blank matrix samples, and S is the slope of the calibration curve. The limit of detection (LOD) for the mussel matrix was

		found to be 0.197 µg/100 g for DTX1, 0.591 µg/100 g for DTX 2, and 0.159 µg/100 g for OA. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends an LOD for methods for the determination of DSTs of 1 µg/100 g for OA and DTX1, and 3 µg/100 g for DTX2.
8. Limit of Quantitation / Sensitivity	Y	Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity (VII. #5), 10 samples (5 from Maine and 5 from Washington, alternating by source location) were each fortified at concentrations spanning 50-150% of the range of interest (4, 8, 16, 32, and 48 µg/100 g). Matrix fortification and extraction were conducted as described above for accuracy/trueness. LOQs for each toxin of interest were determined based on the standard deviation of the response and the slope from the linear range data. The equation was expressed as: LOQ = 10σ/S, where σ is the standard deviation of the response from five blank matrix samples, and S is the slope of the calibration curve. The limit of quantitation (LOQ) was determined to be 0.597 µg/100 g for DTX1, 1.79 µg/100 g for DTX 2, and 0.482 µg/100 g for OA. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends an LOQ for methods for the determination of DSTs 2 µg/100 g for OA and DTX1, and 6 µg/100 g for DTX2.
9. Ruggedness	Y	To test method ruggedness, the effect of the hexane washing step was assessed on accuracy/trueness using both MeOH and matrix-matched standard curves. This was specifically requested by two NSSP laboratories that intend to implement this method, if approved. Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness (VII. #2), 2 sub-samples from each of 10 extracts from previously spiked samples used to assess accuracy/trueness (5 from Maine and 5 from Washington, alternating by source location), individually spiked at one of five concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were hydrolyzed following the procedure above. For each sample, one hydrolyzed extract was put through the above-described hexane washing step, while the other was filtered directly into an LC vial for analysis. Each sample was injected in duplicate and quantified using both MeOH and matrix-matched standard curves. For the non-hexane washed treatment, a corresponding non-hexane washed standard curve was prepared. As another measure of method ruggedness, 10 extracts from previously spiked samples used to assess accuracy/trueness (5 from Maine and 5 from Washington, alternating by source location), individually spiked at one of five concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were re-analyzed using both the current analytical column and conditions (“HPLC”, Waters Xbridge Premier BEH C18 VanGaurd FIT, 3.5 µm particle size, 2.1 × 100

		mm; flow rate 0.300 mL/min; 5 µL injection) and the column validated in proposal #17-103 (“UPLC”, Waters BEH C18, 1.7 µm, 1.0 mm × 150 mm; flow rate 0.120 mL/min; 5 µL injection), which is smaller in both particle size, diameter, and flow rate, but runs at a higher pressure requiring a UPLC system. For analysis of data for the ruggedness testing of the hexane washing step, a paired t-test was used to test for significant differences in accuracy between the two treatments, following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness (VII. #2), as the variance ratio was within -2 to +2 for all three toxins indicating that the variance was homogeneous. The paired t-test indicated significant differences ($P<0.0001$) in accuracy for all three toxins using both a MeOH and matrix-matched standard curve with samples analyzed without the hexane washing step being consistently higher. Average % accuracy was within the acceptable range for all three toxins when the hexane wash step was used (97% and 90% for DTX1, 105% and 101% for DTX2, and 91% and 84% for OA – for MeOH and matrix-matched standard curves, respectively). Without the hexane wash step, OA had acceptable recoveries both with MeOH and matrix-matched standard curves (110% and 101%, respectively). DTX1 had acceptable recoveries only when a matrix-matched standard was used (111%). Recoveries fell outside of acceptable ranges for DTX1 when a MeOH standard curve was used (120%). For DTX2, both MeOH and matrix-matched standard curves had accuracies outside of acceptable ranges (128% and 124%, respectively). For the analysis of results for the comparison of the column used in the current SLV compared to the column used in SLV #17-103 a paired t-test was used to test for significant differences in accuracy between the two columns, following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness (VII. #2), as the variance ratios were within -2 to +2 for all three toxins indicating that the variances were homogeneous. The paired t-test indicated no significant differences in accuracy between the different columns for DTX1 using either a MeOH or matrix-matched standard curve. For DTX2, no significant differences were detected when using a MeOH standard curve, but a significant difference was detected ($P=0.00046$) when using a matrix-matched standard curve. For OA, significant differences were detected for both standard curve options ($P=0.0036$ and $P=0.0024$ for MeOH and matrix-matched standard curves, respectively). However, accuracies were within acceptable ranges for all three toxins (82-103% and 80-101% for MeOH and matrix-matched standard curves, respectively, for the new column, compared to 86-101% and 77-96% for MeOH and matrix-matched standard curves, respectively, for the column used in SLV #17-103). Full data provided in the SLV proposal report. In the current study, the effects of removing the hexane washing step were more pronounced than those

		observed during ISSC proposal #17-103, with significant differences observed for all three toxins, using both MeOH and matrix-matched standard curves. Further, by excluding this clean-up step, several toxins and treatments now showed recoveries outside of acceptable ranges, likely due to the lack of removal of lipophilic matrix enhancers. Results for the re-analysis of a sample set previously tested for accuracy using both the current (HPLC) analytical column and the previous (UPLC) analytical column, using both MeOH and matrix-matched standard curves, were comparable and within acceptable ranges indicating that the previously tested UPLC column would also be acceptable for use with the modified method.
10. Matrix Effects	Y	While the ISSC requires that matrix effects be assessed as part of the single laboratory validation process, they do not prescribe how this should be done or how the data should be treated. To test for potential differences in matrix effects between the 20 individual blank mussel samples collected for this study, each was extracted, hydrolyzed, and hexane washed following the above procedure. Each sample was first screened for DSTs using the LC-MS/MS method to assure that each matrix sample did not contain any naturally accumulated toxins. Once this was confirmed, each extract was fortified at 10 concentrations: 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 ng/mL (equivalent to 0.49-250 µg/100g) of each toxin using 2 µg/mL stocks. Curves were analyzed over a 3-day period with neat (MeOH) standard curves run before and after each day's sample queue. The 10 East coast and 10 West coast matrix curves were alternated to avoid any bias and the slope of each was compared to the average slope of the bracketing MeOH curves. As a further test, hydrolyzed and hexane washed extracts for all 10 East coast and all 10 West coast matrix samples were pooled. The slope of each matrix curve was compared to the average slope of the bracketing MeOH curves. All calibration curves were shown to be linear ($R2 \geq 0.99$) within the range of 0.391-200 ng/mL for all 20 individual matrix sources. However, the difference in slopes compared to the corresponding MeOH curve varied for both matrix and toxin showing both enhancement and suppression (93-116% for DTX1, 92-105% for DTX2, and 92-109% for OA). No trends were seen between East and West coast matrix sources. As a further test, hydrolyzed and hexane washed extracts for all 10 East coast and all 10 West coast matrix samples were pooled and used to prepare 10-point standard curves as described above. Slopes compared to a MeOH standard curve were 100%, 93%, and 93% for pooled East coast blank mussel samples and 99%, 93%, and 91% for pooled West coast blank mussel samples for DTX1, DTX2, and OA, respectively. Full data provided in the SLV proposal report. Although no acceptability criteria have been established for matrix effects, the slopes for all 20 individual matrix sources were within 10% for OA and DTX2 and within 16% for DTX1. These results were similar for pooled

		matrix samples for clam from proposal #17-103 (slopes within 10% for OA and DTX2 and within 19% for DTX1). But due to the variability observed within matrix sources used in this study (93-116% for DTX1, 92-105% for DTX2, and 92-109% for OA), it was decided for the matrix matched standard curves to be used in the SLV, 10 blank mussel samples would be pooled, 5 randomly selected from the East coast and 5 randomly selected from the West coast. For routine analysis in a particular region, it is recommended that at least 5 pooled blank matrix samples be used from the region of sample collection to account for any potential variability between matrix sources.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	The purpose of this proposal was to modify the existing LC-MS/MS method originally validated under ISSC proposal #17-103 to address several deficiencies identified during method verification. Therefore, direct comparability of the methods was not appropriate as the method was never established outside of the validating laboratory and not in routine use. To establish comparability in ISSC proposal #17-103, multiple naturally contaminated certified reference materials (CRMs) were analyzed and compared to the reference values provided by the manufacturer, as recommended by the ISSC Laboratory Committee. As a measure of method comparability for the current SLV, these same CRMs were purchased again (5 individual vials of frozen CRM-DSP-Mus-c and 5 separate extractions of lyophilized NRC-FDMT1). The lyophilized CRM contains a number of additional lipophilic shellfish toxins, including azaspiracids, pectenotoxins, and yessotoxins; therefore, this material was also used as a secondary measure of method specificity. Each was quantified with both MeOH and matrix-matched standard curves and compared to both the reported values and values obtained in proposal #17-103. As a further test of method comparability, we participated in the WEPAL-QUASIMEME BT11 (2025) DSP shellfish toxins study. WEPAL-QUASIMIEME is part of Stichting Wageningen Research, Netherlands, and provides globally accredited proficiency testing (PT) programs including one for DSP testing by LC-MS/MS (BT11). The scope for the QUASIMEME BT11 program included total (free plus esterified) OA, total DTX1, total DTX2, and total combined OA+DTX1+DTX2 (in OA eq.). Three blinded samples (frozen shellfish homogenates) were provided to our laboratory; unblinded sample information, available after the testing period had closed, identified the homogenates as mussels (*Mytilus edulis*) from Ireland (sample QST371BT), cockles (*Cerastodema edule*) from England (sample QST372BT), and mussels (*Mytilus edulis*) from the United Kingdom (sample QST373BT). Samples were analyzed using both matrix-matched and neat (MeOH) standard curves, with hexane sample clean up, but only results for the matrix-matched standard curve were provided to the study organizers through their on-line portal. Statistical analysis was performed by the QUASIMEME study organizers using data from all participating laboratories (40 labs in 2025) and is

		recorded in the WEPAL-QUASIMIEME BT11 DSP Shellfish Toxins Final Report 2025.1 Version 1: 22 July 2025 (accessible to study participants). The data provided using this SLV method is recorded in the study as Q4122A. The study uses a z'-score to evaluate inter-laboratory performance. Statistical explanations of this test and data are described in the Final QUASIMEME 2025 Report. For the CRM analysis, percent accuracy for all three toxins compared to the informational total toxin values (post-hydrolysis) contained in the certificates of analysis ranged from 99-114% when using the MeOH standard curve and 93-108% when using the matrix-matched standard curve. Percent accuracy for the same CRM lots tested during ISSC proposal #17-103 ranged from 92-111%, using a MeOH standard curve only. For the data values for OA, DTX1, DTX2, and total DSTs provided by our laboratory to the QUASIMEME study, all were assessed as satisfactory performance with z'-score's less than 2. Full data provided in the SLV proposal report. The satisfactory performance results for all summitted data from the QUASIMEME study provides additional support that this method is suitable for the detection and quantitation of OA, DTX1, and DTX2 as results were comparable to validated methods currently in use in EU laboratories.
D. Other Information		
1. Cost of the Method	Y	Approximately $14/sample in reagents and consumable supplies.
2. Special Technical Skills Required to Perform the Method	Y	General laboratory skills (i.e., ability to accurately pipette small volumes, etc.). Experience with operation and general maintenance of liquid chromatography mass spectrometry equipment is required.
3. Special Equipment Required and Associated Cost	Y	Capital equipment purchases: 1. Ultra-high Performance Liquid chromatograph (UHPLC) or High-Performance Liquid Chromatograph (HPLC), capable of running in gradient mode [examples: Waters Corporation Acquity UPLC system (Manchester, UK) (approx. $60,000, new with government (GSA) discount), Shimadzu Corporation Nexera UHPLC system (Kyoto, Japan) (approx. $80,000, new with government (GSA) discount)] 2. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities [example: Sciex QTrap 5500+ equipped with a Turbo V ionization source (Framingham, MA) (approx. $320,000, new with government (GSA) discount)]
4. Abbreviations and Acronyms Defined	Y	OA: Okadaic acid; DTX1: Dinophysistoxin-1 ; DTX2: Dinophysistoxin-2; LC-MS/MS: Liquid Chromatography Tandem Mass Spectrometry
5. Details of Turn Around Times (time involved to complete the method)	Y	Analysis of 10 samples, including extraction, hydrolysis, sample analysis, and quantitation can be accomplished in approx. 6 -7 hours. Analysis time increases by 2.5 hours for every 10 additional samples.

6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	The quality systems for the FDA Human Foods Program laboratory in which this work was performed followed the Divisions of Analytical and Bioanalytical Chemistry Laboratory Quality Control Plan (Version 2.3, Dated February 12, 2024). This plan covers QC protocols for the Laboratory Environment, Materials, Equipment, Procedures, Training, Documentation, Reporting, Monitoring and Verification, as well as Corrective Actions.
Submitters Signature	Date:	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. Accuracy/Trueness - Closeness of agreement between a test result and the accepted reference value.
2. Analyte/measurand - The specific organism or chemical substance sought or determined in a sample.
3. Blank - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.

4. Comparability – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. Fit for purpose – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. HORRAT value – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. Limit of Detection – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. Limit of Quantitation/Sensitivity – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. Linear Range – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. Measurement Uncertainty – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. Matrix – The component or substrate of a test sample.
12. Method Validation – The process of verifying that a method is fit for purpose.[1]
13. Precision – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. Repeatability – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. Reproducibility – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. Quality System – The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision-making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. Recovery – The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. Ruggedness – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. Specificity – the ability of a method to measure only what it is intended to measure.[1]
18. Working Range – the range of analyte or measurand concentration over which the method is applied.

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6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Single Laboratory Validation (SLV) Proposal for Submission to the Interstate Shellfish Sanitation Conference (ISSC) For Method Approval

Name of the New Method:

Modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

Name of Method Developers:

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A. NEED FOR THE NEW METHOD

1. Need for Which Method has been Developed:

Shellfish contaminated with natural toxins can cause consumer illnesses. The Food and Drug Administration (FDA) has established guidance levels for five groups of natural toxins in shellfish responsible for the following illnesses: amnesic shellfish poisoning (ASP), azaspiracid shellfish poisoning (AZP), diarrhetic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). While management programs have existed in the United States for PSP, NSP, and ASP for many years, the need for management programs for lipophilic shellfish toxins, including DSP and AZP, are a relatively recent occurrence. The first shellfish harvesting closure due to DSP toxins (DSTs) in excess of the regulatory guidance level of 16 µg OA eq./100 g shellfish (0.16 ppm) occurred on the Texas Gulf Coast in 2008. The first harvesting closure, and illnesses, in the Puget Sound region occurred in 2011 and the first closure in the New England region occurred in 2015. In response to these events, a method for the detection and quantitation of DSTs based on liquid chromatography tandem mass spectrometry (LC-MS/MS) was validated and approved by the ISSC for use in clams in 2017 (Proposal #17-103). A checklist for the evaluation of this method in NSSP labs was approved in 2023 (Proposal #19-136). However, after subsequent failure of verification efforts in several NSSP labs, issues were noted with the method that were preventing its widespread use. This report describes the validation of a modified LC-MS/MS method for DSTs for use in the NSSP for the management of this toxin group in mussels. These modifications

specifically address deficiencies in the original method for clams identified during method verification. If approved, this modified method will subsequently be extended to additional matrices, including clams and oysters.

2. Purpose and Intended Use of the Method:

The intention is for this method to be an Approved Method for Biotoxin Testing for DSP toxins under the NSSP and that it should be added as a new listing in Section IV: Guidance Documents, Chapter III: Laboratory, @.02 Methods, .01 Approved NSSP Laboratory Tests, Table 2 Approved Methods for Marine Biotoxin Testing under the heading: Biotoxin Type: Diarrhetic Shellfish Poisoning (DSP) titled **Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) – Matrix Matched (Mussels only)**, and the applications should be (1) Growing Area Survey and Classification, (2) Dockside Testing Program, and (3) Controlled Relaying.

3. Need for the New Method in the NSSP, Noting Any Relationships to Existing Methods:

In 2011, the European Union (EU) adopted LC-MS/MS as the reference method for lipophilic shellfish toxins, which include DSTs, and developed a harmonized SOP summarizing several similar methods that had been individually validated in various EU labs.[1] However, because the EU protocol is a summary of several distinct methods, measures a number of additional lipophilic toxins not regulated in the US such as pectenotoxins and yessotoxins, and measures both free and esterified DSTs separately, it was necessary to pick a single method from the options in the EU SOP that measured total DSTs to validate for use in the NSSP. ISSC Proposal #17-103 was the first attempt at this effort, and although the method was found to be fit for purpose for the measurement of DSTs in clams in the developing laboratory, verification efforts by several NSSP labs failed due primarily to differences in the performance of the liquid chromatographs and mass spectrometers available in those laboratories. Specifically, the #17-103 method required the use of an ultra-performance liquid chromatograph (UPLC), which utilizes LC columns of smaller diameter and particle size running at elevated pressures and used a Sciex 5500 QTrap® triple quadrupole mass spectrometer. A combination of not having access to a UPLC system and differences in the performance of available mass spectrometers from other manufacturers, specifically increased susceptibility to matrix effects, led to the failure of participating NSSP labs from being able to verify the approved method. This new SLV proposal uses an HPLC column that can be used on both UPLC and standard HPLC systems and introduces the optional use of a matrix-matched standard curve to normalize responses between different brands and models of mass spectrometers. This new SLV was also validated using mussels while the original used clams. Once approved, this modified method will be extended to include additional matrices such as clams, oysters, etc.

4. Type of Method (Chemical, Molecular, or Culture):

Chemical Confirmatory Method: Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) specifically measures total (free plus esterified) okadaic acid, dinophysistoxin-1, and dinophysistoxin-2.

B. METHOD DOCUMENTATION

1. Method Title:

Modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish.

2. Method Scope:

This method is fully validated for the determination of diarrhetic shellfish poisoning toxins in mussels.

3. References:

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4. Principle:

Liquid chromatography tandem mass spectrometry (LC-MS/MS) is an analytical technique that provides both physical separation (liquid chromatography) and mass analysis (mass spectrometry) of sample components. In this work, a Shimadzu Nexera Ultra-High Performance Liquid Chromatography system was coupled with a Sciex 5500+ QTrap® triple quadrupole mass spectrometer by electrospray ionization for targeted quantitation of DSP toxins. Structurally informative transitions were specified for multiple reaction monitoring (MRM) for each toxin of interest. In MRM mode, an ion of interest (precursor ion) is preselected in the first quadrupole and fragmented in the second quadruple. Multiple product ions resulting from fragmentation of the precursor ion are then mass analyzed in the third quadrupole. Two analyte specific transitions, one for quantitation and one for confirmation, are specified for each analyte and monitored in both the calibration standard solutions and in the extracted shellfish matrices. Due to differing susceptibility to matrix enhancement between different manufacturers and models of mass spectrometers, this method includes the optional use of a matrix matched standard curve as well as a liquid/liquid partitioning sample clean-up step using hexane. A linear fit is applied to the peak area data

for the quantitation ion collected for the calibration standards, and the equation for this line is utilized to calculate the concentration of each analyte in the spiked matrix samples. The enhanced resolving power afforded by LC and the selectivity gained by tandem mass spectrometry permitted the accurate detection and quantitation of DSP toxins in complex shellfish matrices. Total toxicity can be estimated as the sum of the molar concentrations of detected analogs multiplied by the relevant specific toxicity equivalency factors (TEFs)[2-3]. The currently accepted toxicity equivalency factors for DSP toxins are as follows: OA – 1, DTX1 – 1, and DTX2 – 0.6. To date, only OA and DTX1 have been found in shellfish responsible for illnesses or involved in harvesting closures the U.S.

5. Analytes of Interest:

Diarrhetic shellfish poisoning toxins: okadaic acid (OA), dinophysistoxin-1 (DTX1), and dinophysistoxin-2 (DTX2). Shellfish metabolites (fatty acid acyl-esters for all 3 toxins, collectively referred to as DTX3) are included through the use of a required alkaline hydrolysis step.

6. Proprietary Aspects: None

7. Cost of the Method:

Capital equipment purchases:

1. Ultra-high Performance Liquid chromatograph (UHPLC) or High-Performance Liquid Chromatograph (HPLC), capable of running in gradient mode [examples: Waters Corporation Acquity UPLC system (Manchester, UK) (approx. $60,000, new with government (GSA) discount), Shimadzu Corporation Nexera UHPLC system (Kyoto, Japan) (approx. $80,000, new with government (GSA) discount)]

2. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities [example: Sciex QTrap 5500+ equipped with a Turbo V ionization source (Framingham, MA) (approx. $320,000, new with government (GSA) discount)]

Cost per sample (approx. for consumables): $14/sample

8. Sample Turn Around Time: Analysis of 10 samples, including extraction, hydrolysis, sample analysis, and quantitation can be accomplished in approx. 8 -12 hours. Analysis time increases by 2.5 hours for every 10 additional samples.

9. Equipment Required:

1. Instrumentation for sample preparation: knives for shucking, stainless steel laboratory spatulas, sieve for draining
2. 4 oz. plastic screw top specimen cups (if a sample of unextracted homogenate is to be saved) (e.g., Covidien #17099)
3. Balance, accuracy to the nearest 0.01 g
4. Blender, food processor, or laboratory homogenizer (e.g., Polytron Ultraturax equipped with a generator appropriate for shellfish (e.g. 20 mm saw toothed))

5. Vortex mixer (either single or multi-tube)
6. Centrifuge, capable of 2000 x *g*, with adaptors for 50 mL and 15 mL centrifuge tubes
7. Heat block or water bath capable of maintaining 76 ± 2 °C
8. Volumetric flasks or graduated cylinders capable of accurately measuring 20, 100, 500, and 1000 mL
9. Disposable 50 mL centrifuge tubes
10. Disposable glass Pasteur pipettes with bulbs
11. 20 mL glass scintillation vials with coned polyethylene lined caps (if a sample of non-hydrolyzed methanolic extract is to be saved) (e.g., Wheaton #986560)
12. Disposable 16 × 100 mm glass screw cap tubes with phenolic PTFE lined caps (e.g., Fisher #14-959 35AA (tubes), Corning #9998-15 (caps))
13. 0.01, 0.05, 0.1, 0.5, and 1 mL positive displacement microdispensers or syringes (e.g., Drummond #3-000-510, 3-000-575, 3-000-590 or Hamilton #80530, 80630, 81330, 81365)
14. 1-3 mL disposable syringes (e.g., Becton Dickinson #309602)
15. PTFE syringe tip membrane filters, 13 mm, pore size 0.2 µm (e.g., Pall #PN4542)
16. LC-MS autosampler vials with appropriate caps (e.g., Agilent #5182-0715)
17. Reversed Phase HPLC Column (e.g., Waters Xbridge Premier HPLC BEH C18 VanGuard Fit, 3.5 µm particle size, 2.1 × 100 mm, or Waters Acquity UPLC BEH C18, 1.7 µm particle size, 1.0 × 150 mm)
18. Standard High-Performance (HPLC) or Ultra-Performance (UPLC) Liquid chromatograph capable of running in gradient mode
19. Mass Spectrometer, equipped with an electrospray ionization source and multiple reaction monitoring scan mode capabilities

10. Reagents Required:

1. Certified Reference Standards

Okadaic acid: e.g. (NRC-CRM-OAd) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-1: e.g. (NRC-CRM-DTX1c) NRC-CNRC Institute for Marine Biosciences, Canada
Dinophysistoxin-2: e.g. (NRC-CRM-DTX2c) NRC-CNRC Institute for Marine Biosciences, Canada

Stock toxin solutions in MeOH were stored at ≤ −20 °C according to manufacturer's recommendations.

2. Chemicals

Acetonitrile, HPLC Optima Grade
Methanol, HPLC Optima Grade
Water, HPLC Optima Grade
Ammonium formate (≥98% purity)
Formic Acid (≥98% purity)
Hydrochloric acid (37%/12 N)
Sodium hydroxide (≥98% purity)
Hexanes (Certified ACS, ≥98.5%)

11. Solution Preparation

1. Extraction solvent: 100% methanol

2. Hydrochloric Acid 2.5 M: Add 60 mL of water to a 100 mL volumetric flask or graduated cylinder. To the water, add 20 mL of 12 N hydrochloric acid (37%) and dilute with additional water to 100 mL. Place in an appropriate glass jar for storage marked with the date of creation. This solution may be stored at room temperature and can be used for up to three months.

3. Sodium hydroxide 2.5 M: Dissolve 10 grams of sodium hydroxide in 75 mL of water in a glass beaker and add to a 100 mL volumetric flask or graduated cylinder. Make up to 100 mL with water and transfer to an appropriate storage vessel marked with the date of creation. This solution may be stored at room temperature and used for up to 3 months.

12. Matrix or Matrices of Interest: Mussels

13. Sample Collection

For this study, mussels were collected from different growing areas at different times of the year (Table 1). Shellfish (≥12 animals, ≥100 g shucked meat) were collected fresh, shucked, drained of excess liquid, and homogenized thoroughly using a blender/food processor and/or stick homogenizer. Homogenized samples were stored frozen at ≤-20°C until analysis.

Table 1. Sources of blank shellfish matrices for spiking experiments

Source	Mussel (*Mytilus edulis*)	
A	Provided by Bigelow Analytical Services, Maine	10 samples collected between March 2023 and April 2024 from various growing areas in Maine
B	Provided by the Washington Department of Health Public Health Laboratory, Washington	10 samples collected between August 2021 and April 2022 from various growing areas in Washington state

14. Safety Requirements:

Proper precautions should be taken to avoid inhalation of harmful reagents or contact with skin or eyes. Analysts should wear a lab coat, gloves and safety glasses when working with chemicals. Chemical reagents that are flammable and/or toxic should be used within a chemical fume hood to protect laboratory workers.

15. Other Information:

Technical Skills Required: General laboratory skills (i.e., ability to accurately pipette small volumes, etc.). LC-MS Operator must be trained in the operation and maintenance of the specific liquid chromatography-mass spectrometry system used.

16. Abbreviation and acronyms:

DTX1: Dinophysistoxin-1
DTX2: Dinophysistoxin-2
OA: Okadaic acid
LC-MS/MS: Liquid-Chromatography Tandem Mass-Spectrometry

C. STEP BY STEP PROCEDURE.

1. Shellfish Preparation:

1. Clean outside of the shellfish with fresh water.
2. Cut the adductor muscles to open and rinse the inside with fresh water to remove any debris.
3. Remove the meat from the shell and drain tissue using a sieve to remove salt water.
4. Combine at least 12 animals and homogenize thoroughly for 60 – 120 seconds using a blender/food processor or a laboratory homogenizer.

2. Extraction Procedure:

1. Accurately weigh 2.00 g ± 0.05 g of tissue homogenate into a 50 mL disposable centrifuge tube.
2. Add 9.0 mL of methanol and mix thoroughly using a vortex mixer for 3 min.
3. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
4. Transfer the supernatant to a clean 20 mL graduated cylinder, volumetric flask, or glass scintillation vial.
5. Re-extract the residual tissue pellet with an additional 9.0 mL of methanol and mix thoroughly using a vortex mixer for 3 min.
6. Centrifuge at 2000 *g* for 10 min at approximately 20 °C.
7. Transfer the supernatant to a 20 mL volumetric flask or graduated cylinder and combine with the supernatant previously extracted. Adjust total extract volume to 20 mL with 100% methanol.
8. Transfer the 20 mL of total extract back into the 20 mL scintillation vial for storage (if desired).

3. Hydrolysis Procedure: Total DSP toxins (i.e. free toxin plus toxin fatty acid esters) must be quantified when performing this method. To do this, all samples must go through an alkaline hydrolysis step prior to analysis.

1. Accurately transfer a 2 mL aliquot of the 20 mL methanolic extract to a 16 × 100 mm glass tube with a phenolic PTFE lined screw cap using a positive displacement microdispenser or syringe.
2. Add 250 µL of 2.5 M NaOH to the 2 mL extract aliquot.
3. Mix with a vortex mixer for 10 seconds and record the total weight of the tube.
4. Ensure caps are securely fastened and heat the mixture at 76 °C for 40 minutes. [Note: 76 °C is above the boiling point for methanol, therefore sample loss will occur if tube caps do not fit well and are not securely fastened.]

5. Dry the water from the outside of the tubes, allow the tubes to cool to room temperature (approx. 5-10 minutes), and then re-weigh each tube. If the weight has dropped by more than 0.1 g, replace lost weight using 100% MeOH.
6. Neutralize samples with 250 µL of 2.5 M HCL, mix by vortexing for 10 seconds.

4. Sample Clean-up Procedure:

Partitioning of the methanolic shellfish extracts with hexane is performed to remove nonpolar lipids such as triglycerides, which could contaminate the source region of the instrument and/or affect the responses of the DSTs[4].

1. Add 7 mL of hexanes directly to the 2.5 mL hydrolyzed methanolic extract.
2. Mix by vortexing for 3 minutes.
3. Partition by centrifuging at 2,000 *g* for 10 min.
4. Using a disposable glass pasture pipette, collect the hexanes (top layer) and discard to an appropriate waste container.
5. Transfer approximately 1 mL of the methanolic extract (bottom layer) into a disposable syringe equipped with a 0.2 µm PTFE syringe tip filter.
6. Filter directly into an LC-MS certified glass vial and cap.

5. Quality Control Procedures:

1. When possible, a divert valve is used to divert LC flow at the beginning and end of each chromatographic run. In preliminary studies, when a divert valve was not used, the ion source region and curtain plate needed to be cleaned between each batch (approximately every 24 hours) to maintain adequate sensitivity. [Divert valve was used in the SLV]
2. A new calibration curve is prepared each day of analysis or once every 24 hrs. for long analytical runs. Each calibration curve is derived from six calibration points, and the linear regression should yield a correlation coefficient (R^2) greater than or equal to 0.99. If analyzing a large number of samples, the use of bracketing calibration curves can be used.
    a. If using bracketing standard curves, the calibration curve should be split into two sets of vials (i.e., each vial only pierced once).
    b. The variation in the individual calibration curve slopes between the first and second set of calibration standards should not exceed 25%[1].
    c. The variation in retention time of the standards in bracketing calibration curves should not exceed 3%[1].
    d. The data from bracketing curves should be averaged and fitted with a line using least squares linear regression. The average calibration curves should yield an R^2 greater than or equal to 0.98[1].
3. Reagent Blanks (methanol solvent) are analyzed after the high calibration standard and periodically (at minimum every 10 samples) after fortified samples to ensure that analyte carryover is not occurring. [Carryover defined as toxins detected > LOD].
4. A Procedural Blank (methanol carried through sample preparation process at the same time as the samples) is performed with each batch of samples.

5. When bracketing standard curves are not used, a continuing calibration verification (CCV) standard can be used; a separate mid-scale calibration standard is analyzed bracketing at most every 10 samples to assure that no retention time shifts (possibly due to column failure) or loss in signal intensity (due to fouling of the column or mass spectrometer) has occurred.
    a. The variation in retention times of the CCV(s) does not exceed 3% compared to that of the standard curve.
    b. The peak areas of the CCV(s) do not vary by >15% compared to the corresponding standard in the calibration curve.
6. The retention time of analytes in all matrix solutions are within 3% that of the toxin standards.
7. Each chromatographic peak is defined by at least 10 data points.
8. To confirm the presence of each DST, two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio (S/N) is used for quantitation, and the transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation must be ≥ 3. The same quantitation and confirmation transitions are used for standards, CCVs, and samples.
9. Ion ratios between the quantitative and confirmation ion transitions of samples, CCVs, and calibration standards should be within ±20% of the relative ion abundance of the neat toxin standards.
10. Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (R_s) of OA/DTX2 are calculated using the equation below, where RT is retention time and W is peak width. R_s must be ≥ 1 for correct identification[1].

$$R_S = \frac{2\times(RT_2 - RT_1)}{W_1 - W_2}$$

6. LC-MS/MS Procedure:

1. Instrumentation Used for Validation: Sciex QTrap 5500+ equipped with a Turbo V ionization source (Framingham, MA) and a Shimadzu Corporation Nexera UHPLC system (Kyoto, Japan).

2. LC Parameters:

HPLC column used for validation: Waters Xbridge Premier BEH C18 VanGuard FIT, 3.5 µm particle size, 2.1 × 100mm
Column Temperature: 40 °C
Autosampler Temperature: 10 °C
Injection Volume: 5µL

Note: Waters BEH C18, 1.7 µm, 1.0 mm × 150 mm [flow rate 0.120 mL/min; column temperature 40 °C; 5 µL injection] that was used in proposal #17-103 (UHPLC only) was also tested during this study (see ruggedness section) and was found to provide equivalent results.

3. LC Gradient:

Mobile phase A: 2mM ammonium formate and 50 mM formic acid in 100% water.

Preparation of 1000 mL: Dissolve 128 ± 10 mg ammonium formate in water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with water and add 1.9 mL of formic acid. Fill to mark with water.

Mobile phase B: 2mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water.

Preparation of 1000 mL: Dissolve 128 ± 10 mg ammonium formate in 48.1 mL water and transfer into a 1000 mL volumetric flask; fill approximately half-way to the mark with acetonitrile and add 1.9 mL of formic acid. Fill to mark with acetonitrile.

For the gradient in Table 2, LC flow is diverted to waste from time 0.0–4.5 min and from 7.75–15.0 min. This was approximately one minute before the first peak and one minute after the last peak to preserve instrument integrity from over spraying matrix into the source.

Table 2. LC Gradient

Time (min)	Flow Rate (mL/min)	%A	%B
0.0	0.300	50	50
2.0	0.300	50	50
6.0	0.300	30	70
8.0	0.300	1.0	99
10.0	0.300	1.0	99
10.5	0.300	50	50
15.0	0.300	50	50

4. MS Ion Source Parameters:

Turbo V ion source parameters were optimized in negative ionization mode for all analytes under the acidic chromatographic conditions listed above (Table 3). These parameters will vary between different instrument platforms or ionization sources.

Table 3. Turbo V ion source parameters

Source Temperature	550 °C
Ion Spray Voltage	−4500 V
Curtain Gas	25 au
Gas 1	40 au
Gas 2	40 au

5. MRM Parameters: Manual or automatic compound optimization must be performed to determine the optimal settings for the MRM parameters as these may vary between different instrument platforms.

Instruments must be correctly calibrated for negative ionization mode as OA, DTX1, DTX2 are optimally monitored at this polarity. The precursor and product ion mass-to-charge (m/z) is confirmed by acquiring full scan MS1 and MS/MS spectra for each toxin. At least two product ions must be monitored for each toxin, one for quantitation and one for confirmation. Dwell times for transitions are set such that at least 10 data points are acquired across each peak. Declustering Potential (DP), Entrance Potential (EP), Collision gas (CAD), Collison Energy (CE), and Collision Cell Exit Potential (CXP) must be optimized for each MRM transition monitored. Table 4 lists the compound-dependent parameters optimized for a Sciex QTRAP 5500+ detector. Note: To confirm the presence of each DST, two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio (S/N) is used for quantitation, and the transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation must be ≥ 3. For this study, the m/z 113.1 product ion was used for confirmation, but both the m/z 113.1 and 151.1 ions were monitored and either could be used.

Table 4. MRM Parameters used for Method Validation on a Sciex QTrap 5500+ Mass Spectrometer

Compound	Polarity	Q1 (*m/z*)	Q3 (*m/z*)	Dwell Time (ms)	DP (V)	EP (V)	CAD Gas	CE (V)	CXP (V)
OA	Negative	803.5	255.2	100	−110	−10	High	−70	−15
OA	Negative	803.5	113.1	100	−110	−10	High	−100	−19
OA*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	255.2	100	−110	−10	High	−70	−15
DTX2	Negative	803.5	113.1	100	−110	−10	High	−100	−19
DTX2*	Negative	803.5	151.1	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	255.2	100	−110	−10	High	−70	−15
DTX1	Negative	817.5	113.1	100	−110	−10	High	−100	−19
DTX1*	Negative	817.5	151.1	100	−110	−10	High	−70	−15

*One additional confirmatory ion transition was monitored for method validation purposes.

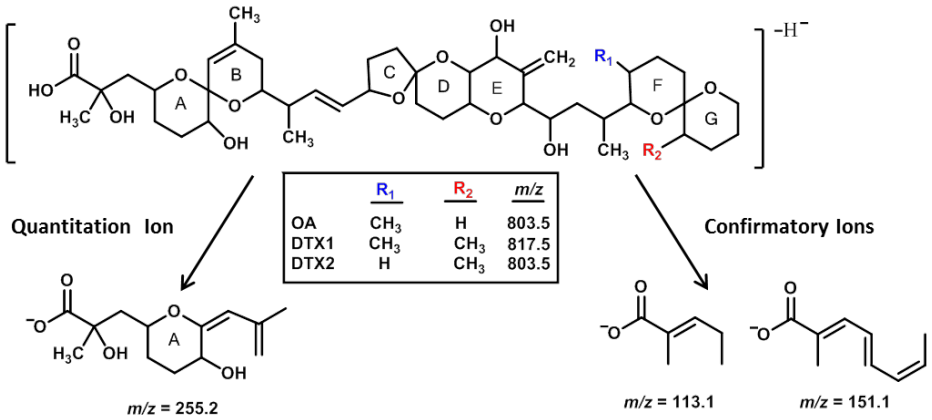


Figure 1. Structure of precursor ions and proposed product ion structures for DSTs.

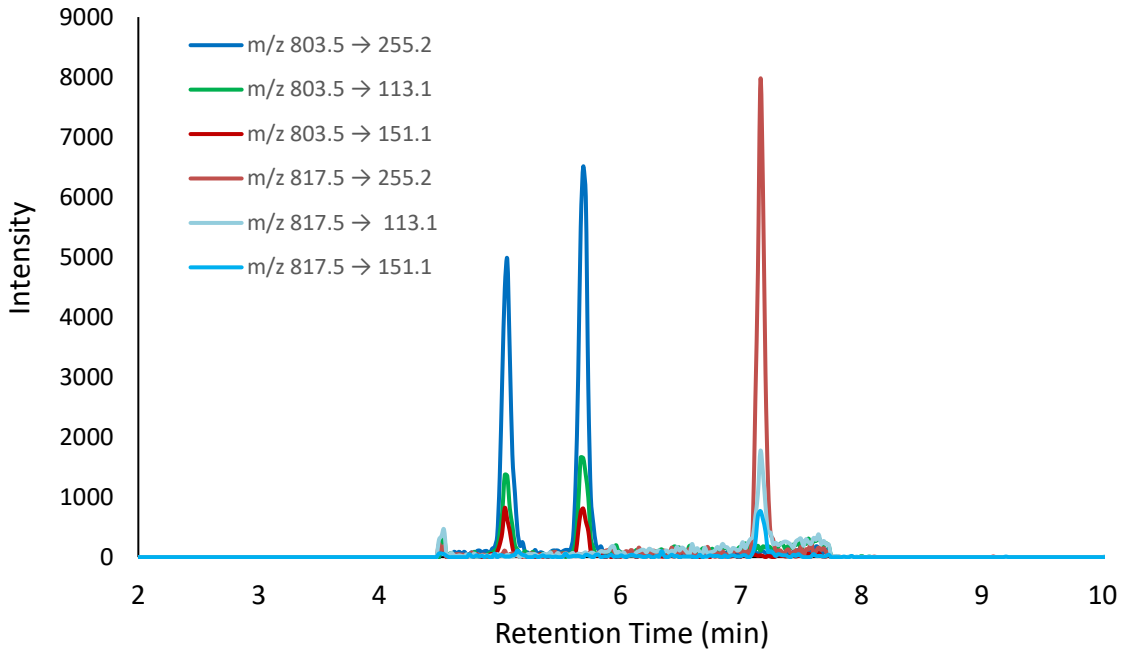


Figure 2. MRM chromatogram for the 12.8 ng/mL spike of OA, DTX1, and DTX2 into blank mussel matrix (12.8 ng/mL in extract is equivalent to 16 µg/100g in shellfish tissue prior to extraction).

D. METHOD VALIDATION

Preparation of Stock Solutions for Validation:

Certified reference materials from the National Research Council Canada were supplied in sealed glass ampoules containing at least 0.5 mL of each toxin at a certified concentration that differs between toxins and lots. Thus, different volumes of each toxin standard, in 0.5 mL aliquots transferred using a 0.5 mL positive displacement Hamilton syringe, were transferred to either 20 mL glass scintillation vials or 1 ml amber glass vials with PTFE lined caps and diluted with methanol to achieve stock standard solutions for each toxin at a concentration of 2 µg/mL (except for the AZAs which are only available in concentrations <2 µg/ml), as seen in Table 5 below. All stock solutions were stored at −20 °C.

Table 5. Toxin Stock Solution Preparation for Mussel Validation Study

Certified Reference Material	Certified Concentration (µg/mL)	Lot #	Volume (mL)	Solvent (ml)	Total Volume	Final Concentration (µg/mL)
CRM-DTX1-c	7.8 ± 0.5	20191010	4.5	14.4	18.9	2
CRM-DTX2-c	3.83 ± 0.18	20221102	4.5	13.05	17.55	
CRM-OA-d	8.4 ± 0.4	20141119	9.5	8.69	18.19	

CRM-AZA 1-c	1.54 ± 0.06	20220525	0.5	0.11	0.61	1
CRM-AZA 2-c	1.43 ± 0.06	20220623	0.5	0.095	0.595	1
CRM AZA 3-b	1.25 ± 0.05	20150527	0.5	0.09	0.59	1
CRM-PTX2-b	5.13 ± 0.13	20120516	0.5	0.6	1.1	2
CRM-YTX-c	5.0 ± 0.2	20151125	0.5	0.725	1.225	2

Validation Criteria:

Matrix Effects (performed as pre-validation), Accuracy / Trueness, Measurement Uncertainty, Precision, Recovery, Specificity, Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity, Ruggedness, and Comparability were assessed for the method.

1. Matrix Effects and Preparation of Optional Matrix Matched Standard Curve

Methods:

As one of the goals of this SLV study was to evaluate the optional use of a matrix-matched standard curve, matrix effects were the first parameter evaluated. This was performed prior to the validation as this information was required to determine how to prepare the matrix-matched curve both for the SLV and for routine analysis. While the ISSC requires that matrix effects be assessed as part of the single laboratory validation process, they do not prescribe how this should be done or how the data should be treated. To test for potential differences in matrix effects between the 20 individual blank mussel samples collected for this study (Table 1), each was extracted, hydrolyzed, and hexane washed following the below procedure. Each sample was first screened for DSTs using the LC-MS/MS method to assure that each matrix sample did not contain any naturally accumulated toxins. Once this was confirmed, each extract was fortified at 10 concentrations: 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 ng/mL (equivalent to 0.49-250 µg/100g) of each toxin using 2 µg/mL stocks. Curves were analyzed over a 3-day period with neat (MeOH) standard curves run before and after each day's sample queue. The 10 East coast and 10 West coast matrix curves were alternated to avoid any bias and the slope of each was compared to the average slope of the bracketing MeOH curves. As a further test, hydrolyzed and hexane washed extracts for all 10 East coast and all 10 West coast matrix samples were pooled.

Procedure:

1. For each sample (matrix source), ≥12 animals were rinsed, shucked, drained, and homogenized in a commercial food processor.
2. 2.0 ± 0.05 g of each homogenate was transferred to a 50 mL disposable centrifuge tube using a clean stainless-steel laboratory spatula.
3. 9.0 mL of 100% methanol was added to the centrifuge tube.
4. Samples were mixed for 3 min using a vortex mixer.
5. Samples were centrifuged at 2000 *g* for 10 min at 20 °C.
6. The supernatant was transferred to a 20 mL glass scintillation vial.
7. The residual tissue pellet was re-extracted with 9.0 mL of methanol, followed by vortexing for 3 min.
8. Samples were centrifuged at 2000 *g* for 10 min at 20 °C

9. The supernatant was transferred to a clean 25 mL graduated cylinder, combined with the first extract, and the total extract volume was adjusted to 20 mL by adding 100% methanol. The adjusted extract was then transferred back into the original 20 mL glass scintillation vial.
10. A 2 mL aliquot of the 20 mL extract was transferred to a 16 × 100 mm glass tube using a 1 mL positive displacement Hamilton syringe and 250 µL of 2.5 M NaOH was added. Each tube was sealed with a phenolic PTFE lined screw cap and vortexed to mix for 30 seconds. Tube weights were recorded, then placed in a 76 ± 2 °C water bath for 40 minutes.
11. Sample tubes were dried, allowed to cool to room temperature for 5–10 minutes, and re-weighted to assess any evaporative sample loss. [Note: No samples required volume adjustment during these experiments.] Samples were then neutralized with 2.5 M HCL, followed by vortex mixing for 30 seconds.
12. 7 mL of hexane was added to each 2.5 mL hydrolyzed methanolic extract and vortexed for 1 minute to mix. Samples were partitioned by centrifuging at 2,000 *g* for 10 min at 20 °C. Using a disposable glass Pasteur pipette, the upper hexane layer was removed and discarded into an appropriate waste container, and approximately 1 mL of the remaining methanolic extract (bottom layer) was transferred into a 1 mL disposable syringe equipped with a 13 mm, 0.2 µm syringe tip filter using a clean disposable glass Pasteur pipette. Each filtrate was collected directly into an LC-MS certified glass vial for analysis.
13. If found to be negative, aliquots of each extract, as well as aliquots from East and West coast (n=10 each) pooled extracts, were fortified at 10 concentrations: 0.391, 0.781, 1.56, 3.13, 6.25, 12.5, 25, 50, 100, and 200 ng/mL (equivalent to 0.49-250 µg/100g) of each toxin using 2 µg/mL stocks.

Results:

The slope of each matrix curve was compared to the average slope of the bracketing MeOH curves and reported in Table 6 along with the R^2 of the linear regression for each curve. All calibration curves were shown to be linear ($R^2 \geq 0.99$) within the range of 0.391–200 ng/mL for all 20 individual matrix sources. However, the difference in slopes compared to the corresponding MeOH curve varied for both matrix and toxin showing both enhancement and suppression (93-116% for DTX1, 92-105% for DTX2, and 92-109% for OA). No trends were seen between East and West coast matrix sources. As a further test, hydrolyzed and hexane washed extracts for all 10 East coast and all 10 West coast matrix samples were pooled and used to prepare 10-point standard curves as described above. Slopes compared to a MeOH standard curve were 100%, 93%, and 93% for pooled East coast blank mussel samples and 99%, 93%, and 91% for pooled West coast blank mussel samples for DTX1, DTX2, and OA, respectively (Table 7).

Discussion:

Although no acceptability criteria have been established for matrix effects, the slopes for all 20 individual matrix sources were within 10% for OA and DTX2 and within 16% for DTX1. These results were similar for pooled matrix samples for clam from proposal #17-103 (slopes within 10% for OA and DTX2 and within 19% for DTX1). But due to the variability between individual analytes observed within matrix sources used in this study (93-116% for DTX1, 92-105% for DTX2, and 92-109% for OA), it was decided for the matrix matched standard curves to be used in this SLV, 10 blank mussel samples would be pooled, 5 randomly selected from the East coast and 5 randomly selected from the West coast. For routine analysis

in a particular region, it is recommended that at least 5 pooled blank matrix samples be used from the region of sample collection to account for any potential variability between matrix sources.

Table 5. Matrix effects in 20 blank mussel samples collected for SLV study.

Toxin		DTX1		DTX2		OA	
Day	Matrix	% Slope	R^2	% Slope	R^2	% Slope	R^2
1	1 (E)	107	0.9993	95	0.9998	105	0.9998
	2 (W)	108	0.9995	105	0.9999	108	0.9998
	3 (E)	109	0.9998	101	0.9996	109	0.9999
	4 (W)	116	0.9979	99	0.9989	105	0.9992
2	5 (E)	105	0.9989	99	0.9998	103	0.9999
	6 (W)	104	0.9989	96	0.9981	101	0.9997
	7 (E)	95	0.9985	103	0.9998	102	0.9992
	8 (W)	104	0.9996	96	0.9999	98	0.9983
	9 (E)	113	0.9998	102	0.9992	108	0.9995
	10 (W)	105	0.9990	100	0.9997	100	0.9999
	11 (E)	114	0.9995	102	0.9996	106	0.9998
	12 (W)	113	0.9998	103	1.000	101	0.9998
3	13 (E)	101	0.9999	94	0.9991	96	0.9999
	14 (W)	100	0.9999	97	1.000	96	0.9998
	15 (E)	96	0.9998	92	0.9999	98	1.000
	16 (W)	97	0.9998	95	0.9999	93	0.9988
	17 (E)	93	1.000	94	0.9998	92	0.9992
	18 (W)	99	0.9998	95	0.9999	98	0.9996
	19 (E)	99	0.9999	96	0.9996	95	1.000
	20 (W)	109	1.000	104	0.9998	107	0.9995

Table 6. Matrix effects in pooled east coast and west coast mussel samples collected for SLV study.

Toxin		DTX1		DTX2		OA	
Pooled Matrix	Sample Set	% Slope	R^2	% Slope	R^2	% Slope	R^2
East Mix	Odd # from Table 5	100	1.000	93	0.9993	93	0.9998
West Mix	Even # from Table 5	99	0.9996	93	0.9998	91	0.9976

2. Accuracy/Trueness and Measurement Uncertainty

Methods:

Following the NSSP Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Accuracy/Trueness and Measurement Uncertainty (VII. #1), a total of 20 mussel samples were used, 10 samples from Maine and 10 from Washington (Table 1). Each sample was first screened for DSTs using the described LC-MS/MS method to assure that each matrix sample did not contain any naturally accumulated toxins. Once these samples were established to be free of contamination, each was spiked at one of five concentrations ranging from 8 µg/100 g to 32 µg/100 g. Concentrations for spiking were selected to cover one half to two times the regulatory limit for DSTs in shellfish which is 16 µg of toxin per 100 g of shellfish tissue. Source locations were alternated (ME, WA, ME, WA, etc.) for all 20 samples to avoid any bias. All samples were quantified using both neat (MeOH) and matrix-matched standard curves, prepared as described above.

Procedure:

1. For each sample, ≥12 animals were rinsed, shucked, drained, and homogenized in a commercial food processor.
2. 2.0 ± 0.05 g of each homogenate was transferred to a 50 mL disposable centrifuge tube using a clean stainless-steel laboratory spatula.
3. Individual DSP toxin standards were added into the tube, using positive displacement pipettes, so that the final concentration of each toxin was 8, 12, 16, 24, or 32 µg/100 g. Samples were vortexed gently to mix.
4. 9.0 mL of 100% methanol was added to the centrifuge tube
5. Samples were mixed for 3 min using a vortex mixer
6. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
7. The supernatant was transferred to a 20 mL glass scintillation vial.
8. The residual tissue pellet was re-extracted with 9.0 mL of methanol, followed by vortexing for 3 min.
9. Samples were centrifuged at 2000 *g* for 10 min at 20 °C
10. The supernatant was transferred to a clean 25 mL graduated cylinder, combined with the first extract, and the total extract volume was adjusted to 20 mL by adding 100% methanol. The adjusted extract was then transferred back into the original 20 mL glass scintillation vial.
11. A 2 mL aliquot of the 20 mL extract was transferred to a 16 × 100 mm glass tube using a 1 mL positive displacement Hamilton syringe and 250 µL of 2.5 M NaOH was added. Each tube was sealed with a phenolic PTFE lined screw cap and vortexed to mix for 30 seconds. Tube weights were recorded, then placed in a 76 ± 2 °C water bath for 40 minutes.
12. Sample tubes were dried, allowed to cool to room temperature for 5–10 minutes, and re-weighted to assess any evaporative sample loss. [Note: No samples required volume adjustment during these experiments.] Samples were then neutralized with 2.5 M HCL, followed by vortex mixing for 30 seconds.
13. 7 mL of hexane was added to each 2.5 mL hydrolyzed methanolic extract and vortexed for 1 minute to mix. Samples were partitioned by centrifuging at 2,000 *g* for 10 min at 20 °C. Using a disposable glass Pasteur pipette, the upper hexane layer was removed and discarded into an

appropriate waste container, and approximately 1 mL of the remaining methanolic extract (bottom layer) was transferred into a 1 mL disposable syringe equipped with a 13 mm, 0.2 µm syringe tip filter using a clean disposable glass Pasteur pipette. Each filtrate was collected directly into an LC-MS certified glass vial for analysis.

Results:

Accuracy/trueness data for OA, DTX1, and DTX2 in mussel are reported in Tables 7, 8, and 9, respectively. The average accuracy for all samples combined was 100% for DTX1, 105% for DTX2, and 94% for OA with measurement uncertainties of 0.50, 0.50, and 0.54 µg/100g, respectively, when quantified using a MeOH standard curve. Accuracies were 91% for DTX1, 99% for DTX2, and 85% for OA with measurement uncertainties of 0.50, 0.50, and 0.47 µg/100g, respectively, when quantified using a matrix-matched standard curve.

Discussion:

FDA typically considers acceptable accuracy/trueness (as % recovery) to be 60-115% for concentrations in the range of 10 µg/kg (equivalent to 1 µg/100g) and 80-110% for concentrations in the range of 100 µg/kg (equivalent to 10 µg/100g)[5]. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends recoveries between 60-115% or 70-130% depending on the toxin and method for the DST group[2]. Based on this, the method was found to have acceptable accuracy when using both MeOH and matrix-matched standard curves. Recoveries for all three toxins were higher when using a MeOH curve, most likely due to matrix enhancement. Measurement uncertainties did not differ when using either MeOH or matrix-matched standard curves.

Table 7. Accuracy/Trueness for DTX1 in Mussel

Sample	Spike Concentration (µg/100 g shellfish)	Determined Concentration (µg/100 g shellfish)		Accuracy (%)	
		MeOH	Matrix Matched	MeOH	Matrix Matched
1	8	8.3	6.6	104.3	82.9
2	12	12.4	11.4	103.5	95.4
3	16	18.0	15.8	112.4	98.7
4	24	24.8	23.2	103.4	96.9
5	32	35.1	32.1	109.8	100.2
6	8	8.5	7.7	105.7	96.8
7	12	11.1	10.2	92.9	85.3
8	16	15.9	14.7	99.2	91.9
9	24	23.2	21.7	96.5	90.2
10	32	31.2	29.1	97.5	90.9
11	8	6.8	6.1	84.8	76.7
12	12	11.6	10.6	96.3	88.7
13	16	15.8	14.7	98.9	91.7
14	24	26.2	24.5	109.4	102.2
15	32	31.5	29.5	98.4	92.1
16	8	8.3	7.6	103.7	94.6
17	12	11.3	10.4	94.1	86.7
18	16	14.7	13.6	91.9	85.1
19	24	22.2	20.7	92.4	86.2
20	32	31.6	29.6	98.7	92.4
Average % Accuracy				**99.7**	**91.3**
Measurement Uncertainty (µg/100g)				**0.50**	**0.50**

Table 8. Accuracy/Trueness for DTX2 in Mussel

Sample	Spike Concentration (µg/100 g shellfish)	Determined Concentration (µg/100 g shellfish)		Accuracy (%)	
		MeOH	Matrix Matched	MeOH	Matrix Matched
1	8	9.2	7.9	115.4	98.9
2	12	12.5	12.0	104.3	100.2
3	16	18.3	16.7	114.6	104.5
4	24	26.8	25.6	111.7	106.5
5	32	34.0	31.9	106.4	99.8
6	8	8.1	7.3	101.7	90.7
7	12	11.1	10.6	92.1	88.7
8	16	16.5	15.3	103.4	95.5
9	24	23.5	22.5	98.1	93.6
10	32	32.1	30.1	100.2	94.0
11	8	7.9	7.7	99.4	96.8
12	12	12.6	12.2	105.3	102.0
13	16	16.5	15.9	102.9	99.4
14	24	26.7	25.7	111.1	106.9
15	32	34.3	33.0	107.2	103.0
16	8	8.1	7.9	101.9	99.2
17	12	12.2	11.8	101.7	98.4
18	16	17.2	16.6	107.2	103.5
19	24	25.2	24.2	104.9	100.9
20	32	34.3	32.9	107.1	102.9
Average % Accuracy				**104.8**	**99.3**
Measurement Uncertainty (µg/100g)				**0.50**	**0.40**

Table 9. Accuracy/Trueness for OA in Mussel

Sample	Spike Concentration (µg/100 g shellfish)	Determined Concentration (µg/100 g shellfish)		Accuracy (%)	
		MeOH	Matrix Matched	MeOH	Matrix Matched
1	8	8.4	6.6	105.6	82.6
2	12	11.0	9.9	92.0	82.9
3	16	17.0	14.4	106.2	90.1
4	24	24.2	21.9	100.9	91.2
5	32	33.2	29.2	103.8	91.3
6	8	6.3	6.0	78.4	75.0
7	12	10.8	9.7	90.1	81.1
8	16	13.9	12.8	86.9	80.2
9	24	22.7	20.5	94.6	85.5
10	32	31.2	28.3	97.6	88.5
11	8	6.3	6.1	78.9	76.3
12	12	10.3	9.6	85.6	80.4
13	16	14.4	13.3	90.0	83.3
14	24	25.3	23.1	105.6	96.3
15	32	31.8	28.9	99.5	90.4
16	8	8.6	8.2	107.8	102.1
17	12	9.9	9.3	82.1	77.3
18	16	13.2	12.3	82.6	76.7
19	24	22.1	20.3	92.3	84.4
20	32	30.2	27.5	94.5	85.9
Average % Accuracy				**93.7**	**85.1**
Measurement Uncertainty (µg/100g)				**0.54**	**0.47**

3. Recovery and Precision

Methods:

Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Recovery and Precision (VII. #3), 10 samples (5 from Maine and 5 from Washington, alternating by source location) were each fortified at concentrations of 8, 16, and 32 µg/100 g, representing 1/2X, 1X, and 2X the regulatory level of 0.16 mg okadaic acid (OA) equivalents/kg (0.16 ppm). To assess method precision (as intermediate precision[5]), all samples were analyzed as replicate injections on separate days. Matrix fortifications and extractions were conducted as described above for accuracy/trueness. Results from the first injection were used to assess recovery, while the results from replicate injections were used to assess precision. All samples were quantified using both MeOH and matrix-matched standard curves, prepared as described above.

As another measure of method precision (as repeatability[5]), %RSDs were calculated for five replicate extractions of two different naturally contaminated certified reference materials (CRMs) purchased from the National Research Council (NRC) Canada. Full details for the analysis of the CRMs is provided in the section on Comparability (Section 7).

Results:

Recovery data for the three DSTs in mussel are reported in Tables 10, 11, and 12. Recoveries ranged from 102-103% for DTX1, 105-106% for DTX2, and 92-96% for OA when using a MeOH standard curve. Recoveries ranged from 89-95% for DTX1, 95-99% for DTX2, and 80-86% for OA when using a matrix-matched standard curve. One-way analysis of variance testing for significant differences between spike concentrations was not significant with the exception of DTX1 using the matrix-matched standard curve only (p=0.042). Tukey's test for honestly significant differences showed that the significant variance was between the low (8 µg/100 g) and the high (32 µg/100g) concentrations only.

Precision data for the modified LC-MS/MS method is reported in Tables 13, 14, and 15. Following the SOP, a nested analysis of variance (F test) was performed, and significant differences were found for all treatments with the exception of DTX2 using a matrix matched standard curve only (data not shown). This indicates that the precision of the method was not consistent across the range of concentrations tested, potentially due to additional sources of variation not accounted for with this test. It should be noted that variation in matrix effects was observed between individual samples in the initial matrix effects study, which led to the recommendation of pooling blank matrices for use in the matrix matched standard curves. As an alternative approach, Bartlett's test of homogeneity of variances was used, which differs from the F test in that it pools the data across replicates and samples and compares differences in the variance over the three spike levels. Using this approach, Bartlett's test was not significant for DTX1 using either standard curve but was still significant for DTX2 using the MeOH curve only (p=0.0057) and was weakly significant for OA using both the MeOH and matrix matched standard curves (p=0.021 and p=0.034, respectively). The SOP does not provide guidance for the calculation of precision if the initial F test fails. Therefore, instead of calculating a single precision score across the range of concentrations tested, as indicated in the SOP, the precision of the method, measured as the relative standard deviation (%RSD), was calculated individually for each spike level. Using this approach, the %RSD ranged from 5.6-8.3 for DTX1, 4.4-8.4 for DTX2, and 9.4-15.8 for OA when using a MeOH standard curve; %RSD ranged from 4.6-7.1 for DTX1, 4.6-5.5 for DTX2, and 5.5-10.0 for OA when using a matrix-matched standard curve.

%RSDs for five replicate extractions of the two naturally contaminated CRMs ranged from 1.39-4.72 for all toxins when using a MeOH standard curve and 1.41-2.46 when using a matrix-matched standard curve (Table 25).

Discussion:

FDA typically considers acceptable recoveries and precision to be 60-115% and <22%, respectively, for concentrations in the range of 10 µg/kg (equivalent to 1 µg/100g), and 80-110% and <11%, respectively, for concentrations in the range of 100 µg/kg (equivalent to 10 µg/100g)[5]. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends recoveries between 60-115% or 70-130% depending on the toxin and method for the DST group[2]. In this study, recoveries using both MeOH and matrix matched standard curves were well within these guidelines. The significant difference in variance between the low and high fortification levels (for DTX1 using a matrix matched standard curve only) was not surprising considering most validation guidelines have a broader range of acceptable recoveries and precision at lower spike levels.

For precision (as intermediate precision), replicate injections on separate days of the ten samples used to assess recovery had <10 %RSD for both MeOH and matrix matched standard curves, for all toxins and concentrations, with the exception of OA and only at the lowest fortification level (%RSD 15.8 for the 8 µg/100 spike level) and only for the MeOH standard curve. For repeatability, five replicate extractions of two naturally contaminated CRMs had <5 %RSD for all three toxins. FDA method validation guidelines recommend %RSDs of <22% acceptable for concentrations in the range of 1 µg/100g and <11% in the range of 10 µg/100g.

While the use of both MeOH and matrix-matched standard curves produced acceptable recoveries, it should be noted that recoveries were higher for all three toxins when using a MeOH standard curve, again likely due to matrix enhancement, and variance was lower when using a matrix-matched standard curve compared to using MeOH.

Table 10. Percent Recovery for DTX1 in Mussel

Sample	Low (8µg/100g)		Medium (16µg/100g)		High (32µg/100g)	
	MeOH	Matrix Matched	MeOH	Matrix Matched	MeOH	Matrix Matched
1	104%	83%	103%	90%	103%	94%
2	103%	82%	104%	91%	107%	98%
3	115%	93%	112%	99%	112%	102%
4	109%	87%	109%	95%	110%	100%
5	106%	97%	91%	84%	101%	94%
6	101%	93%	99%	92%	97%	90%
7	92%	84%	90%	83%	98%	91%
8	102%	93%	104%	96%	103%	96%
9	91%	82%	101%	94%	99%	93%
10	107%	97%	105%	97%	101%	95%
Average Recovery	**103%**	**89%**	**102%**	**92%**	**103%**	**95%**
Standard Deviation	**7.22**	**6.19**	**7.01**	**5.25**	**5.03**	**3.73**

MeOH Standard Curve
One-way ANOVA: F-value 0.122, p-value: **0.885 (not significant)**
No significant differences were found between recoveries for different spike concentrations.

Matrix Matched Standard Curve
One-way ANOVA: F-value 3.566, p-value**: 0.042 (significant)**
The variance was significantly different for low versus high spike concentrations only, as determined by Tukey's Honestly Significant Difference.

Table 11. Percent Recovery for DTX2 in Mussel

Sample	Low (8µg/100g)		Medium (16µg/100g)		High (32µg/100g)	
	MeOH	Matrix Matched	MeOH	Matrix Matched	MeOH	Matrix Matched
1	115%	99%	106%	97%	107%	100%
2	114%	98%	102%	93%	111%	104%
3	114%	97%	115%	105%	111%	104%
4	115%	99%	112%	102%	106%	100%
5	102%	91%	98%	91%	96%	90%
6	107%	96%	103%	95%	102%	95%
7	98%	87%	103%	95%	100%	94%
8	110%	99%	106%	98%	102%	95%
9	89%	87%	100%	96%	103%	98%
10	99%	96%	111%	106%	111%	106%
Average Recovery	**106%**	**95%**	**106%**	**98%**	**105%**	**99%**
Standard Deviation	**8.92**	**4.70**	**5.33**	**5.02**	**5.08**	**5.02**

MeOH Standard Curve
One-way ANOVA: F-value 0.125, p-value **0.883 (not significant)**
No significant differences were found between recoveries for different spike concentrations.

Matrix Matched Standard Curve
One-way ANOVA: F-value 1.728, p-value **0.197 (not significant)**
No significant differences were found between recoveries for different spike concentrations.

Table 12. Percent Recovery for OA in Mussel

Sample	Low (8µg/100g)		Medium (16µg/100g)		High (32µg/100g)	
	MeOH	Matrix Matched	MeOH	Matrix Matched	MeOH	Matrix Matched
1	106%	83%	100%	84%	100%	88%
2	114%	90%	102%	87%	68%	59%
3	112%	88%	106%	90%	99%	87%
4	103%	80%	109%	92%	104%	91%
5	78%	75%	81%	75%	95%	86%
6	84%	80%	87%	80%	100%	91%
7	79%	76%	91%	84%	98%	89%
8	80%	76%	91%	84%	96%	87%
9	71%	63%	98%	88%	96%	87%
10	95%	85%	95%	86%	100%	90%
Average Recovery	**92%**	**80%**	**96%**	**85%**	**96%**	**86%**
Standard Deviation	**15.59**	**7.69**	**8.67**	**4.92**	**10.00**	**9.59**

MeOH Standard Curve
One-way ANOVA: F-value 0.388, p-value: **0.716 (not significant)**
No significant differences were found between recoveries for different spike concentrations.

Matrix Matched Standard Curve
One-way ANOVA: F-value 1.806, p-value: **0.184 (not significant)**
No significant differences were found between recoveries for different spike concentrations.

Table 13. Precision for Duplicate Injections for DTX1 in Mussel

Sample	Low (8µg/100g)		Medium (16µg/100g)		High (32µg/100g)	
	MeOH	Matrix Marched	MeOH	Matrix Matched	MeOH	Matrix Matched
1a	8.3	6.6	16.5	14.4	33.1	30.1
1b	8.4	6.7	16.2	14.1	35.3	32.2
2a	8.2	6.5	16.6	14.5	34.3	31.3
2b	9.2	7.5	16.9	14.7	34.4	31.4
3a	9.2	7.5	18.0	15.8	35.7	32.6
3b	9.1	7.3	19.3	17.1	36.4	33.2
4a	8.7	7.0	17.4	15.2	35.1	32.1
4b	9.0	7.2	17.3	15.1	35.0	32.0
5a	8.5	7.7	14.6	13.5	32.2	30.0
5b	7.8	7.2	14.7	13.6	31.2	29.1
6a	8.1	7.4	15.9	14.7	31.0	28.9
6b	8.4	7.7	15.9	14.7	32.0	29.8
7a	7.4	6.7	14.4	13.3	31.2	29.1
7b	7.1	6.5	14.1	13.1	30.5	28.4
8a	8.2	7.5	16.6	15.4	32.9	30.6
8b	8.3	7.6	15.8	14.6	33.0	30.7
9a	7.3	6.6	16.2	15.1	31.8	29.9
9b	6.7	6.0	15.6	14.5	30.2	28.3
10a	8.6	7.8	16.7	15.5	32.3	30.4
10b	8.2	7.4	17.7	16.4	31.9	30.0
%RSD	**8.3**	**7.1**	**7.9**	**6.8**	**5.6**	**4.6**

Bartlett test of homogeneity of variances:

Data: Accuracy by Concentration for DTX1

MeOH: Bartlett's K-squared = 2.96, df = 2**,** p-value: **0.227 (not significant)**

Matrix Matched: Bartlett's K-squared = 2.81, df = 2, p-value: **0.245 (not significant)**

Result: The variance is not significantly different between concentrations for DTX1 using either MeOH or matrix matched standard curves.

Table 14. Precision for Duplicate Injections for DTX2 in Mussel

Sample	Low (8µg/100g)		Medium (16µg/100g)		High (32µg/100g)	
	MeOH	Matrix Marched	MeOH	Matrix Matched	MeOH	Matrix Matched
1a	9.2	7.9	17.0	15.5	34.2	32.1
1b	10.3	8.9	17.6	16.0	34.2	32.1
2a	9.1	7.8	16.4	14.8	35.4	33.3
2b	8.9	7.6	17.9	16.4	34.3	32.2
3a	9.1	7.8	18.3	16.7	35.5	33.3
3b	9.2	7.9	17.6	16.0	35.1	33.0
4a	9.2	7.9	17.9	16.3	34.0	31.9
4b	9.2	7.9	17.8	16.2	34.4	32.3
5a	8.1	7.3	15.7	14.5	30.8	28.8
5b	8.3	7.4	16.7	15.5	30.9	29.0
6a	8.5	7.6	16.5	15.3	32.5	30.5
6b	8.3	7.4	17.1	15.8	34.2	32.2
7a	7.9	7.0	16.4	15.2	32.1	30.1
7b	8.3	7.4	15.9	14.6	31.9	29.9
8a	8.8	7.9	16.9	15.7	32.5	30.5
8b	8.6	7.7	15.7	14.5	32.6	30.6
9a	7.1	6.9	16.0	15.3	33.0	31.5
9b	7.6	7.3	16.1	15.4	32.0	30.5
10a	7.9	7.7	17.7	17.0	35.5	33.8
10b	8.1	7.9	17.0	16.3	34.6	33.0
%RSD	**8.4**	**5.5**	**4.8**	**4.6**	**4.4**	**4.6**

Bartlett test of homogeneity of variances:

Data: Accuracy by Concentration for DTX2

MeOH: Bartlett's K-squared = 10.3, df = 2**,** p-value: **0.00574 (significant)**

Matrix Matched: Bartlett's K-squared = 0.688, df = 2, p-value**: 0.709 (not significant)**

Result: The variance is not significantly different between concentrations for DTX2 when using a matrix matched standard curve but a significant difference was found when using a MeOH curve.

Table 15. Precision for Duplicate Injections for OA in Mussel

Sample	Low (8µg/100g)		Medium (16µg/100g)		High (32µg/100g)	
	MeOH	Matrix Marched	MeOH	Matrix Matched	MeOH	Matrix Matched
1a	8.4	6.6	16.0	13.5	31.9	28.0
1b	7.7	6.0	15.8	13.3	33.1	29.1
2a	9.1	7.2	16.4	13.8	21.8	18.8
2b	9.5	7.6	16.5	14.0	23.8	20.6
3a	9.0	7.1	17.0	14.4	31.8	27.9
3b	8.4	6.6	17.6	14.9	31.9	28.0
4a	8.2	6.4	17.4	14.8	33.2	29.2
4b	8.5	6.7	16.6	14.0	33.6	29.5
5a	6.3	6.0	13.0	12.0	30.5	27.7
5b	6.7	6.4	13.4	12.4	30.0	27.2
6a	6.7	6.4	13.9	12.8	32.0	29.0
6b	6.9	6.6	14.3	13.2	31.5	28.6
7a	6.4	6.1	14.5	13.4	31.2	28.3
7b	6.9	6.6	14.1	13.0	30.7	27.9
8a	6.4	6.1	14.6	13.5	30.8	27.9
8b	6.5	6.2	14.9	13.8	31.2	28.3
9a	5.6	5.1	15.6	14.1	30.9	27.9
9b	5.5	5.0	14.8	13.4	30.1	27.2
10a	7.6	6.8	15.2	13.8	32.0	29.0
10b	7.6	6.8	16.1	14.6	31.8	28.8
%RSD	**15.8**	**9.7**	**8.5**	**5.5**	**9.4**	**10.0**

Bartlett test of homogeneity of variances:

Data: Accuracy by Concentration for OA

MeOH: Bartlett's K-squared = 7.68, df = 2**,** p-value = **0.0215 (significant)**

Matrix Matched: Bartlett's K-squared = 6.76, df = 2, p-value = **0.034 (significant)**

Result: The variance is significantly different between concentrations for OA when using both matrix matched and MeOH standard curves.

4. Specificity

Methods:

Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Specificity (VII. #4), all three DSTs were analyzed in the presence of the potentially co-occurring lipophilic toxins azaspiracids (AZA-1-3), pectenotoxin (PTX-2), and yessotoxin (sources in Table 5). Three aliquots of a single blank mussel tissue matrix were used. One aliquot served as a control blank, one sample contained an action level spike (16 µg/100 g) of each DST, and one contained the same concentration of DSTs plus a 16 µg/100 g spike each of AZA-1, AZA-2, AZA-3, PTX-2, and YTX. Five technical replicates of each aliquot, excluding the negative control blank, were analyzed. The specificity index (SI) is reported for each DST in Tables 16, 17, and 18. A one-way analysis of variance was used to test for significant differences from an SI of 1.

Results:

The specificity index for all three DSTs was between 0.96-1.01 using both MeOH and matrix-matched standard curves. The one-way analysis of variance found that the SI was not significantly difference from 1 for DTX1 and OA but was significant for DTX2 (P=0.020). The average difference between samples containing the additional lipophilic shellfish toxins was only 0.60 ± 0.43 and 0.72 ± 0.53 µg/100g for MeOH and matrix-matched standard curves, respectively. In addition, the freeze-dried mussel CRM material analyzed as part of the comparability testing (5 replicates), which is reported to also contain domoic acid and multiple congeners of azaspiracids, yessotoxins, pectenotoxins, and the cyclic imines spirolides, pinnatoxins, and gymnodimines, was also used to assess method specificity. Despite the presence of these multiple potentially co-occurring and interfering compounds, the LC-MS/MS method produced results for DSTs that were between 99-105% and 93-108%, for MeOH and matrix-matched standard curves, respectively, compared to the informational values for total (post-hydrolysis) DSTs reported by NRC (Table 21).

Discussion:

Despite the significant finding for DTX2, the combined data shows that the method is specific for DSTs and does not suffer from interference from potentially co-occurring lipophilic shellfish toxins. The significant finding for DTX2 is believed to be due to the high precision of the 5 technical replicates, as the average difference between samples with and without the potential interferants was very small (0.60 ± 0.43 and 0.72 ± 0.53 µg/100g for MeOH and matrix-matched standard curves, respectively).

Table 16. Specificity Index for DTX1

DTX1				
Replicate	**Concentration of aliquot spiked with targeted analyte (µg/100g)**		**Concentration of aliquot spiked with targeted analyte and suspected interfering toxins (µg/100g)**	
	MeOH	Matrix Matched	MeOH	Matrix Matched
1	19.06	17.56	19.88	18.34
2	19.05	17.55	19.50	17.98
3	18.86	17.37	19.64	18.11
4	18.96	17.47	18.62	17.15
5	19.34	17.83	19.43	17.91
Average	**19.05**	**17.56**	**19.41**	**17.90**
Specificity Index for MeOH standard curve: **0.98**; p-value: **0.151 (not significant)**				
Specificity Index for matrix matched standard curve: **0.98**; p-value: **0.151 (not significant)**				

Table 17. Specificity Index for DTX2

DTX2				
Replicate	**Concentration of aliquot spiked with targeted analyte (µg/100g)**		**Concentration of aliquot spiked with targeted analyte and suspected interfering toxins (µg/100g)**	
	MeOH	Matrix Matched	MeOH	Matrix Matched
1	17.69	16.89	18.84	18.05
2	17.68	16.88	17.83	17.03
3	17.80	17.00	17.84	17.04
4	17.37	16.56	18.23	17.43
5	17.58	16.77	18.92	18.14
Average	**17.62**	**16.82**	**18.33**	**17.54**
Specificity Index for MeOH standard curve: **0.96**; p-value: 0.020 (significant)				
Average difference for MeOH standard curve**: 0.60 ± 0.43**				
Specificity Index for matrix matched standard curve: **0.96**; p-value: 0.020 (significant)				
Average difference for matrix matched standard curve**: 0.72 ± 0.53**				

Table 18. Specificity Index for OA

OA				
Replicate	**Concentration of aliquot spiked with targeted analyte (µg/100g)**		**Concentration of aliquot spiked with targeted analyte and suspected interfering toxins (µg/100g)**	
	MeOH	Matrix Matched	MeOH	Matrix Matched
1	17.83	15.86	17.60	15.65
2	17.87	15.90	18.75	16.71
3	18.83	16.79	17.55	15.60
4	18.15	16.16	18.01	16.02
5	17.69	15.73	17.87	15.90
Average	**18.07**	**16.09**	**17.96**	**15.98**
Specificity Index for MeOH standard curve: **1.01**; p-value: **0.703 (not significant)**				
Specificity Index for matrix matched standard curve: **1.01**; **p-value: 0.703 (not significant)**				

5. Working and Linear Ranges, Limit of Detection, Limit of Quantitation / Sensitivity

Methods:

Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity (VII. #5), 10 samples (5 from Maine and 5 from Washington, alternating by source location) were each fortified at concentrations spanning 50-150% of the range of interest (4, 8, 16, 32, and 48 µg/100 g). Matrix fortification and extraction were conducted as described above for accuracy/trueness. LODs and LOQs were determined based on the standard deviation of the response and the slope from the linear range data. The equations for each are expressed as: LOD = 3.3σ/S and LOQ = 10σ/S, where σ is the standard deviation of the response from five blank matrix samples, and S is the slope of the calibration curve.

Results:

Linear range data for OA, DTX1, and DTX2 extracted from fortified mussel is shown in Figures 3, 4, and 5. Coefficients of determination were greater than 0.98 for all three toxins showing linearity across the range of interest. Linearity data were also processed as suggested in SLV SOP #5 for each toxin by plotting the log of the response per unit toxin versus the log concentration of spiked toxin along with the 95% confidence intervals of the mean response across all concentrations (shown in Figures 6, 7, and 8). With the exception of a single data point (1/10 OA spikes at 4 µg/100g which was the lowest concentration tested), all other data points fell within the 5% brackets, indicating that between 4 µg/100 g and 48 µg/100 g the mass spectrometric response is linearly proportional to the concentration within the working range of the analysis for all three toxins. The limit of detection (LOD) for the mussel matrix was found to be 0.197 µg/100 g for DTX1, 0.591 µg/100 g for DTX 2, and 0.159 µg/100 g for OA. The limit of quantitation (LOQ) was determined to be 0.597 µg/100 g for DTX1, 1.79 µg/100 g for DTX 2, and 0.482 µg/100 g for OA.

Discussion:

The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends an LOD and LOQ for methods for the determination of DSTs of 1 and 2 µg/100 g, respectively, for OA and DTX1, and 3 and 6 µg/100 g, respectively, for DTX2. Altogether, this data shows that the method is linear within the range of interest, sufficiently sensitive, and fit for its intended purpose.

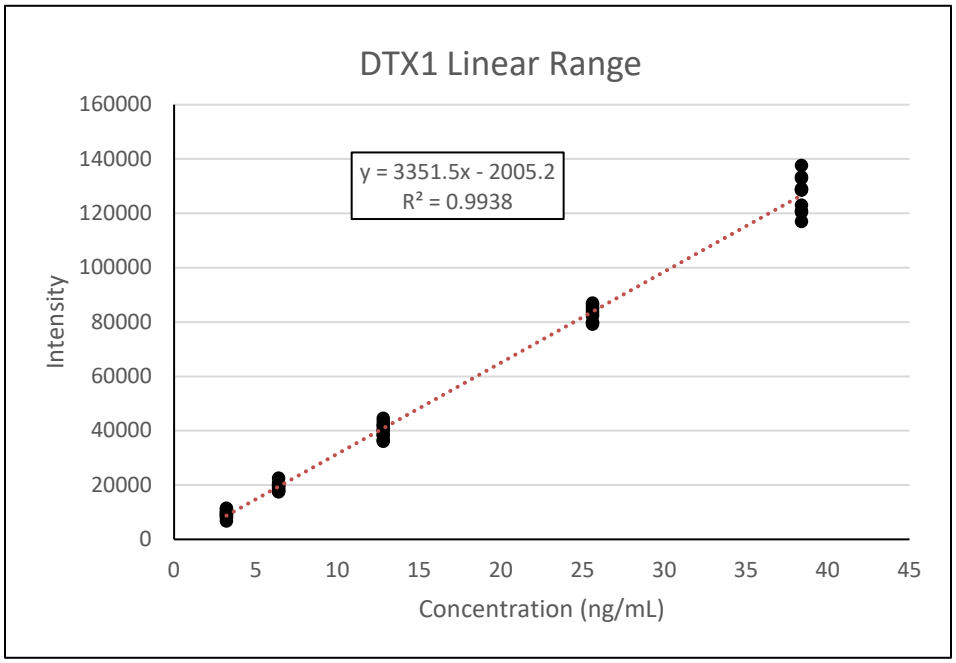


Figure 3. DTX1 response over a range of 3.2–38.4 ng/mL (equivalent to 4-48 µg/100g) in fortified mussel (n = 10).

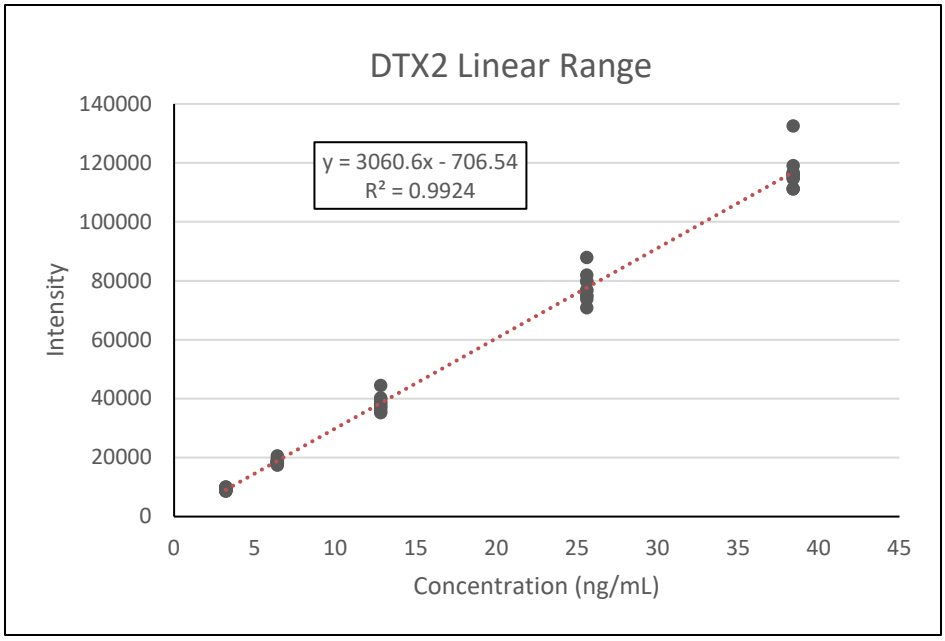


Figure 4. DTX2 response over a range of 3.2–38.4 ng/mL (equivalent to 4-48 µg/100g) in fortified mussel (n = 10).

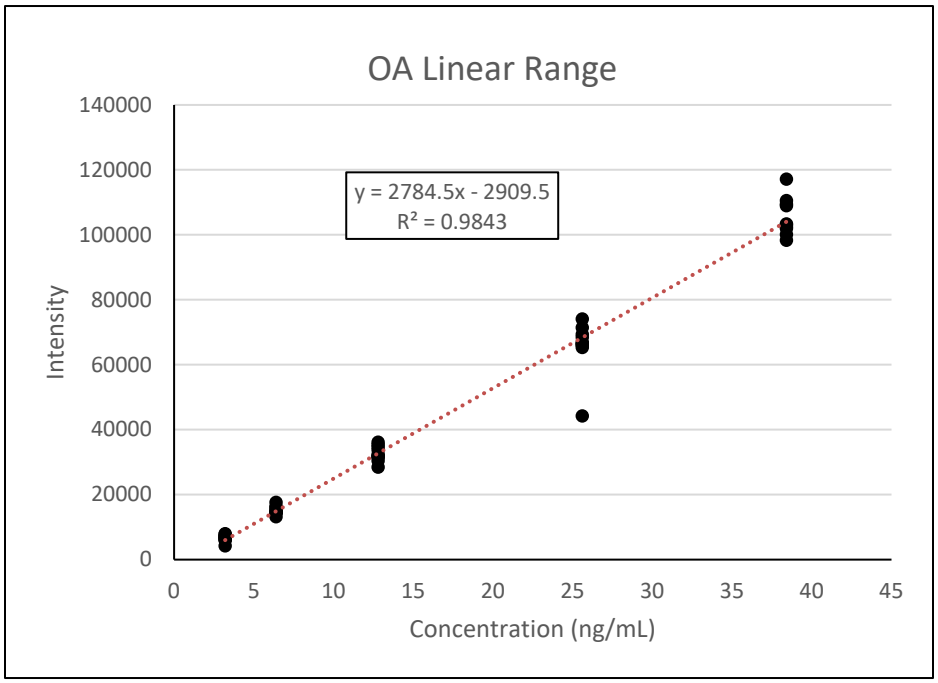


Figure 5. OA response over a range of 3.2–38.4 ng/mL (equivalent to 4-48 µg/100g) in fortified mussel (n = 10).

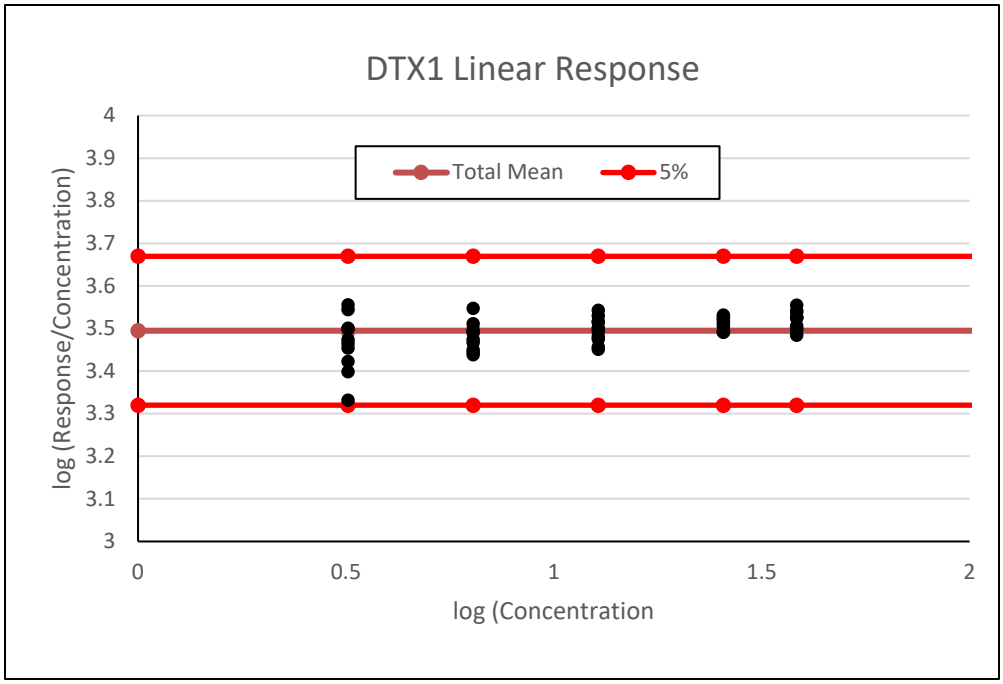


Figure 6. Linearity data for DTX1 plotted as the log of the response per unit concentration versus the log of the spiked concentration.

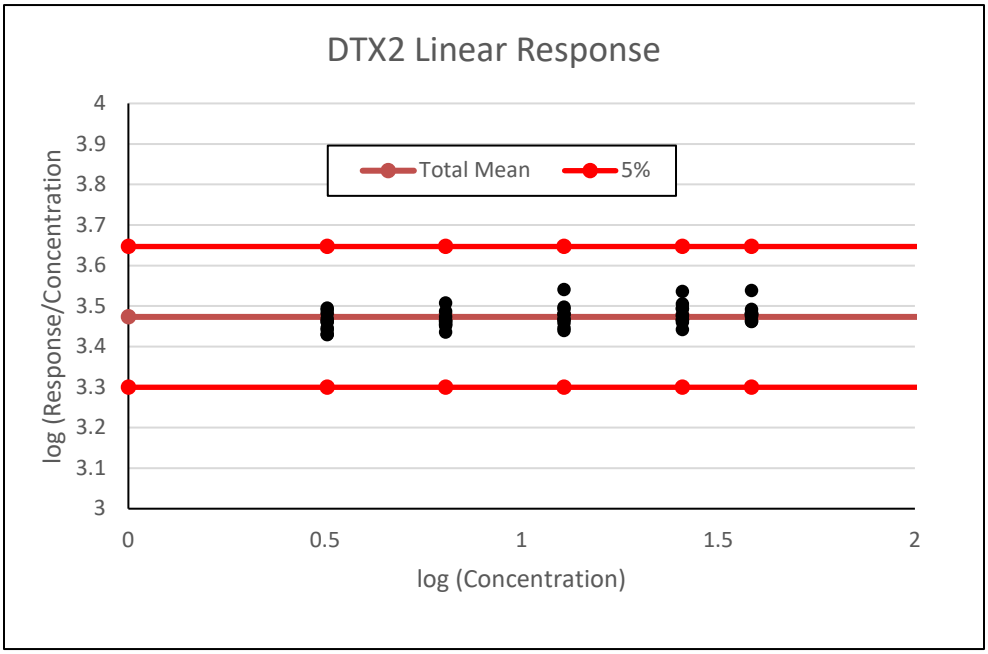


Figure 7. Linearity data for DTX2 plotted as the log of the response per unit concentration versus the log of the spiked concentration.

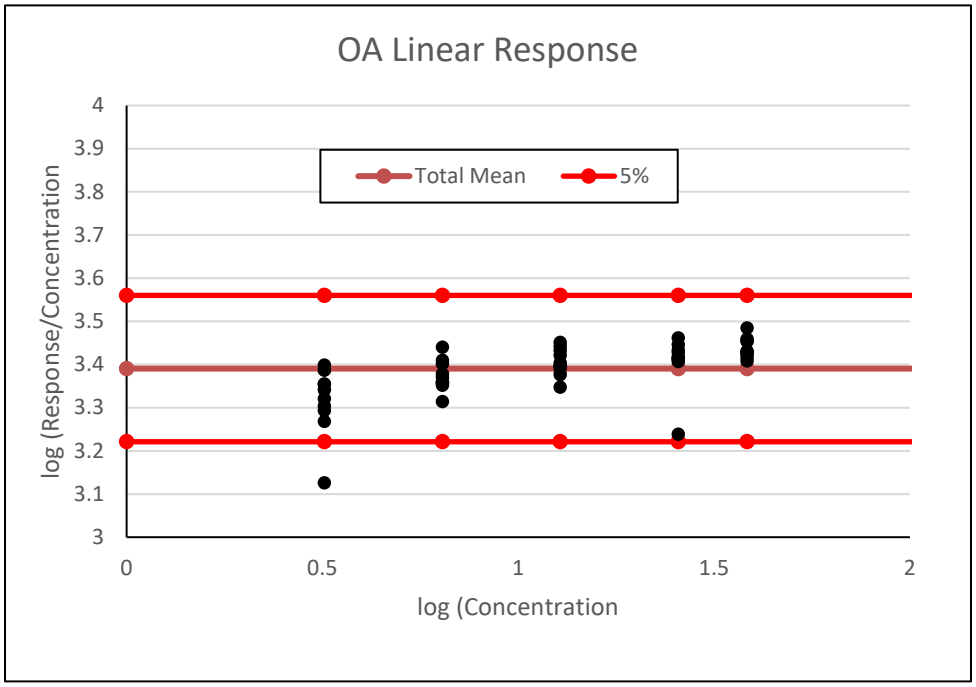


Figure 8. Linearity data for OA plotted as the log of the response per unit concentration versus the log of the spiked concentration.

Limits of Detection (LOD) and Quantitation (LOQ):

LODs and LOQs are based on the standard deviation of the response and the slope. The equations for each are expressed as:

$$LOD = \frac{3.3\sigma}{S} \qquad LOQ = \frac{10\sigma}{S}$$

	LOD (µg/100g)			LOQ (µg/100g)		
	OA	DTX2	DTX1	OA	DTX2	DTX1
Matrix Matched (Mussel)	0.148	0.201	0.558	0.448	0.608	1.69

	LOD (µg/100g)			LOQ (µg/100g)		
	OA	DTX2	DTX1	OA	DTX2	DTX1
Non-Matrix Matched (MeOH)	0.159	0.197	0.591	0.482	0.597	1.79

Where σ = the standard deviation of the response from five blank matrix samples
S = the slope of the calibration curve

6. Ruggedness

Methods:

To test method ruggedness, the effect of the hexane washing step was assessed on accuracy/trueness using both MeOH and matrix-matched standard curves. This was specifically requested by two NSSP laboratories that intend to implement this method, if approved. Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness (VII. #2), 2 sub-samples from each of 10 extracts from previously spiked samples used to assess accuracy/trueness (5 from Maine and 5 from Washington, alternating by source location), individually spiked at one of five concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were hydrolyzed following the procedure above. For each sample, one hydrolyzed extract was put through the above-described hexane washing step, while the other was filtered directly into an LC vial for analysis. Each sample was injected in duplicate and quantified using both MeOH and matrix-matched standard curves. For the non-hexane washed treatment, a corresponding non-hexane washed standard curve was prepared.

As another measure of method ruggedness, 10 extracts from previously spiked samples used to assess accuracy/trueness (5 from Maine and 5 from Washington, alternating by source location), individually spiked at one of five concentrations bracketing the regulatory guidance level (8, 12, 16, 24, and 32 µg/ 100 g), were re-analyzed using both the current analytical column and conditions ("HPLC", Waters Xbridge Premier BEH C18 VanGaurd FIT, 3.5 µm particle size, 2.1 × 100 mm; flow rate 0.300 mL/min; 5 µL injection) and the column validated in proposal #17-103 ("UPLC", Waters BEH C18, 1.7 µm, 1.0 mm × 150 mm; flow rate 0.120 mL/min; 5 µL injection), which is smaller in both particle size, diameter, and flow rate, but runs at a higher pressure requiring a UPLC system. The LC and detector (Sciex QTrap 5500 coupled to a Waters Corporation Acquity UPLC system) used in ISSC proposal #17-103 were no longer available so only the columns were directly compared.

Results:

Data for the ruggedness testing of the hexane washing step are presented in Tables 19, 20, and 21. Following the Marine Biotoxin and Non-MPN Based Microbiological Methods SOP for Ruggedness (VII. #2), a paired t-test was used to test for significant differences in accuracy between the two treatments as the variance ratio was within -2 to +2 for all three toxins indicating that the variance was homogeneous. The paired t-test indicated significant differences ($P<0.0001$) in accuracy for all three toxins using both a MeOH and matrix-matched standard curve with samples analyzed without the hexane washing step being consistently higher. Average % accuracy was within the acceptable range for all three toxins when the hexane wash step was used (97% and 90% for DTX1, 105% and 101% for DTX2, and 91% and 84% for OA – for MeOH and matrix-matched standard curves, respectively). Without the hexane wash step, OA had acceptable recoveries both with MeOH and matrix-matched standard curves (110% and 101%, respectively). DTX1 had acceptable recoveries only when a matrix-matched standard was used (111%). Recoveries fell outside of acceptable ranges for DTX1 when a MeOH standard curve was used (120%). For DTX2, both MeOH and matrix-matched standard curves had accuracies outside of acceptable ranges (128% and 124%, respectively).

Results for the comparison of the column used in the current SLV compared to the column used in SLV #17-103 can be found in Tables 22, 23, and 24. Following the Marine Biotoxin and Non-MPN Based

Microbiological Methods SOP for Ruggedness (VII. #2), a paired t-test was used to test for significant differences in accuracy between the two columns as the variance ratios were within -2 to +2 for all three toxins indicating that the variances were homogeneous. The paired t-test indicated no significant differences in accuracy between the different columns for DTX1 using either a MeOH or matrix-matched standard curve. For DTX2, no significant differences were detected when using a MeOH standard curve, but a significant difference was detected (P=0.00046) when using a matrix-matched standard curve. For OA, significant differences were detected for both standard curve options (P=0.0036 and P=0.0024 for MeOH and matrix-matched standard curves, respectively). However, accuracies were within acceptable ranges for all three toxins (82-103% and 80-101% for MeOH and matrix-matched standard curves, respectively, for the new column, compared to 86-101% and 77-96% for MeOH and matrix-matched standard curves, respectively, for the column used in SLV #17-103).

Discussion:

In both this validation in mussels, and the previous validation in clams (ISSC proposal #17-103), there was interest from participating ISSC labs in excluding the hexane washing step originally proposed by the method developers[4]. Therefore, the effect of the hexane wash step was evaluated as part of proposal #17-103 and while a significant difference was found in the measured concentrations with and without this clean-up step for all three toxins (using a MeOH standard curve only), the mean of the differences was small (range 1.4-1.8 µg/100 g). Based on this data, the hexane washing step was listed as optional in the method checklist (proposal # 19-136). In the current study, the effects of removing the hexane washing step were more pronounced with significant differences observed for all three toxins, using both MeOH and matrix-matched standard curves. Further, by excluding this clean-up step, several toxins and treatments now showed recoveries outside of acceptable ranges, likely due to the lack of removal of lipophilic matrix enhancers.

Results for the re-analysis of a sample set previously tested for accuracy using both the current (HPLC) analytical column and the previous (UPLC) analytical column, using both MeOH and matrix-matched standard curves, were also comparable and within acceptable ranges indicating that the previously tested UPLC column would also be acceptable for use with the modified method.

Note: The same samples and standards analyzed using the current analytical (HPLC) column (5 µL injection, flow rate 300 µL/min) had significantly lower peak intensities (approx. 3-fold) compared to samples analyzed using the previous UPLC column (5 µL injection, flow rate 120 µL/min). While the results were comparable and the method was still sufficiently sensitive for its intended use, use of the previous UPLC column demonstrated greater method sensitivity while also using less solvent. This was also evidenced by comparing the LOD and LOQs between the two SLV studies where SLV #17-103 had a method sensitivity 2-15-fold lower, depending on the toxin [LOD: 0.066, 0.013, 0.041; LOQ: 0.20, 0.039, 0.13 µg/100g for OA, DTX2, and DTX1, respectively (for clams)].

Table 19. Ruggedness Testing for DTX1 in Mussel (Effect of Hexane Wash Step)

DTX1		Hexane Wash				No Hexane Wash			
Sample	Spike (µg/100g)	Determined Concentration (µg/100g) & % Accuracy				Determined Concentration (µg/100g) & % Accuracy			
		MeOH	% Acc.	Matrix Matched	% Acc.	MeOH	% Acc.	Matrix Matched	% Acc.
1a	8	6.8	85.0	6.1	76.3	8.7	108.8	8.0	100.0
1b	8	7.1	88.8	6.4	80.0	8.9	111.3	8.1	101.3
2a	12	11.6	96.7	10.6	88.3	14.4	120.0	13.3	110.8
2b	12	11.4	95.0	10.5	87.5	14.3	119.2	13.3	110.8
3a	16	15.8	98.8	14.7	91.9	18.9	118.1	17.6	110.0
3b	16	15.8	98.8	14.7	91.9	18.9	118.1	17.6	110.0
4a	24	26.2	109.2	24.5	102.1	32.3	134.6	30.3	126.3
4b	24	25.5	106.3	23.8	99.2	31.6	131.7	29.5	122.9
5a	32	31.5	98.4	29.5	92.2	37.3	116.6	35.0	109.4
5b	32	32.0	100.0	30.0	93.8	38.7	120.9	36.3	113.4
6b	8	8.3	103.8	7.6	95.0	10.4	130.0	9.6	120.0
6b	8	8.3	103.8	7.6	95.0	10.7	133.8	9.9	123.8
7a	12	11.3	94.2	10.4	86.7	14.5	120.8	13.4	111.7
7b	12	11.8	98.3	10.8	90.0	14.4	120.0	13.4	111.7
8a	16	14.7	91.9	13.6	85.0	17.4	108.8	16.2	101.3
8b	16	15.0	93.8	13.9	86.9	18.2	113.8	17.0	106.3
9a	24	22.2	92.5	20.7	86.3	26.9	112.1	25.1	104.6
9b	24	22.5	93.8	21.0	87.5	28.3	117.9	26.5	110.4
10a	32	31.6	98.8	29.6	92.5	36.8	115.0	34.5	107.8
10b	32	29.1	90.9	27.2	85.0	38.0	118.8	35.7	111.6
Average Accuracy		**96.9%**		**89.6%**		**119.5%**		**111.2%**	
Skewness		**0.41**		**0.41**		**0.41**		**0.41**	
Variance		**77.89**		**69.52**		**112.96**		**100.82**	

Methanol Standard Curve:
Variance Ratio: 1.45 (not significant)
Paired t-test (two tailed): P-value 1.685×10^{-8} (significant)

Matrix Matched Standard Curve:
Variance Ratio: 1.45 (not significant)
Paired t-test (two tailed): P-value 1.685×10^{-8} (significant)

Table 20. Ruggedness Testing for DTX2 in Mussel (Effect of Hexane Wash Step)

DTX2		Hexane Wash				No Hexane Wash			
Sample	Spike (µg/100g)	Determined Concentration (µg/100g) & % Accuracy				Determined Concentration (µg/100g) & % Accuracy			
		MeOH	% Acc.	Matrix Matched	% Acc.	MeOH	% Acc.	Matrix Matched	% Acc.
1a	8	7.9	98.8	7.7	96.3	9.7	121.3	9.5	118.8
1b	8	7.9	98.8	7.7	96.3	9.9	123.8	9.6	120.0
2a	12	12.6	105.0	12.2	101.7	15.3	127.5	14.8	123.3
2b	12	12.5	104.2	12.1	100.8	15.3	127.5	14.8	123.3
3a	16	16.5	103.1	15.9	99.4	20.7	129.4	20.0	125.0
3b	16	17.3	108.1	16.7	104.4	21.2	132.5	20.4	127.5
4a	24	26.7	111.3	25.7	107.1	32.5	135.4	31.2	130.0
4b	24	26.8	111.7	25.8	107.5	32.0	133.3	30.7	127.9
5a	32	34.3	107.2	33.0	103.1	41.2	128.8	39.6	123.8
5b	32	34.1	106.6	32.8	102.5	40.5	126.6	38.9	121.6
6b	8	8.1	101.3	7.9	98.8	10.6	132.5	10.3	128.8
6b	8	8.5	106.3	8.3	103.8	10.7	133.8	10.3	128.8
7a	12	12.2	101.7	11.8	98.3	15.1	125.8	14.6	121.7
7b	12	12.3	102.5	11.9	99.2	15.6	130.0	15.1	125.8
8a	16	17.2	107.5	16.6	103.8	20.0	125.0	19.3	120.6
8b	16	16.5	103.1	15.9	99.4	20.3	126.9	19.6	122.5
9a	24	25.2	105.0	24.2	100.8	31.2	130.0	30.0	125.0
9b	24	25.3	105.4	24.3	101.3	30.6	127.5	29.4	122.5
10a	32	34.3	107.2	32.9	102.8	39.6	123.8	38.0	118.8
10b	32	33.4	104.4	32.1	100.3	40.1	125.3	38.5	120.3
Average Accuracy		**104.9%**		**101.4%**		**128.3%**		**123.8%**	
Skewness		**0.40**		**0.40**		**0.36**		**0.36**	
Variance		**92.85**		**85.04**		**126.93**		**116.26**	

Methanol Standard Curve:
Variance Ratio: 1.37 (not significant)
Paired t-test (two tailed): P-value 1.371×10^{-9} (significant)

Matrix Matched Standard Curve:
Variance Ratio: 1.37 (not significant)
Paired t-test (two tailed): P-value 1.371×10^{-9} (significant)

Table 21. Ruggedness Testing for OA in Mussel (Effect of Hexane Wash Step)

OA		Hexane Wash				No Hexane Wash			
Sample	Spike (µg/100g)	Determined Concentration (µg/100g) & % Accuracy				Determined Concentration (µg/100g) & % Accuracy			
		MeOH	% Acc.	Matrix Matched	% Acc.	MeOH	% Acc.	Matrix Matched	% Acc.
1a	8	6.3	78.8	6.1	76.3	7.3	91.3	7.0	87.5
1b	8	5.8	72.5	5.6	70.0	7.2	90.0	6.9	86.3
2a	12	10.3	85.8	9.6	80.0	11.8	98.3	11.0	91.7
2b	12	9.9	82.5	9.3	77.5	12.6	105.0	11.7	97.5
3a	16	14.4	90.0	13.3	83.1	17.6	110.0	16.2	101.3
3b	16	14.2	88.8	13.1	81.9	17.8	111.3	16.4	102.5
4a	24	25.3	105.4	23.1	96.3	29.6	123.3	26.9	112.1
4b	24	25.2	105.0	23.0	95.8	30.4	126.7	27.7	115.4
5a	32	31.8	99.4	28.9	90.3	36.9	115.3	33.4	104.4
5b	32	31.9	99.7	28.9	90.3	36.4	113.8	33.0	103.1
6b	8	8.6	107.5	8.2	102.5	8.9	111.3	8.4	105.0
6b	8	6.9	86.3	6.6	82.5	9.2	115.0	8.7	108.8
7a	12	9.9	82.5	9.3	77.5	12.9	107.5	12.0	100.0
7b	12	10.5	87.5	9.8	81.7	13.0	108.3	12.1	100.8
8a	16	13.2	82.5	12.3	76.9	17.3	108.1	15.9	99.4
8b	16	13.8	86.3	12.8	80.0	16.8	105.0	15.5	96.9
9a	24	22.1	92.1	20.3	84.6	26.5	110.4	24.1	100.4
9b	24	21.5	89.6	19.6	81.7	27.5	114.6	25.0	104.2
10a	32	30.2	94.4	27.5	85.9	36.8	115.0	33.3	104.1
10b	32	30.7	95.9	27.9	87.2	36.7	114.7	33.2	103.8
Average Accuracy		**90.6%**		**84.1%**		**109.7%**		**101.2%**	
Skewness		**0.48**		**0.40**		**0.39**		**0.36**	
Variance		**85.78**		**85.04**		**116.73**		**116.26**	

Methanol Standard Curve:
Variance Ratio: 1.36 (not significant)
Paired t-test (two tailed): P-value 2.646×10^{-8} (significant)

Matrix Matched Standard Curve:
Variance Ratio: 1.37 (not significant)
Paired t-test (two tailed): P-value 2.646×10^{-8} (significant)

Table 22. Column Comparability (Ruggedness) Testing for DTX1 in Mussel

DTX1		Current Column				Column from #17-103			
Sample	Spike (µg/100g)	Determined Concentration (µg/100g) & % Accuracy				Determined Concentration (µg/100g) & % Accuracy			
		MeOH	% Acc.	Matrix Matched	% Acc.	MeOH	% Acc.	Matrix Matched	% Acc.
1a	8	5.9	73.2	5.6	69.6	7.5	93.8	7.0	87.3
1b	8	6.7	84.2	6.4	80.2	6.5	81.0	6.2	76.5
2a	12	10.8	90.3	10.3	86.1	11.2	93.1	10.2	84.1
2b	12	10.9	91.2	10.4	87.0	12.2	101.8	11.1	91.4
3a	16	14.2	88.5	13.5	84.5	15.3	95.5	13.7	84.7
3b	16	14.4	90.2	13.8	86.0	16.8	105.0	15.0	92.7
4a	24	23.5	98.1	22.5	93.6	25.9	108.1	22.8	94.0
4b	24	24.6	102.6	23.5	98.0	26.4	110.1	23.2	95.7
5a	32	30.5	95.2	29.1	90.9	31.6	98.6	27.5	85.4
5b	32	29.2	91.1	27.8	87.0	33.5	104.6	29.2	90.4
6b	8	8.7	108.3	8.3	103.2	8.3	103.9	7.7	95.9
6b	8	8.6	107.0	8.2	102.0	8.3	104.3	7.8	96.2
7a	12	12.4	103.0	11.8	98.2	10.5	87.6	9.6	79.4
7b	12	12.1	101.0	11.6	96.3	11.8	98.7	10.7	88.8
8a	16	15.0	93.7	14.3	89.4	14.1	88.0	12.6	78.4
8b	16	14.1	88.0	13.4	83.9	15.4	96.4	13.8	85.5
9a	24	24.2	101.0	23.1	96.4	23.5	97.8	20.6	85.3
9b	24	23.3	96.9	22.2	92.5	23.4	97.7	20.6	85.2
10a	32	31.0	97.0	29.6	92.6	30.9	96.6	27.0	83.7
10b	32	31.4	98.2	30.0	93.8	32.5	101.7	28.4	88.0
Average Accuracy		**94.9%**		**90.6%**		**98.2%**		**87.4%**	
Skewness		**-0.71**		**-0.73**		**-0.61**		**-0.10**	
Variance		**68.46**		**62.61**		**52.35**		**33.24**	

Methanol Standard Curve:
Variance Ratio: 0.76 (not significant)
Paired t-test (two tailed): P-value = 0.099 (not significant)

Matrix Matched Standard Curve:
Variance Ratio: 0.53 (not significant)
Paired t-test (two tailed): P-value = 0.089 (not significant)

Table 23. Column Comparability (Ruggedness) Testing for DTX2 in Mussel

DTX2		Current Column				Column from #17-103			
Sample	Spike (µg/100g)	Determined Concentration (µg/100g) & % Accuracy				Determined Concentration (µg/100g) & % Accuracy			
		MeOH	% Acc.	Matrix Matched	% Acc.	MeOH	% Acc.	Matrix Matched	% Acc.
1a	8	7.4	92.9	7.5	93.5	7.4	92.8	7.3	91.0
1b	8	7.1	88.9	7.2	89.7	7.6	94.8	7.5	92.8
2a	12	10.8	90.1	10.7	89.2	11.8	98.1	11.3	93.7
2b	12	11.8	98.3	11.6	97.0	12.2	101.7	11.7	97.1
3a	16	16.0	100.0	15.7	97.9	16.2	101.0	15.4	95.4
3b	16	15.2	95.2	14.9	93.2	16.2	101.4	15.4	95.7
4a	24	26.1	108.7	25.3	105.2	25.7	107.1	24.2	99.9
4b	24	24.8	103.5	24.1	100.3	26.0	108.4	24.5	101.1
5a	32	33.5	104.6	32.3	101.0	31.5	98.5	29.5	91.6
5b	32	32.1	100.2	31.0	96.7	32.7	102.2	30.7	95.0
6b	8	8.8	110.4	8.8	110.2	7.9	99.1	7.8	96.7
6b	8	8.5	106.9	8.5	106.8	8.3	104.2	8.2	101.4
7a	12	12.9	107.2	12.7	105.5	11.3	94.6	11.0	90.5
7b	12	12.0	100.4	11.9	99.0	11.5	95.6	11.1	91.5
8a	16	17.1	106.8	16.7	104.3	16.2	101.0	15.4	95.5
8b	16	16.6	104.0	16.3	101.6	16.0	99.8	15.2	94.3
9a	24	26.8	111.7	26.0	108.2	25.0	104.1	23.5	97.2
9b	24	26.6	110.9	25.8	107.4	24.8	103.5	23.4	96.7
10a	32	35.7	111.7	34.5	107.7	32.9	102.9	30.8	95.6
10b	32	35.2	109.9	33.9	106.0	33.9	106.0	31.7	98.4
Average Accuracy		**103.1%**		**101.0%**		**100.8%**		**95.6%**	
Skewness		**-0.64**		**-0.45**		**-0.19**		**0.20**	
Variance		**51.07**		**40.03**		**18.03**		**10.04**	

Methanol Standard Curve:
Variance Ratio: 0.35 (not significant)
Paired t-test (two tailed): P-value = 0.11 (not significant)

Matrix Matched Standard Curve:
Variance Ratio: 0.25 (not significant)
Paired t-test (two tailed): P-value = 0.00046 (significant)

Table 24. Column Comparability Testing (Ruggedness) for OA in Mussel

OA		Current Column				Column from #17-103			
Sample	Spike (µg/100g)	Determined Concentration (µg/100g) & % Accuracy				Determined Concentration (µg/100g) & % Accuracy			
		MeOH	% Acc.	Matrix Matched	% Acc.	MeOH	% Acc.	Matrix Matched	% Acc.
1a	8	4.9	61.2	5.1	63.3	5.5	68.5	5.4	66.7
1b	8	4.9	61.7	5.1	63.8	4.8	59.7	4.8	59.3
2a	12	9.2	76.8	9.2	76.6	9.7	81.2	9.0	74.3
2b	12	8.4	70.2	8.4	70.2	10.2	84.9	9.4	77.3
3a	16	12.7	79.4	12.5	78.2	13.7	85.7	12.3	76.5
3b	16	12.5	78.4	12.4	77.2	14.0	87.2	12.5	77.7
4a	24	21.4	89.3	20.8	86.8	23.4	97.7	20.5	84.9
4b	24	21.2	88.3	20.6	85.8	24.3	101.4	21.3	88.0
5a	32	27.4	85.7	26.5	82.9	29.0	90.8	25.3	78.4
5b	32	27.5	85.9	26.6	83.1	29.1	91.1	25.4	78.6
6b	8	7.3	91.0	7.3	91.7	7.3	91.2	6.9	85.7
6b	8	6.6	82.9	6.7	84.0	7.2	90.0	6.8	84.8
7a	12	9.6	79.6	9.5	79.2	9.8	81.6	9.0	74.5
7b	12	10.0	83.2	9.9	82.6	9.9	82.6	9.1	75.4
8a	16	13.0	81.5	12.8	80.2	12.6	79.0	11.4	70.9
8b	16	13.1	81.9	12.9	80.5	12.3	76.7	11.1	68.9
9a	24	22.0	91.7	21.4	89.0	21.7	90.4	19.1	78.8
9b	24	22.0	91.8	21.4	89.2	22.3	93.1	19.6	81.1
10a	32	28.9	90.5	28.0	87.5	29.2	91.1	25.4	78.7
10b	32	28.2	88.2	27.3	85.3	29.7	92.8	25.8	80.1
Average Accuracy		**82.0%**		**80.9%**		**85.8%**		**77.0%**	
Skewness		**-1.19**		**-1.06**		**-1.11**		**-0.80**	
Variance		**81.10**		**60.91**		**94.67**		**47.24**	

Methanol Standard Curve:
Variance Ratio: 1.16 (not significant)
Paired t-test (two tailed): P-value = 0.0036 (significant)

Matrix Matched Standard Curve:
Variance Ratio: 0.53 (not significant)
Paired t-test (two tailed): P-value = 0.0024 (significant)

7. Comparability

Methods:

The purpose of this proposal was to modify the existing LC-MS/MS method originally validated under ISSC proposal #17-103 to address several deficiencies identified during method verification. Therefore, direct comparability of the methods was not appropriate as the method was never established outside of the validating laboratory and not in routine use. To establish comparability in ISSC proposal #17-103, multiple naturally contaminated certified reference materials (CRMs) were analyzed and compared to the reference values provided by the manufacturer. As a measure of method comparability for the current SLV, these same CRMs were purchased again (5 individual vials of frozen CRM-DSP-Mus-c and 5 separate extractions of lyophilized NRC-FDMT1). The lyophilized CRM contains a number of additional lipophilic shellfish toxins, including azaspiracids, pectenotoxins, and yessotoxins; therefore, this material was also used as a secondary measure of method specificity. Each was quantified with both MeOH and matrix-matched standard curves and compared to both the reported values and values obtained in proposal #17-103.

As a further test of method comparability, we participated in the WEPAL-QUASIMEME BT11 (2025) DSP shellfish toxins study. WEPAL-QUASIMIEME is part of Stichting Wageningen Research, Netherlands, and provides globally accredited proficiency testing (PT) programs including one for DSP testing by LC-MS/MS (BT11). The scope for the QUASIMEME BT11 program included total (free plus esterified) OA, total DTX1, total DTX2, and total combined OA+DTX1+DTX2 (in OA eq.). Three blinded samples (frozen shellfish homogenates) were provided to our laboratory; unblinded sample information, available after the testing period had closed, identified the homogenates as mussels (*Mytilus edulis*) from Ireland (sample QST371BT), cockles (*Cerastodema edule*) from England (sample QST372BT), and mussels (*Mytilus edulis*) from the United Kingdom (sample QST373BT). Samples were analyzed using both matrix-matched and neat (MeOH) standard curves, with hexane sample clean up, but only results for the matrix-matched standard curve were provided to the study organizers through their on-line portal. Statistical analysis was performed by the QUASIMEME study organizers using data from all participating laboratories (40 labs in 2025) and is recorded in the WEPAL-QUASIMIEME BT11 DSP Shellfish Toxins Final Report 2025.1 Version 1: 22 July 2025 (accessible to study participants). The data provided using this SLV method is recorded in the study as Q4122A. The study uses a z'-score to evaluate inter-laboratory performance. Statistical explanations of this test and data are described in the Final 2025 Report.

Results:

Data for the CRM analysis is presented in Table 25. Percent accuracy for all three toxins compared to the informational total toxin values (post-hydrolysis) contained in the certificates of analysis ranged from 99-114% when using the MeOH standard curve and 93-108% when using the matrix-matched standard curve. Percent accuracy for the same CRM lots tested during ISSC proposal #17-103 ranged from 92-111%, using a MeOH standard curve only. Although all samples had acceptable accuracy, samples analyzed using a MeOH standard curve had consistently higher values, with the exception of one sample, compared to the matrix-matched standard curve which is consistent with the previous observation of matrix enhancement.

For the data values for OA, DTX1, DTX2, and total DSTs provided by our laboratory to the QUASIMEME study, all were assessed as satisfactory performance with z'-scores less than 2 (Table 26). Table 27 shows the data we submitted to the study, using a matrix matched standard curve, compared to the assigned values determined by the study organizers, with a calculated % error. Table 29 shows the same unknown sample data but using a methanol calibration curve to calculate the sample concentrations (these values were not submitted to the QUASIMEME study but were used for this in-house comparison after the final report was provided to us).

Discussion:

FDA typically considers acceptable accuracy (as % recovery) to be 60-115% for concentrations in the range of 10 µg/kg (equivalent to 1 µg/100g) and 80-110% for concentrations in the range of 100 µg/kg (equivalent to 10 µg/100g)[5]. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends recoveries between 60-115% or 70-130% depending on the toxin and method for the DST group[2]. Results for all three toxins were within acceptable ranges for the CRM materials analyzed with the modified LC-MS/MS method for mussels using matrix-matched standard curves. When using the MeOH standard curve, results for OA and DTX2 were slightly above the acceptable range based on FDA guidelines (113% and 114% for OA and DTX2, respectively) but were both within acceptable limits based on the Codex recommendations. Further, results for this SLV were comparable to the results for these same lots of CRMs analyzed previously as part of SLV #17-103.

The satisfactory performance results for all summitted data from the QUASIMEME study provides additional support that this method is suitable for the detection and quantitation of OA, DTX1, and DTX2 as results were comparable to validated methods currently in use in EU laboratories. These data also provide support that mussel matrix-matched calibration curves may be suitable for evaluating other matrices as unknown sample QST372BT was cockles (*Cerastodema edule*) from England.

Table 25. Method comparability data for certified reference naturally contaminated mussel tissues in both frozen and lyophilized forms as tested by LC-MS/MS (n=5 each). (Informational values for total DSTs post-hydrolysis used)

DTX1					
Frozen CRM (110 µg/100g)	**MeOH**	**Matrix Matched**	**Lyophilized CRM (85 µg/100g)**	**MeOH**	**Matrix Matched**
1	103.9%	98.6%	**1**	97.8%	107.0%
2	104.3%	99.1%	**2**	94.6%	104.0%
3	103.9%	98.7%	**3**	93.8%	103.2%
4	107.3%	101.8%	**4**	104.3%	113.0%
5	108.7%	103.2%	**5**	102.4%	111.2%
Average Accuracy	**105.6%**	**100.2%**		**98.6%**	**107.7%**
%RSD	**2.12**	**2.09**		**4.72**	**1.67**

DTX2					
Frozen CRM (220 µg/100g)	**MeOH**	**Matrix Matched**	**Lyophilized CRM (541 µg/100g)**	**MeOH**	**Matrix Matched**
1	115.6%	110.7%	**1**	107.3%	103.2%
2	111.5%	106.8%	**2**	102.2%	98.4%
3	111.8%	107.0%	**3**	104.0%	100.1%
4	112.1%	107.3%	**4**	104.1%	100.2%
5	115.2%	110.2%	**5**	108.6%	104.5%
Average Accuracy	**113.2%**	**108.4%**		**105.2%**	**101.2%**
%RSD	**1.76**	**1.74**		**2.50**	**2.46**

OA					
Frozen CRM (240 µg/100g)	**MeOH**	**Matrix Matched**	**Lyophilized CRM (391 µg/100g)**	**MeOH**	**Matrix Matched**
1	116.5%	107.0%	**1**	102.2%	95.2%
2	112.2%	103.0%	**2**	97.8%	91.1%
3	113.9%	104.6%	**3**	99.3%	92.5%
4	114.2%	104.9%	**4**	101.1%	94.2%
5	113.3%	104.0%	**5**	100.0%	93.1%
Average Accuracy	**114.0%**	**104.7%**		**100.1%**	**93.2%**
%RSD	**1.39**	**1.41**		**1.68**	**1.69**

Table 26. Z-scores provided by QUASIMEME study organizers for SLV generated data

2025 BT-11 Lipophilic Shellfish Toxins Proficiency Tests z'-score Mussel Matched Matrix Curve					
Sample	Lab	Total OA	Total DTX1	Total DTX2	Total-hy-OA+DTX1+DTX2
QST371BT	FDA-Q4122A	0.20	NA	-0.80	0.20
QST372BT	FDA-Q4122A	0.60	0.80	0.00	0.70
QST373BT	FDA-Q4122A	0.50	NA	0.60	0.50
Satisfactory Performance		½z_i' ½ < 2			
Questionable Performance		2 < ½z_i' ½ < 3			
Unsatisfactory Performance		½z_i' ½> 3			

Table 27. QUASIMEME assigned values vs. SLV generated values using a mussel matrix matched curve.

2025 BT-11 Lipophilic Shellfish Toxins Proficiency Test Submitted Data Mussel Matched Matrix Curve					
Sample	Lab	Total OA	Total DTX1	Total DTX2	Total-hy-OA+DTX1+DTX2
		µg/100 g	µg/100 g	µg/100 g	µg/100 g (OA eq.)
QST371BT	QUASIMEME	6.13	ND	1.00	6.48
	FDA-Q4122A	6.42	ND	0.74	6.86
	%error	**4.75**		**-26.41**	**5.91**
QST372BT	QUASIMEME	15.80	17.50	2.25	35.1
	FDA-Q4122A	17.78	21.17	2.23	40.29
	%error	**12.52**	**20.99**	**-0.75**	**14.79**
QST373BT	QUASIMEME	9.47	ND	12.90	17.40
	FDA-Q4122A	10.51	ND	14.53	19.23
	%error	**11.03**		**12.60**	**10.52**

Table 28. QUASIMEME assigned values vs. SLV values using a neat (MeOH) standard curve.

2025 BT-11 Lipophilic Shellfish Toxins Proficiency Test Submitted Data Neat (MeOH) Standard Curve					
Sample	Lab	Total OA	Total DTX1	Total DTX2	Total-hy-OA+DTX1+DTX2
		µg/kg	µg/kg	µg/kg	µg/kg (OA eq.)
QST371BT	QUASIMEME	6.13	ND	1.00	6.48
	FDA-Q4122A	6.76	ND	1.45	7.63
	%error	**10.22**		**45.00**	**17.69**
QST372BT	QUASIMEME	15.80	17.50	2.25	35.10
	FDA-Q4122A	18.34	21.02	2.85	41.07
	%error	**16.10**	**20.11**	**26.67**	**17.02**
QST373BT	QUASIMEME	9.47	ND	12.90	17.40
	FDA-Q4122A	10.92	0.70	14.30	20.20
	%error	**15.36**		**10.85**	**16.12**

Summary and Conclusions:

The SLV data presented here establishes that the modified LC-MS/MS method is fit for the purpose of quantifying DTX1, DTX2 and OA in mussels at the regulatory level of 16 µg/100 g using both a MeOH and matrix-matched standard curve.

Average accuracy at five concentrations ranging from 8 to 32 µg/100 g were 100% for DTX1, 105% for DTX2, and 94% for OA with measurement uncertainties of 0.50, 0.50, and 0.54 µg/100g, respectively, when quantified using a MeOH standard curve. Accuracies were 91% for DTX1, 99% for DTX2, and 85% for OA with measurement uncertainties of 0.50, 0.50, and 0.47 µg/100g, respectively, when quantified using a matrix-matched standard curve.

Average recoveries ranged from 102-103% for DTX1, 105-106% for DTX2, and 92-96% for OA when using a MeOH standard curve. Recoveries ranged from 89-95% for DTX1, 95-99% for DTX2, and 80-86% for OA when using a matrix-matched standard curve. One-way analysis of variance testing for significant differences between spike concentrations was not significant with the exception of DTX1 using the matrix matched standard curve only ($p=0.042$). Tukey's test for honestly significant differences showed that the significant variance was between the low (8 µg/100 g) and the high (32 µg/100g) concentrations only. FDA typically considers acceptable recoveries to be 60-115% for concentrations in the range of 10 µg/kg (equivalent to 1 µg/100g), and 80-110% for concentrations in the range of 100 µg/kg (equivalent to 10 µg/100g)[5]. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends recoveries between 60-115% or 70-130% depending on the toxin and method for the DST group[2]. In this study, recoveries using both MeOH and matrix-matched standard curves were well within these guidelines. The significant difference in variance between the low and high fortification levels (for DTX1 using a matrix matched standard curve only) was not surprising considering most validation guidelines have a broader range of acceptable recoveries and precision at lower spike levels.

For precision (as intermediate precision), both the nested ANOVA (F test) as well as Bartlett's test of homogeneity of variances indicated that the precision was not consistent across all concentrations for all

toxins. The exact reason for this is not clear but additional sources of variation not accounted for in these tests, such as differences in matrix effects between individual samples, could potentially explain these results. Therefore, precision was calculated for each spike concentration separately. The %RSD ranged from 5.6-8.3 for DTX1, 4.4-8.4 for DTX2, and 9.4-15.8 for OA across the range of concentrations tested when using a MeOH standard curve. %RSDs ranged from 4.6-7.1 for DTX1, 4.6-5.5 for DTX2, and 5.5-10.0 for OA across the range of concentrations tested when using a matrix-matched standard curve. FDA typically considers acceptable method precision to be <22% for concentrations in the range of 10 µg/kg (equivalent to 1 µg/100g), and <11% for concentrations in the range of 100 µg/kg (equivalent to 10 µg/100g)[5]. Replicate injections on separate days for all toxins and concentrations had <10 %RSD for both MeOH and matrix matched standard curves, with the exception of OA at the lowest fortification level (%RSD 15.8 for the 8 µg/100 spike level) and the MeOH standard curve only. For repeatability, five replicate extractions of two naturally contaminated CRMs had <5 %RSD for all three toxins.

Acceptable linearity in response was observed for all three toxins over a concentration range spanning 4–48 µg toxin/100 g mussel tissue (50-150% of the range of interest), and the method was found to be sufficiently sensitive for its intended use. The Codex Alimentarius Standard for Recommended Methods of Analysis and Sampling (CXS 234-1999) recommends an LOD and LOQ for methods for the determination of DSTs of 1 and 2 µg/100 g, respectively, for OA and DTX1, and 3 and 6 µg/100 g, respectively, for DTX2[2]. Surprisingly, samples analyzed using the previously evaluated UPLC column from SLV #17-103 had higher responses, at a lower flow rate, compared to the HPLC column tested in this SLV. This suggests that instrument parameters, such as flow rate, can likely be optimized if method sensitivity needs to be increased.

The method was found to be specific for DSTs, as results were not affected in the presence of multiple potentially co-occurring lipophilic shellfish toxins such as azaspiracids, pectenotoxins, and yessotoxin. Unlike SLV #17-103 where ruggedness testing found that removal of the hexane washing step had no negative effects on the performance of the method, during this study, removal of this sample clean-up step did negatively impact the results as recoveries for DTX2, using both MeOH and matrix-matched standard curves, and DTX1, using a MeOH standard curve only, were above acceptable ranges. Based on the results of this SLV, we cannot conclude that the hexane washing step is optional and recommend that individual laboratories evaluate accuracy and recovery with and without the hexane wash as part of the method verification process. Lastly, use of the UPLC analytical column tested as part of SLV #17-103 provided comparable results to the HPLC column evaluated here. As noted above, this UPLC column, while using a lower flow rate, also had higher peak intensities and therefore greater method sensitivity.

As a measure of method comparability, multiple aliquots of both naturally contaminated CRMs available from NRC (both frozen and lyophilized) were analyzed using both matrix-matched and neat (MeOH) standard curves and compared to the informational values for total DST's provided by the manufacturer and also to data generated in proposal #17-103 using the previous UPLC column. Results for all three toxins were within acceptable ranges for the CRM materials analyzed with the modified LC-MS/MS method for mussels using matrix-matched standard curves. When using the MeOH standard curve, results for OA and DTX2 were slightly above the acceptable range based on FDA guidelines (113% and 114% for OA and DTX2, respectively, with acceptable % recovery in this concentration range being 80-110%), but were both within acceptable limits based on the Codex recommendations (60-115% and 70-130% depending on toxin and method). Results for this SLV were comparable to the results for these same lots of CRMs analyzed previously as part of SLV #17-103 (92-111%, using a MeOH standard curve only. As another measure of method comparability, we participated in the WEPAL-QUASIMEME BT11 (2025) DSP shellfish toxins study, which is used as a measure of proficiency by many European labs. The satisfactory performance results (z-scores <1 for all toxins, with satisfactory results defined as z-score <2) for all summitted data provides additional support that this method is comparable to validated reference methods being run in the EU.

Overall, the method presented here was found to be accurate, precise, specific, sensitive, and fit for the purpose of managing DSP toxins in mussels under the NSSP. Further, data presented here provide options that laboratories could consider when implementing this method (i.e. MeOH vs. matrix-matched standard curves, more than one equivalent analytical column, etc.) but these options will need to be carefully verified in participating NSSP laboratories as differences in available instrumentation have previously been shown to affect the performance of the method.

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SHELLFISH LABORATORY EVALUATION CHECKLIST
Diarrhetic Shellfish Poisoning Toxins (DSP) LC-MS/MS

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ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "✓"**

C – Critical K - Key O - Other NA - Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 7, 8		1.1.1 Written Plan adequately covers all the following: (check those that apply)
			a. Organization of the laboratory
			b. Staff training requirements
			c. Standard operating procedures
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established
			e. Laboratory safety
			f. Internal performance assessment
			g. External performance assessment
C	**5**		**1.1.2 QA Plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
C	**3, 13**		**1.2.5 LC-MS Operator must be trained in the operation and maintenance of the specific liquid chromatography-mass spectrometry system used.**
		1.3 Work Area	
O	1		1.3.1 Adequate for workload and storage.
O	1		1.3.2 Clean and well lighted.
O	1		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment	
C	**3, 13**		**1.4.1 A heat block or water bath capable of heating samples to 76 ± 2 °C.**
K	2		1.4.2 Balances provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	7, 8		1.4.3 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.4 Refrigerator temperature is maintained between 0 and 4 ºC.
K	7		1.4.5 Refrigerator temperature is monitored at least once daily on working days. Results are recorded and records maintained.
K	2		1.4.6 Freezer temperature is maintained at -10 ºC or below.

K	7		1.4.7 Freezer temperature is monitored at least once daily on working days. Results are recorded and records maintained.
C	**10**		**1.4.8 All in-service thermometers are properly calibrated and immersed.**
K	4		1.4.9 All glassware is clean.
K	3, 12, 13		1.4.10 An ultra-performance liquid chromatography (UPLC) system equipped with the following is used: a. mobile phase system capable of running in gradient mode of and delivering a pulse-free flow of 0.12 mL/min appropriate for the analytical column in use b. solvent degasser (optional) c. autosampler (refrigerated preferred) with loop suitable for five (5) µL injections d. column compartment capable of controlling temperature at 40 ºC e. a data collection system (e.g., computer, integrator)
C	**3, 13**		**1.4.11 A mass spectrometer equipped with the following is used:** **a. an electrospray ionization source operating in negative ion mode and** **b. multiple reaction monitoring scan mode capability.** **c. if a divert valve is used to divert LC flow at the beginning and end of each chromatographic run, the switching time should be at least one minute before the first peak elution and at least one minute after the last peak elution.**
K	2		1.4.12 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3, 13		1.4.13 A centrifuge capable of generating 2000 x g and holding 15 mL and 50 mL polypropylene tubes is used.
		1.5	**Reagents and Reference Solution Preparation and Storage**
C	**3, 13**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	7		1.5.2 Water contains < 100 CFU/ml determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	7		1.5.23 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared, and expiration date.
C	**3, 13**		**1.5.34 The mobile phase system used to analyze DSP toxins consists of: A: 2 mM ammonium formate and 50 mM formic acid in water B: 2 mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water**
O	2		1.5.45 Mobile phase is degassed manually before use if the UPLC system does not have a degasser or if the degasser is not in use.
C	**3, 13**		**1.5.56 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
C	**6**		**1.5.67 All primary standards are stored appropriately as per supplier recommendations.**

C	**6**		**1.5.78 All standards used are within their expiration date.**
C	**2, 3, 13**		**1.5.89 All standards are prepared either gravimetrically or using appropriate positive displacement pipettes or syringes.**
C	**3, 13**		**1.5.910 Working standards are made up from primary standard by dilution with either~~the~~ toxin-free~~,~~ extraction solvent (i.e., 100% methanol), or blank composite matrix comprised of ≥ 5 previously tested matrix sample extracts containing DSP toxins < LOD, per the laboratory's method verification.**
		1.6 Collection and Transportation of Samples	
O	5, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	5, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**5, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**
K	2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
		PART II – EXAMINATION OF SHELLFISH FOR DSP TOXINS	
		2.1 Preparation of Sample	
C	**2**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., three (3) geoduck gut balls).**
O	5		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	5		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**

O	5		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for five (5) minutes.
K	5		2.1.8 Pieces of shell and drainage are discarded.
C	**2, 5**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction	
K	2		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**3, 13**		**2.2.2 Two (2.00) ± 0.05 g of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**3, 13**		**2.2.3 The sample homogenate is extracted with 9 mL of 100% methanol by ~~and~~ vortexing ~~ed~~ for 3 minutes ~~to mix~~.**
K	3, 13		2.2.4 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant decanted into a clean container (e.g. new polypropylene tube or glass vial).
C	**3, 13**		**2.2.5 The tissue pellet is re-extracted with nine (9) mL of 100% methanol by vortexing for 3 minutes ~~and homogenized to mix~~.**
K	3, 13		2.2.6 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant combined with the supernatant collected in 2.2.4.
K	3, 13		2.2.7 The total extract volume is adjusted to 20 mL with 100% methanol.
K	3, 13		2.2.8 The crude extract is hydrolyzed or stored in the freezer at < -10 °C.
		2.3 Sample Hydrolysis and Cleanup	
K	3, 13		2.3.1 A two (2) mL aliquot of the sample extract is transferred to an appropriately size* glass tube with a phenolic PTFE lined screw cap using a positive displacement pipette or syringe. *Note: A 16 x 100 mm tube will have sufficient volume to perform the hydrolysis and hexane wash steps and fit in a standard 15 mL centrifuge tube adaptor.
K	3, 13		2.3.2 The sample extract is hydrolyzed by adding 250 µL of 2.5 M NaOH and the sample is homogenized with a vortex mixer for 30 seconds.
C	**3, 13**		**2.3.3 Sample tube caps are securely fastened to prevent extract loss, and the weight of the sample tube is recorded. The sample tube is heated at 76 °C for 40 minutes, then allowed to cool to room temperature, dried, and re-weighed. If the weight has dropped by more than 0.1 g, lost volume is replaced using 100% MeOH.**
K	3, 13		2.3.4 Samples are neutralized with 250 µL of 2.5 M HCL and vortexed to mix.
		2.3.5 – 2.3.7 Hexane Sample Clean-up: Applicable as determined during the laboratory's method verification.	
C ~~O~~	**3, 13**		**2.3.5 Nonpolar lipids are removed by adding seven (7) mL of hexanes to the hydrolyzed sample extract and vortexing to mix. ~~(2.3.5 – 2.3.7 Preferred).~~**

K	3, 13		2.3.6 The sample extract/hexane mixture is partitioned by centrifuging for 10 minutes at 2000 x g. ~~(2.3.5 – 2.3.7 Preferred).~~
K	3, 13		2.3.7 The hexane layer is removed with a glass pipette and one (1) mL of the hydrolyzed methanolic extract is removed and filtered into an LC-MS glass autosampler vial using a 0.2 µm PTFE syringe tip filter. ~~(2.3.5 – 2.3.7 Preferred).~~
K	2, 3, 13		2.3.8 The cleaned-up extract is loaded into the autosampler immediately for analysis.
		2.4 Analysis	
C	**3, 13**		**2.4.1 Analytes are detected in standards and samples using the mass transitions in the table (negative ion mode). Note: To confirm the presence of each toxin, only two mass transitions must be observed above the LOD. The transition that yields the highest signal-to-noise ratio (S/N) is used for quantitation, the transition with the second highest S/N is used for confirmation.**
K	3, 13		2.4.2 Other system parameters such as collision energy are optimized for the specific system using standards before analysis.
C	**3, 13**		**2.4.3 A standard calibration curve of at least six (6) concentrations is performed at the beginning of each run. An additional curve is required if a run lasts longer than 24 hours.**
K	3, 13		2.4.4 Five (5) µL of extract is injected for analysis.
K	2, 12		2.4.5 Samples are stored in the sample compartment of the autosampler at ≤ 10°C during analysis. Otherwise the samples must be analyzed within 24 hours if the autosampler is held at room temperature.
K	3, 13		2.4.6 A column heater is used and the temperature is maintained at 40 °C, with a tolerance as specified by the manufacturer, during the analysis.
C	**3, 13**		**2.4.7 ~~An~~The following analytical column, or equivalent, is used for analyte separation: for UPLC, Acquity ~~UPLC~~ BEH C18 1.0 × 150 mm, 1.7 µm particle size, for HPLC or UPLC, Waters Xbridge Premier BEH C18 (VanGuard FIT) 2.1 × 100mm, 3.5 µm particle size. ~~(or equivalent) analytical column is used for analyte separation~~**
C	**3, 13**		**2.4.8 Analytes are separated on the LC column using gradient elution.**

Compound	Q1 (m/z)	Q3 (m/z)
OA	-803.5	-255.2
OA	-803.5	-113.1
OA	-803.5	-151.1
DTX2	-803.5	-255.2
DTX2	-803.5	-113.1
DTX2	-803.5	-151.1
DTX1	-817.5	-255.2
DTX1	-817.5	-113.1
DTX1	-817.5	-151.1

K	2		2.4.9 The column is stored following the manufacturer's instructions when not in use.
K	**2**		2.4.10 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.
C	**3, 13**		**2.4.11 A matrix or procedural blank (i.e. NRC CRM Zero-Mus or equivalent negative matrix, or methanol carried through sample preparation process at the same time as the samples) must~~should~~ be included in each analytical batch.**
K~~C~~	3, 13		2.4.12 Standard and/or quality control (e.g., CCV) vial septa are pierced only once.
		2.5 System Suitability	
C	**3, 13**		**2.5.1 The retention time of analytes in all samples are within 3% that of one of the intermediate toxin standards, measured from the apex of the peak.**
C	**3, 13**		**2.5.2 Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (Rs) of OA/DTX2 should ≥ 1 when calculated using the equation below (RT is retention time and W is peak width at baseline; Peak 1 is OA and Peak 2 is DTX2).** $$Rs = 2 \times (RT2 - RT1)/(W1 \mp W2)$$
K	2, 3, 13		2.5.3 Each chromatographic peak must be defined by at least 10 data points.
C	**3, 13**		**2.5.4 Reagent blanks (methanol) are analyzed after the high calibration standard, periodically (as determined by the laboratory's internal verification), and after fortified samples to ensure that analyte carryover is not occurring. Analyte carryover is defined as a confirmed peak > LOD.**
C	**3, 13**		**2.5.5 To confirm the presence of each DST, two (2) mass transitions must be observed above the limit of detection (LOD).** **The transition yielding the highest signal-to-noise ratio (S/N) is used for quantitation (i.e. 817.5 → 255.2 for DTX-1, 803.5 → 255.2 for OA and DTX-2). The transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation is ≥ 3.**
C	**3, 13**		**2.5.6 The ratio of the abundance of the confirmation ion transition to the quantitation ion transition is calculated for each toxin. These ion ratios must be within ± 20% of that of the toxin standards in order to confirm toxin identity.**
C	**3, 13**		**2.5.7 When ≥ 10 samples are analyzed, analysts must show that the calibration has not significantly drifted using an option provided below:**

			OPTION 1 – USE OF BRACKETING CALIBRATION CURVES
C	**3, 13**		**2.5.7.1 A second standard curve is analyzed at the end of the analytical sequence and the averaged peak areas are used for the linear regression.**
C	**3, 13**		**2.5.7.2 The linear regression of the averaged calibration curves must yield an $R^2 \geq 0.98$. Results are recorded and records are maintained.**
C	**3, 13**		**2.5.7.3 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The average of bracketing calibration curves yields an $R^2 < 0.98$.** **b) The difference in the slope between bracketing calibration curves exceeds 25%.** **c) The difference in retention times of the standards in the bracketing standard curves exceeds 3%.**

			OPTION 2 – USE OF A CONTINUING CALIBRATION VERIFICATION (CCV) STANDARD
C	**3, 13**		**2.5.7.4 The linear regression of the single calibration curve must yield an $R^2 \geq 0.99$.**
C	**3, 13**		**2.5.7.5 A continuing calibration verification (CCV) standard, matching on of the intermediate standards from the calibration curve, is analyzed after every 10 samples and at the end of a run.**
C	**3, 13**		**2.5.7.6 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The calibration curve yields an $R^2 < 0.99$.** **b) The retention time of a CCV sample exceeds 3% of the corresponding standard.*** **c) The peak area of a CCV sample exceeds ± 15% compared to the corresponding standard in the calibration curve.*** *** Samples immediately preceding and post the failed CCV shall be reanalyzed with a new standard curve.**
~~**C**~~	~~**3**~~		~~**2.5.8 Repeated injections of calibration or control samples at a concentration near the action level agree within ± 10% (as determined through the use of the coefficient of variation).**~~

C	3, 11, 13	2.6	**Calculation of Sample Toxicity**
			2.6.1 The toxicity of the individual toxins is calculated as follows: $\mu gOAeq/g \frac{ug}{g} toxin = \mu MC \times \frac{805.0}{1000mL} \times \frac{V}{W} \times X$ Hyd $\times X$ ReTx **where:** **µMC = the Ceoncentration of toxin in µg/ml of the extract injected, in µM, determined using the standard curve** **805.0 = molecular weight of OA (g/mol)** **V = Ttotal volume of homogenate and extraction solvent (mL)** **W = Wweight (g) of tissue homogenate extracted** **Hyd = Ddilution factor for hydrolysis (1.25)** **ReTx = Rrelative toxicity of toxin vs. Okadaic Acid** **Relative Toxicity Values**

Toxin	Molecular Weight (g/mol)	ReTx
OA	805	1
DTX1	819	1
DTX2	805	0.6

The individual toxicities for each toxin are summed to obtain the overall sample toxicity in ppm OA equivalents.

C	9		**2.6.2 Any value at or above 0.16 ppm OA equivalents (mg/kg or µg/g) of the sum of any analytes present is actionable. Shellfish Program Management is made aware of positive result. Laboratory action is_____ .**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice. 21 CFR 58.
3. Interstate Shellfish Sanitation Conference (ISSC), Proposal 17-103 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish
4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
5. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
6. Consult reference standard product literature.
7. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
8. American Public Health Association. 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.

9. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington,

D.C. and Columbia, S.C.

10. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.

11. The EFSA Journal. 2009. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. 1306, 1-23.

12. Deeds, J.R. and M.D. Celiz, Personal Communication, Addendum to proposal 19-136 DSP LC MS/MS Additional Ruggedness Testing; effect of the sample storage temperature during analysis (24 hrs), Email Received by ISSC Checklist Subcommittee 2/18/2021.

13. Interstate Shellfish Sanitation Conference (ISSC), Proposal 26-XXX Modified Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish

LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
DIARRHETIC SHELLFISH POISON (DSP) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the DSP Component

1. **Conforms Status: The DSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The DSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The DSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥4**.
   - b. or the total # of Key nonconformities is **≥6.**
   - c. or the total # of Critical, Key, or Other is **≥12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *10 – DSP LC-MS/MS Checklist*, Rev. June 2024

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Vibrio MPN with Biochemical or PCR Confirmation Checklist
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to adopt the checklist in the attached supplemental documentation.
12. Public Health Significance	There is no checklist to evaluate the Vibrio MPN followed by biochemical or PCR confirmation of isolates, even though these methods are included as approved methods in the NSSP. The proposed checklist will provide Laboratory Evaluation Officers an appropriate and standardized tool by which to evaluate laboratories implementing these methods.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835 TEL.
240- 402-4960/9258/7629, 301-796-0788
HFPSHELLFISHLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Vibrio MPN with Biochemical or PCR Confirmation

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Vibrio parahaemolyticus MPN with Biochemical Confirmation (FDA BAM Chapter 9) [PART III]
	Vibrio vulnificus MPN with Biochemical Confirmation (FDA BAM Chapter 9) [PART III]
	Vibrio parahaemolyticus MPN with PCR Confirmation (FDA BAM Chapter 9) [PART IV]
	Vibrio vulnificus MPN with PCR Confirmation (FDA BAM Chapter 9) [PART IV]

PART I – QUALITY ASSURANCE				
CODE	**REF**	**ITEM**		
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (check those items which apply).
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The laboratory follows all internal policies and procedures while implementing the documented quality system**
K	6		1.1.3	The Laboratory participates in a Vibrio proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and at least three (3) months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well-lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air is fewer than 15 colonies per plate for a 15 minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of at least 0.1 pH units.

K	9		1.4.2	The pH electrodes being used consist of a pH half-cell and reference half-cell or equivalent combination electrode free from Ag/AgCl or contains an ion exchange barrier preventing passage of Ag ions into the solution which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment *(Indicate the adjustment used).*
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.
K	6		1.4.5	The pH meter manufacturer instructions are followed for calibration, a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded.
K	4, 14		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope *(Indicate the method used).*
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are verified monthly according to manufacturer specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12**		**1.4.10**	**Refrigerator temperatures are maintained between 2 and 8 ºC.**
C	**2**		**1.4.12**	**Freezer temperatures are maintained at -15 ºC or below.**
K	6		1.4.13	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12**		**1.4.14**	**The temperature of the incubator is maintained at 35 ± 2.0 ºC, except when incubating mCPC or CC plates, which require a temperature range of 35–40 ºC.**
C	**6**		**1.4.15**	**Working thermometers used in the air incubators are graduated in at least 0.5 ºC increments.**
K	5, 8		1.4.16	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.19	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.20**	**All working thermometers are appropriately immersed.**
C	**5**		**1.4.21**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**5, 6**		**1.4.22**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records (certificates of calibration) are maintained.**

K	3		1.4.23 Standards thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**5**		**1.4.24 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (Indicate the thermometer type used.)**
K	3, 8		1.4.25 The accuracy of working thermometers is verified annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Discard working temperature-sensing devices that differ by >1 ºC from the reference/ standards device. Results are recorded and records maintained
O	8		1.4.26 Appropriate pipet aids are available and used to inoculate samples.
		1.5 Labware and Glassware Washing	
K	5		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and sample.
O	5		1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method of preparation is used to ensure the appropriate volumes of diluent.
C	**5**		**1.5.5 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1 mL aliquots; nor, are pipettes larger than 1.1 mL used to deliver 0.1 mL aliquots.**
K	5		1.5.6 In washing reusable pipets, glassware and labware, a succession of at least three (3) fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**8**		**1.5.7 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.8 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded, and records maintained.**
		1.6 Sterilization and Decontamination	
K	5		1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
K	4		1.6.2 Routine autoclave maintenance is performed, and the records are maintained.
C	**16, 17, 18**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer, or an appropriate working temperature monitoring device.**

K	2, 5, 6		1.6.4	An autoclave standards thermometer (or data logger) has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. If in-house checks for accuracy of the standards thermometer will be conducted at the steam point, calibration of the autoclave standards thermometer at 100 °C is also recommended, but not required.
K	2, 10, 15		1.6.5	The autoclave standards thermometer (or data logger) is checked every five (5) years for accuracy at either 121 °C by a qualified calibration laboratory; or, is checked in-house at the steam point (100 °C) if it has been previously calibrated at both 100 °C and 121 °C. Any change in temperature at the steam point changes the calibrated temperature at 121 °C by the same magnitude. Date of most recent determination: _____
K	2, 8		1.6.6	Working autoclave thermometers (or data loggers) are checked against the autoclave standards thermometer at 121 ºC yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape and/or sterilization chemical indicators (e.g., strips, pouches) is used with each autoclave batch.
K	6, 8		1.6.9	Autoclave sterilization records including the length of sterilization cycle, time in autoclave, and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Indicate the appropriate type or types*)
K	5, 8		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 ºC.
K	8		1.6.11	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	8		1.6.12	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records are maintained.
K	5		1.6.13	Reusable pipets are stored and sterilized in aluminum or stainless-steel containers.
K	5		1.6.14	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 ºC for two (2) hours.
C	**2**		**1.6.15**	**The sterility of reusable items is determined with each load sterilized. Results are recorded and records maintained. Sterility is determined by an appropriate microbiological method, biological indicators and/or chemical integrators. Indicators and/or integrators appropriate for cycle and conditions are used and appropriately placed throughout the autoclave and/or product to verify the sterility of autoclave sterilized consumables. Autoclave sterilized materials are considered sterile as per manufacturer IFU of autoclave packages and/or indicators/integrators used. Upon expiration, the items may be re-packaged, autoclaved, and verified. Results are recorded and records maintained.**

C	**2**		**1.6.16 The sterility of pre-sterilized consumables is determined with each lot received. Sterility is verified by an appropriate microbiological method, or a traceable certification of sterilization is obtained. Results are recorded and records maintained. Certificates are maintained in accordance with the records management plan.**
K	8		1.6.17 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media and Reagent Preparation	
C	**12**		**1.7.1 Media and reagents are prepared from the individual components and pH adjusted appropriately, except in the case of TCBS, which is commercially dehydrated.**
K	6		1.7.2 Media is prepared and sterilized according to manufacturer's instructions.
K	1, 5, 8		1.7.3 Dehydrated media, and media and reagent components are properly stored in a cool, clean, dry place.
K	1		1.7.4 Dehydrated media and media components are labeled with date of receipt, date opened or date prepared, and analyst initials.
C	**1, 2, 7**		**1.7.5 Caked or expired media or components are discarded.**
C	**6**		**1.7.6 Reagent water is distilled or deionized (*indicate appropriate choice*), tested monthly and exceeds 0.5 megohms-cm resistivity (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 ºC. (*Indicate the appropriate water quality descriptor determined*). Results are recorded and the records maintained.**
C	**6**		**1.7.7 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 mg/L). Results are recorded, and records maintained.** **Specify method of determination: _____**
K	6		1.7.8 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded, and records maintained.
K	12		1.7.9 The volume and concentration of media (APW) in the tube is suitable for the amount of sample inoculated.
C	**2**		**1.7.10 Total exposure time of autoclaved media does not exceed 60 minutes. TCBS, CC and mCPC are not autoclaved.**
C	**1**		**1.7.11 Media and diluent sterility is determined for each load sterilized. Results are recorded, and records maintained.**
C	**1**		**1.7.12 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____**

C	**6, 12**		**1.7.13**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media and Reagents		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	2		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	2		1.8.3	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	6		1.8.4	Storage under refrigeration of prepared agar plates in sealed plastic bags shall not exceed two (2) weeks.
K	6		1.8.5	Storage under refrigeration of prepared broth media or reagents with loose fitting closures shall not exceed one (1) month.
K	6		1.8.6	Storage under refrigeration of prepared broth media and diluent with screw-cap closures shall not exceed three (3) months.
K	2, 6		1.8.8	All prepared broth media and diluent stored under refrigeration are warmed to room temperature prior to use, at temperatures that do not exceed the medium's incubation temperature.
		PART II – SHELLFISH SAMPLES		
		2.1 Sample Handling and Receipt		
C	**1, 5, 12**		**2.1.1**	**A representative sample is collected.**
K	5		2.1.2	Shellfish samples are received in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	1, 5		2.1.3	Shellfish samples, as received, are labeled with the collectors' company/processor, collectors' name, type of shellfish, the source (harvest area and sampling station), the time, date, and place (if applicable) of collection or are rejected for regulatory analysis
C	**5, 12**		**2.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
K	5, 15		2.1.5	If ice is used in sample transport, samples are rejected if melt water has come in contact with the samples.
C	**15**		**2.1.6**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 hours. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 hours once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 11		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2, 11		2.2.2	Blades of shucking knives are not corroded.
K	5, 11		2.2.3	The hands of the analyst are thoroughly washed with soap and water, or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2, 11		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5, 11		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5, 11		2.2.6	Shellfish are allowed to drain in a clean container or on clean towels prior to opening.

K	2, 5, 11		2.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**5, 11**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5, 11, 12**		**2.2.9**	**The contents of the shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**12**		**2.2.10**	**A representative sample of at least 12 shellfish are used for analysis.**
C	**2, 11**		**2.2.11**	**The quantity of meat and liquor is sufficient to cover the blender blades or additional shellfish are used in order to ensure sample homogeneity.**
K	5, 12		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	12		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**12, 15**		**2.2.14**	**Samples are blended for 90-120 seconds until homogenous.**
PART III – *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN SHELLFISH BY BIOCHEMICAL CONFIRMATION				
		3.1 APW Enrichment and Colony Isolation: Preparation of Samples for Biochemical Confirmation		
C	**12**		**3.1.1**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**12, 13**		**3.1.2**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used:_____ Specify number of tubes per dilution:_____**
C	**2**		**3.1.3**	**For *V. parahaemolyticus* analysis, a *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**		**3.1.4**	**The process control cultures accompany the samples throughout incubation, isolation and confirmation. Results are recorded, and records are maintained.**
C	**12**		**3.1.5**	**Inoculated APW enrichment tubes are incubated at 35.0 ± 2.0 ºC.**
C	**12**		**3.1.6**	**Tubes are read after 18-24 hours of incubation. Clear tubes are negative. Turbid tubes are positive. Positive tubes are confirmed as *Vibrio parahaemolyticus* or *Vibrio vulnificus* as appropriate.**
K	12		3.1.7	A loopful from the top one (1) cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation.

C	**12**		**3.1.8 TCBS plates are incubated at 35 ± 2 ºC and mCPC or CC plates are incubated at 35-40 ºC for 18-24 hours.**
C	**12**		**3.1.9 Presumptive colonies are selected meeting these phenotypic characteristics:** ***V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, two (2) to three (3) mm in diameter. Interfering large, opaque and yellow colonies are avoided.** ***V. vulnificus* appear on mCPC or CC agar as round, flat, opaque, yellow colonies, one (1) to two (2) mm in diameter. Typical positives have "fried egg" appearance. Purple/blue colonies are avoided.**
K	**12**		**3.1.10 For colony confirmation by biochemical testing, presumptive colonies are streaked for purity on non-selective agar media.**
C	**12**		**3.1.11 Plates are incubated at 35 ± 2 ºC for 18-24 hours.**
		3.2 Biochemical Confirmation of Colonies	
K	12		3.2.1 Biochemical testing media contains 2-3% NaCl for identification of *V. parahaemolyticus* and a minimum of 0.5% NaCl for identification of *V. vulnificus*.
C	**12**		**3.2.2 Biochemical testing is conducted using isolated colonies for AGS, oxidase, motility, and salt tolerance, at a minimum. If a commercial biochemical diagnostic method is used, manufacturer's instructions are followed, with the exception of using 2% NaCl for cell suspensions.** **Specify method used: _____**
C	**12**		**3.2.3 Colonies are confirmed with the following biochemical reactions, or based on diagnostic test kit manufacturer's recommendations:** **a. *V. parahaemolyticus* produce an alkaline slant and acidic butt in AGS, are oxidase positive, are motile, and grow in 3%, 6%, and 8% NaCl, but do not grow in 0% or 10% NaCl.** **b. *V. vulnificus* produce an alkaline slant and acidic butt in AGS, are oxidase positive, are motile, and grow in 3% and 6% NaCl, but do not grow in 0%, 8%, or 10% NaCl.**
		3.3 Computation of Results	
C	**12**		**3.3.1 Upon identification of positive colonies refer to the original positive dilutions of APW and record MPN results. Use the Dilution Selection Tool located in FDA BAM Appendix 2 to determine which dilutions to use in the final MPN.**
K	12, 13		3.3.2 Results are reported as MPN/g of sample or pass/fail in the case of PHP samples.
PART IV – *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN SHELLFISH BY PCR			
		4.1 APW Enrichment and Colony Isolation: Preparation of Samples for PCR	
C	**12**		**4.1.1 The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.**

				For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.
C	**12, 13**		**4.1.2**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used:_____ Specify number of tubes per dilution:_____**
C	**2**		**4.1.3**	**For *V. parahaemolyticus* analysis, a *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**		**4.1.4**	**The process control cultures accompany the samples throughout incubation, isolation and confirmation. Results are recorded, and records are maintained.**
C	**12**		**4.1.5**	**Inoculated APW enrichment tubes are incubated at 35.0 ± 2.0 ºC.**
C	**12**		**4.1.6**	**Tubes are read after 18-24 hours of incubation. Clear tubes are negative. Turbid tubes are positive. Positive tubes are confirmed as *Vibrio parahaemolyticus* or *Vibrio vulnificus* as appropriate.**
K	12		4.1.7	A loopful from the top one (1) cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation.
C	**12**		**4.1.8**	**TCBS plates are incubated at 35 ± 2 ºC and mCPC or CC plates are incubated at 35-40 ºC for 18-24 hours.**
C	**12**		**4.1.9**	**Presumptive colonies are selected meeting these phenotypic characteristics:** ***V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, two (2) to three (3) mm in diameter. Interfering large, opaque and yellow colonies are avoided.** ***V. vulnificus* appear on mCPC or CC agar as round, flat, opaque, yellow colonies, one (1) to two (2) mm in diameter. Typical positives have "fried egg" appearance. Purple/blue colonies are avoided.**
K	**12**		**4.1.10**	**For colony confirmation by PCR, presumptive colonies are streaked for purity on non-selective agar media.**
C	**12**		**4.1.11**	**Plates are incubated at 35 ± 2 ºC for 18-24 hours.**
		4.2 PCR Reagents		
C	**12**		**4.2.1**	**The PCR primers are appropriate for the species targeted:** ***Vibrio parahaemolyticus*** **L-TL 5' aaa gcg gat tat gca gaa gca ctg 3'**

				R-TL 5' gct act ttc tag cat ttt ctc tgc 3' ***Vibrio vulnificus*** **Vvh-785F 5' ccg cgg tac agg ttg gcg ca 3'** **Vvh-1303R 5'cgc cac cca ctt tcg ggc c 3'**
K	19		4.2.2	Lyophilized primers and probes are stored according to manufacturer's instructions.
C	**20**		**4.2.3**	**Lyophilized primers are reconstituted and diluted to produce a 0.01 mM working solution.**
K	20		4.2.4	PCR reagents (dNTPs, buffer, MgCl2, primers) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.
		4.3 Culture Template Preparation		
C	**19**		**4.3.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**19**		**4.3.2**	**Pipet tips have aerosol barriers.**
K	19		4.3.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	19		4.3.4	Aseptic technique is observed throughout the extraction and PCR analysis.
C	**12**		**4.3.5**	**Purified isolates are inoculated into non-selective broth medium and incubated overnight at 35 ± 2 ºC.**
C	**12**		**4.3.6**	**Following incubation, a 1 mL aliquot is centrifuged at 15,000 x g for 3 min. The pellet is washed twice with physiological saline and then resuspended in 1 mL of PCR-grade water.**
K	12		4.3.7	The template is stored at –20C until use, if not analyzed within 24 h.
K	12, 19		4.3.8	Frozen extracts are analyzed within 6 months of frozen storage.
		4.4 Preparation PCR Master Mix		
C	**19**		**4.4.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**12**		**4.4.2**	**For each reaction, the specified amount of each component is added (final concentrations) per reaction:** ***Vibrio parahaemolyticus*** **PCR Buffer 1X** **MgCl2 4 mM** **dNTPs 200 µM** **L-TL primer 1 µM** **R-TL primer 1 µM** **Taq polymerase 5 U** **PCR-grade water to final volume of 49 µL** ***Vibrio vulnificus*** **PCR Buffer 1X** **MgCl2 4 mM** **dNTPs 200 µM** **Vvh-785F primer 1 µM** **Vvh-1303R primer 1 µM** **Taq polymerase 5 U** **PCR-grade water to final volume of 49 µL**
K	2		4.4.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**12**		**4.4.4**	**Forty-nine (49) µL of Master Mix is added to each PCR tube.**
K	19		4.4.5	Master Mix must be used on the day of preparation or stored at –20 °C until time of use.

		4.5 PCR		
K	12		4.5.1	If previously frozen, the culture templates are completely thawed at temperatures no warmer than room temperature.
C	12		**4.5.2**	**One (1) µL of culture template is added to each reaction tube containing 49 µL of Master Mix for a total PCR reaction volume of 50 µL.**
C	**12, 19**		**4.5.3**	**One (1) µL of culture template from the negative process control culture is added to a reaction tube containing 49 µL of Master Mix.**
C	**12, 19**		**4.5.4**	**One (1) µL of culture template from the positive process control culture is added to a reaction tube containing 49 µL of Master Mix.**
C	**12, 19**		**4.5.5**	**One (1) µL of PCR-grade water is added to a reaction tube containing 49 µL of Master Mix as a no template control. A no template control is included with each PCR run.**
C	**12, 19**		**4.5.6**	**One (1) µL of culture template from a positive control culture (prepared separately from the positive process control) is added to a reaction tube containing 49 µL of Master Mix as the positive PCR control. A positive PCR control is included with each PCR run.**
K	2		4.5.7	Immediately prior to loading the reaction tubes into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**12**		**4.5.8**	**After centrifugation, tubes are inserted into the instrument.**
		4.6 PCR Amplification and Product Visualization		
C	**12**		**4.6.1**	**The PCR cycling parameters used are appropriate for the protocol.** ***Vibrio parahaemolyticus*** **Initial denaturation 94°C, 3 min** **25 cycles:** **94°C, 1 min** **60°C, 1 min** **72°C, 2 min** **Final Extension 72°C, 3 min** **Hold at 8°C until ready for gel analysis** ***Vibrio vulnificus*** **Initial denaturation 94°C, 10 min** **25 cycles:** **94°C, 1 min** **62°C, 1 min** **72°C, 1 min** **Final Extension 72°C, 10 min** **Hold at 8°C until ready for gel analysis**
C	**12**		**4.6.2**	**A 1.5-1.8% gel is prepared (w/v) using molecular grade agarose and 1X TBE. 1 µg/ml of ethidium bromide is added.**
C	**12**		**4.6.3**	**1X TBE is used as the running buffer.**
K	12		4.6.4	Electrophoresis is done at a constant voltage of 5-10 V/cm until sufficient separation of the weight maker is achieved to determine product band size.
K	12		Bands are visualized with a UV transilluminator.	
		4.7 PCR Confirmation: Computation of Results		
C	**12**		**4.7.1**	**All runs in which the positive PCR control generates a band of appropriate size (450 bp for *Vibrio parahaemolyticus* and 519 bp for *Vibrio vulnificus*) and the no template control reaction generates no band are considered**

				valid.
C	**12**		**4.7.2**	**All reactions in a valid run which generate a band of appropriate size for the target of interest (450 bp for *Vibrio parahaemolyticus* and 519 bp for *Vibrio vulnificus*) are considered to be positive.**
C	**12**		**4.7.3**	**Upon identification of positive reactions/colonies refer to the original positive dilutions of APW and record MPN results. Use the Dilution Selection Tool located in FDA BAM Appendix 2 to determine which dilutions to use in the final MPN.**
K	12, 13		4.7.4	Results are reported as MPN/g of sample or pass/fail in the case of PHP samples.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
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4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.
5. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
6. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
7. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study*. U.S. Government Printing, Washington, D.C.
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9. Fisher, J. 1985. Measurement of pH. American Laboratory 16:54-60.
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11. American Public Health Association. 2001. *Compendium of Methods for the Microbiological Examination of Foods*. APHA, Washington, D.C..
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15. U.S. Department of Commerce. 1986. *NBS Monograph 174*, Gaithersburg, Maryland.
16. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
17. American Public Health Association (APHA). 2017. *Standard Methods for the Examination of Water and Wastewater*, 23rd Edition, APHA/AWWA/WEF. Pg. 9-8 and 9-10.
18. *Difco Manual*, 11th Edition, 1998, Division of Becton Dickinson and Company, Sparks, Maryland, Pg. 13.
19. U.S. Environmental Protection Agency (EPA). 2004. Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples. EPA 815-B-04-001. Office of Water, Cincinnati, OH.
20. Integrated DNA Technologies. Oligonucleotide Stability Study. 2014.

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
VIBRIO MPN WITH BIOCHEMICAL OR PCR CONFIRMATION COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Vibrio MPN with Biochemical or PCR Confirmation component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The Vibrio MPN with Biochemical or PCR Confirmation component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form ? – *Vibrio MPN Checklist*, July 2026

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Revised Quality Systems Checklist
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to modify the NSSP Quality Systems checklist. Specific edits are reflected in the attached supporting documentation.
12. Public Health Significance	The proposed modification is to improve efficiency in the current NSSP Quality Systems Laboratory Evaluation checklist.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

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HFPSHELLFISHLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Laboratory Quality Assurance

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Conformity is noted by a Yes, No, or not applicable (N/A) for each checklist item. Please note that for all N/A indications, you must document the reason why this requirement is N/A. Record comments related to any requirement on the space provided in the summary of nonconformities. All nonconformities must be identified and explained. Quality System must be in place for onsite laboratory evaluation to be scheduled.

Parts of the Quality Checklist	
Part I	Quality Management: ~~Laboratory Operations and Responsibilities for Quality Systems~~Requirements for the National Shellfish Sanitation Program Laboratory Quality System
Part II	Quality Assurance: The Process of Documenting and Maintaining a Quality System
Part III	Quality Control: Documentation for Quality System Defensibility

PART I – Quality Management: ~~Laboratory Operations and~~ Requirements~~sponsibilities~~ for National Shellfish Sanitation Program Laboratory Quality Systems		
ITEM		
Conformance	**References**	Quality Assurance Document Version:
~~1.1 Components of the Laboratory Quality System~~		
☐ Yes ☐ No ☐ N/A~~Yes~~ ☐ ~~No~~ ☐ ~~N/A~~	1,4,6~~2,5,8~~ ~~1,3,6,91,3,6,9~~	1.1.1~~2~~ The laboratory's organizational roles and responsibilities are clearly defined and communicated to all employees. Employee acknowledgement and understanding are documented. ~~Management and technical structure exist to support the Quality System.~~
	1.1.1 Observations:~~1.1.2 Observations:~~	
☐ Yes ☐ No ☐ N/A~~Yes~~ ☐ ~~No~~ ☐ ~~N/A~~	6~~81,~~ ~~91~~,3,6,9	1.1.2 The laboratory has documentation of designated quality personnel and/or a designated quality manager with the authority and resources required to implement and maintain the Quality System.~~1.1.3 Quality documentation is required by the laboratory. These include a Quality Assurance (QA) Manual (or otherwise named) and Standard Operating Procedures (SOPs) to support the quality assurance process of the laboratory.~~
	1.1.2 Observations:~~1.1.3 Observations:~~	

~~**1.2 Laboratory Management Structure and Quality Systems**~~		
☐ Yes ☐ No ☐ N/A	~~1,3,6,9~~2 ~~,5,8~~1,4, 6	1.1.3~~2.1~~ Management and technical structure exist to support the Quality System. ~~The laboratory's structure is clearly organized with supervisory chain delineated.~~
	1.1~~2~~.~~1~~3 Observations:	
☐ Yes ☐ No ☐ N/A	~~9~~8 6	1.1.4~~2.2~~ The laboratory has ensured that its management and personnel are free from any undue internal and external commercial, financial or ~~and~~ other pressures and influences that may adversely affect the quality of their work.
	1.1.4~~2.2~~ Observations:	

~~**1.3 Laboratory Personnel and Roles in a Quality System**~~		
☐ Yes ☐ No ☐ N/A	~~9~~8~~6~~	1.1.5~~3.2~~ The QAM ~~The laboratory has a~~ policy and ~~the~~ training procedures ~~in the QAM~~ for laboratory personnel ~~that~~ are documented and relevant to the scope of the current activities in the laboratory. The laboratory maintains a personnel file/ record of any relevant authorization(s), qualification~~s~~, training~~s~~, and/or ~~proficiencies~~proficiency for each analyst. ~~If the laboratory intends to add methods to their scope, training SOPs must also be added with successful completion by the analyst(s) that will perform the method(s). In the case of a single person laboratory, method proficiency verificationmust be retained during the life of the methods use in the laboratory.~~
	1.1.5~~3.2~~ Observations:	

PART II – Quality Assurance: The Process of Documenting and Maintaining a Quality System

~~2.1 Quality Assurance Process: QA Manual, SOPs and Document Control~~

~~2.2 Quality Manual Items~~		
☐ Yes ☐ No ☐ N/A	~~1,~~ 9~~8~~6	2.1.1~~2.1~~ Quality Assurance Manual contains: ☐ 1. Table of Contents; ☐ 2. Organizational roles and responsibilities~~chart~~; ☐ 3. A description of the Quality System and procedure for implementation and maintenance; ☐ 4. Policy and procedure for resource management (human resources, competence and training, work environment and safety), description of responsibilities; ☐ 5. Policy and procedures for rejection criteria; ☐ 6. Policy and procedures for calibration of equipment and Equipment file items such as maintenance; ☐ 7. Policy and procedure for traceability and required documentation, ☐ 8. Policy and procedure for internal audits; ☐ 9. Policy and Procedure for data review~~analysis~~ and control of nonconforming work; and ☐ 10. Policy for corrective actions (see 2.1.7~~CAs~~) and preventative actions (see 2.1.8)~~PAs~~).
	2.1~~2~~.1 Observations:	

☐ Yes ☐ No ☐ N/A	~~1,3,6,~~ ~~9~~2,5,~~8~~6	2.1.2 The QA manual includes or references all laboratory SOPs and any supporting procedures, including technical procedures and work instructions. The laboratory has documented that all personnel involved in testing have read and understand the applicable SOPs and associated quality documentation and implement the policies and procedures required for the performance of their technical function.~~2.2.2 The organizational chart clearly depicts laboratory structure with quality and technical personnel listed.~~
	2.1~~2~~.2 Observations:	
☐ Yes ☐ No ☐ N/A	~~1,~~ ~~9~~8~~6~~	2.1.3 SOPs and the QA Manual are controlled documents, such that specific individuals are designated within the laboratory with editorial control. These individuals are identified in the QA Manual. Each time an SOP or the QA manual is changed, the new version will be marked as such and will be distributed to the laboratory with older versions removed from circulation. ~~2.2.5 A policy regarding appropriate equipment file maintenance and retention (e.g., calibration records, maintenance documentation, manuals of operation) is included in the QA Manual.~~
	2.1.3~~2.5~~ Observations:	

☐ Yes ☐ No ☐ N/A	~~1,~~ ~~9~~86	2.~~2~~1.4~~7~~ The SOP for traceability of analytical results is included or referenced in the QA Manual~~l and shall be provided upon request~~. This traceability procedure includes a documented procedure for the unique identification of samples and the process for chain of custody verification.
	2.1.4~~2.7~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1,~~ ~~9~~86	2.~~2~~1.~~8~~5~~The QA Manual has a policy and a procedure for internal quality audits. These audits are planned and scheduled annually or as needed.~~ The policy for internal quality audits states auditors do not audit their own work.~~ In the case of a single person laboratory, FDA LEOs will assist with an audit plan.~~
	2.1.5~~2.8~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1,~~ ~~9~~86	2.1~~2~~.~~10~~6 The QA Manual contains a procedure for the ~~controlof~~control of nonconforming work in the case of: ☐ a. identification, documentation, evaluation, segregation (where practical), disposition of nonconforming sample/analyte/result and customer notification; ☐ b. assigning responsibility for the review and the authority for disposition of nonconforming sample/analyte/result; ☐ c. a nonconforming result correction and the re-verification/ calibration of the affected equipment after the correction to demonstrate conformity (if necessary); and ☐ d. handling a nonconforming result when it is detected, after delivery to the customer.

	2.1.6~~2.10~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1,~~ ~~9~~8 1,4, 6	2.1~~2~~.~~11~~7 The QA manual has a policy and a procedure for developing corrective action(s) to eliminate the cause of identified nonconformities to prevent recurrence. Corrective actions describe the nonconformities, define the process for evaluating the need for actions to ensure that nonconformities do not recur (root cause analysis), explain the process to implement the corrective action(s) needed, and the resultant outcome. There is also a procedure to monitor progress of any ongoing corrective actions and the resolution. ~~The QA manual contains a procedure for preventative actions in which laboratory staff identify potential nonconformities in audit results, quality records, or customer complaints through a review process. Steps are then determined to identify preventive actions to implement. The necessary changes are made to SOPs and this exercise is recorded, and records maintained.~~
	2.1.7~~2.11~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1, 3, 6,~~ ~~9~~2,5,8 1,4,6	2.1~~2~~.~~12~~8 The QA manual contains a procedure for preventive actions in which laboratory staff identify potential nonconformities in audit results, quality records, or customer complaints through a review process. Steps are then determined to identify preventive actions to implement. The necessary changes are made to SOPs and this exercise is recorded, and records maintained.~~The QA manual has a policy and a procedure for developing corrective action(s) to eliminate the cause of identified nonconformities in order to prevent recurrence. Corrective actions describe the nonconformities, define the process for evaluating the need for actions to ensure that nonconformities do not recur (root cause analysis), explain the~~

		~~process to implement the corrective action(s) needed, and the resultant outcome. There is also a procedure to monitor progress of any ongoing corrective actions and the resolution.~~
	2.1.8~~2.12~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1, 3, 4, 6,~~ ~~9~~2,3,5,8 1,2,4,6	2.1~~2~~.9~~13~~ The QA Manual contains a policy stating laboratory management shall ensure and document the competence of staff: ☐ a. I~~i~~ndependently operating equipment resulting in a documented measurement;~~,~~ ☐ b. performing analysis ~~result~~;~~,~~ ☐ c. generating ~~quality~~quality control value/result;~~t,~~ ☐ d. determination of value for sample result, and review/closure of corrective action for efficacy.
	2.1.9~~2.13~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1,~~ ~~9~~81,2,4,6	2.1.10~~2.14~~ The laboratory's sample acceptance procedures include the following: ☐ a. safe handling, transport, and storage to prevent contamination or deterioration and protect sample integrity; ☐ b. handling nonconforming samples and who will be contacted for sample rejection; ☐ c. the policy for sample rejection criteria includes what the laboratory will accept and reject based on NSSP requirements and chain of custody; and ☐ d. these procedures are provided to ~~The~~customers. ~~policy for sample rejection criteria includes what the laboratory will accept and reject based on NSSP requirements and chain of custody.~~

		2.2.104 Observations:
PART III- Quality Control: Documentation for Quality System Defensibility		
☐ Yes ☐ No ☐ N/A	1, 986,7	3.1.1 The laboratory investigates proficiency testing (PT) programs for areas of continual improvement and actively addresses problematic results through the prescribed corrective action process. Suggested change: The laboratory participates in proficiency testing (PT) programs and actively addresses problematic results through the prescribed corrective action processes. If no PT exists, the laboratory evaluates laboratory personnel proficiency by performing sample analysis inter- or intra- laboratory comparisons.
		3.1.1 Observations:
☐ Yes ☐ No ☐ N/A	1, 3, 6, 9, 102,5, 8,91,4, 6,7	3.1.2 Corrections to quality control records, bench sheets and reports follow the requirements below: ☐ a. A single line is drawn through the incorrect information; ☐ b. The correct information is written next to the incorrect information; ☐ c. The person responsible for the correction initialed the information; ☐ d. If not obvious, the reason for correction has been included; and; ☐ e. If corrections are necessary in an electronic document, old information must be retained in some form, the person making the change must be identified, the date of the change noted, and the reason for the change noted.

		3.1.2~~3~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1, 3, 6, 9, 10~~2,5,8~~,9~~1,4,6,7	3.1.3~~4~~ All records and documents must be written in indelible ink. All ~~records,~~records are required to be retained for two years (or length of time as dictated by State law), are legible and stored in such a way that they are readily retrievable to prevent damage or loss.	
		3.1.3~~4~~ Observations:	
☐ Yes ☐ No ☐ N/A	~~1, 3, 6, 9~~2,5,8~~1~~,4,6	3.1.4~~2.1~~ The laboratory will internally verify newly adopted methods following NSSP Method Verification guidance ~~to~~. ~~confirm with objective evidence that the intended protocols are demonstrated and outcomes are fulfilled.~~ Methodologies do not deviate from the ~~verified~~alidated method and the laboratory's internal verification remains on file in the laboratory.	
		3.1~~2~~.~~1~~4 Observations:	

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Rescind the SmartCycler Instrument from the MPN Real-Time PCR Methods for *Vibrio parahaemolyticus* and *Vibrio vulnificus*
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to rescind the Cepheid SmartCycler instrument from the Approved NSSP MPN Real-Time PCR Methods for *Vibrio parahaemolyticus* and *Vibrio vulnificus*. The proposal removes the Cepheid SmartCycler from the method name and also removes SmartCycler specific dyes in line 3.1.3. See supplemental documentation for specific edits.
12. Public Health Significance	There are no laboratories using this instrument in support of the Program. The manufacturer has discontinued the instrument and service to the instrument is no longer supported. In addition, consumables required to run the instrument are no longer produced. There are alternative methods in the Program for the detection and confirmation of Vibrios. Additionally, the ISSC Constitution, Bylaws, and Procedures states in Procedure XV, Section xi. that a method is subject to recantation when the equipment is no longer available. As such, there should be no impact to the Program and the protection of public health.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

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CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] ~~SmartCycler II and~~ AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] ~~SmartCycler II and~~ AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* detection in Oysters [PART IV] QuantStudio DX

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15-minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11**	**Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ 0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22	All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.

K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3**	**The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14	Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.

C	**2**		**1.6.16**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18**	**The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19	Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation		
K	13, 14		1.7.1	Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2	Media components are properly stored in a cool dry place.
O	6		1.7.3	Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**6**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**6**		**1.7.6**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (<0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8	The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9**	**Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4	Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5	Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6	All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II – SAMPLES AND PROCESSING				
		2.1 Sample Collection, Transportation and Receipt		
C	**2, 6**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**

K	5		2.1.2	Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3	Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 6		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2	Blades of shucking knives are not corroded.
K	5		2.2.3	The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6	Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7	Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9**	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10**	**A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11**	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14**	**Samples are blended for 60 to 120 seconds until homogenous.**
		2.3 APW Enrichment		
K	5		2.3.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**2.3.2**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**2.3.3**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution_____**

C	**2, 15, 23**		**2.3.4 For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **An uninoculated APW blank will serve as the uninoculated process control.** **The process control cultures accompany the samples throughout the method. Records are maintained.**
C	**13**		**2.3.5 Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**2.3.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
PART III- PCR METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN OYSTERS AB 7500 FAST			
		3.1 PCR Reagents	
C	**14, 15**		**3.1.1 Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.1.2 Fluorescent probes are stored in light occluding tubes or containers.
C	**14, 15, 18, 19**		**3.1.3 The PCR forward and reverse primers and probes:** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED -5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE -5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.1.4 Lyophilized forward and reverse primers, and probes, are reconstituted with TE buffer to produce a 0.1 mM stock solution.**
C	**14, 18**		**3.1.5 Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.1.6 Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**

C	**21, 22**		**3.1.7**	**Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.1.8**	**PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.2 DNA Extraction		
C	**14, 18**		**3.2.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.2.2**	**Pipet tips have aerosol barriers.**
K	14, 18		3.2.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.2.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.2.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.2.6**	**One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.2.7**	**Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.2.8	A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.2.9**	**After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**
K	14, 18		3.2.10	Frozen extracts are analyzed within 6 months of frozen storage.
		3.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**3.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.3.2**	**For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.3.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.3.4**	**Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.3.5**	**Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.4 PCR		
C	**14, 19**		**3.4.1**	**If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.4.2**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.4.3	Two (2) µL of APW blank extracted from the uninoculated process control is added to a reaction tube or plate well containing 23µL of Master Mix.
K	14, 19		3.4.4	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
K	14, 19		3.4.5	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
C	**14, 19**		**3.4.6**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix as a no template control. A no template control is included with each PCR run.**

C	**14, 19**		**3.4.7**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control. A positive PCR control is included with each PCR run.**
K	14, 19		3.4.8	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.4.9**	**After centrifugation, tubes or plates are inserted into the instrument.**
		3.5 PCR Amplification		
C	**14, 19**		**3.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		3.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.5.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.6 Computation of Results		
K	14, 19		3.6.1	All runs in which the positive PCR control generates a Ct value for the target(s) of interest and the no template control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**3.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**3.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control is considered invalid and should be re-tested.**
C	**13**		**3.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from either the calculator or MPN table in conjunction with dilution selection tool in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.6.8	For APW enrichment, results are reported as MPN/g of sample.

PART IV- PCR METHOD FOR *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* DETECTION IN OYSTERS QUANTSTUDIO DX			
		4.1 PCR Reagents	
C	**14, 15**	**4.1.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15	4.1.2	Fluorescent probes are stored in light occluding tubes or containers.
C	**26**	**4.1.3**	**The PCR forward and reverse primers, and probes target.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3' Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3' Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3' WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3' WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3' Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3' Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3' Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3' Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3' TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3' TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3' Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ **<u>For Vv Real-time PCR Method</u>** vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3' vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3' vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGBNFQ
C	**14, 18**	**4.1.4**	**Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**26**	**4.1.5**	**Storage of thawed working stocks of primers and probes are stored between 2-8°C, not to exceed 2 weeks.**
C	**26**	**4.1.6**	**Storage of aliquoted working stocks of primers and probes stored in -20°C manual defrost freezer does not exceed 1 year.**
C	**26**	**4.1.7**	**Taqman Environmental Mastermix 2.0 is stored in -20°C manual defrost freezer until first use. After first use, it is stored between 2-8°C.**
C	**26**	**4.1.8**	**Internal control (IC) is stored in -20°C manual defrost freezer until first use. After first use, they are stored between 2-8°C.**
		4.2 DNA Extraction	
C	**14, 18**	**4.2.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**	**4.2.2**	**Pipet tips have aerosol barriers.**
K	14, 18	4.2.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18	4.2.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**	**4.2.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**

C	**26**		**4.2.6**	**Two-hundred (200) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**26**		**4.2.7**	**For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.**
K	26		4.2.8	Extracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen extracts are analyzed within 1 month of frozen storage.
C	**26**		**4.2.9**	**A tlh+ trh+ tdh+ *V. parahaemolyticus* (WA4647 or equivalent), a tlh+ tdh+ Orf8+ *V. parahaemolyticus* (BAA-240 or equivalent), and vvha+ *V. vulnificus* (ATCC 27562 or equivalent) cultures are extracted and combined to serve as the positive PCR (amplification) control.**
		4.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**4.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**26**		**4.3.2**	**For each reaction, add the specified amount of water, appropriate primer and probe concentrations, and Taqman Environmental Master Mix.**
K	14, 16, 18		4.3.3	The Master Mix is vortexed to mix constituents and then briefly spun immediately prior to dispensing aliquots to reaction tubes or plates.
C	**14, 16, 18, 26**		**4.3.4**	**Eighteen (18) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**4.3.5**	**Master Mix must be used on the day of preparation or stored at –20°C until time of use.**
		4.4 PCR		
C	**14, 19, 26**		**4.4.1**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 18µL of Master Mix for a total PCR reaction volume of 20µL.**
K	14, 19, 26		4.4.2	Two (2) µL of extracted blank APW from the uninoculated process control is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.3	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.4	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
C	**14, 19, 26**		**4.4.5**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as a no template control.**
C	**14, 19, 26**		**4.4.6**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as the positive PCR (amplification) control.**
K	14, 19, 26		4.4.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 30 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**16**		**4.4.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		4.5 PCR Amplification		
C	**14, 19**		**4.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		4.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**4.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		4.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**4.5.5**	**The analysis settings are adjusted as specified in the protocol.**

		4.6 Computation of Results		
K	14, 19		4.6.1	All runs in which the NTC has no amplification; positive control generates a Ct value for the target(s) of interest; and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	2		**4.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**4.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**4.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		4.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**4.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control (except NTC) is considered invalid and should be re-tested.**
C	**13**		**4.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		4.6.8	For APW enrichment, results are reported as MPN/g of sample.

REFERENCES

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PCR MICROBIOLOGY COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the PCR Microbiology Component:

1. **Does Not Conform Status**: The PCR Microbiology component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥4 or**
   b. The total # of Key nonconformities is **≥13 or**
   c. The total # of Critical, Key and Other is **≥18**
2. **Provisionally Conforms Status**: The PCR Microbiology component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *6 – PCR Microbiology Checklist*, Rev. December 2025

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Rescind the Alkaline Phosphatase Vibrio Probe Method
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists Section IV. Chapter III. @.02 Methods .01 Approved Laboratory Tests
11. Text of Proposal/ Requested Action	Section IV. Chapter II @.02 Methods .01 Approved Laboratory Tests

	Vibrio Type:	**Application: PHP Sample Type: Shucked**	**Application: Reopening**
MPN[1]	***Vibrio vulnificus (V.v.)***	X	
MPN[2]	***Vibrio parahaemolyticus (V.p.)***	X	
~~**PCR[3]**~~	~~***Vibrio parahaemolyticus (V.p.)***~~	~~X~~	
MPN-Real Time PCR[43]	***tdh+ and trh+ Vibrio parahaemolyticus (V.p.)***	X	X
MPN-Real Time PCR[54]	***Vibrio parahaemolyticus (V.p.)***	X	X
~~**Direct Plating Method[6]**~~	~~***Vibrio parahaemolyticus (V.p.)***~~	~~-~~	~~X~~
MPN-Real Time PCR[75]	***Vibrio vulnificus (V.v.)***	X	

Footnotes:
1. MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual (BAM), 7th Edition, May 2004 revision, followed by confirmation using biochemical

	analyses or PCR method from the FDA BAM as described in Hill et al., 1991. ~~or by the DNA–alkaline phosphatase gene probe for vvhA as described by Wright et al., or a method that a State can demonstrate is equivalent.~~ 2. MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7 th Edition, May 2004 revision, followed by confirmation using biochemical analyses or PCR method from the FDA BAM as described in Bej et al., 1999. ~~or the DNA-alkaline phosphatase gene probe for tlh as described by McCarthy et al., or a method that a State can demonstrate is equivalent.~~ ~~3. MPN method in Chapter 9 of the FDA Bacteriological Analytical Manual, 7 th Edition, May 2004 revision, and as described in the "Direct Plating Procedure for the Enumeration of Total and Pathogenic Vibrio parahaemolyticus in Oyster Meats" developed by FDA, Gulf Coast Seafood Laboratoryfollowed by confirmation using conventional PCR, or a method that a State can demonstrate is equivalent.~~ ~~4~~3. MPN-Real Time PCR Method for the tdh and trh Genes for Total V. parahaemolyticus as described in Kinsey et al., 2015. ISSC 2015 Summary of Actions Proposal 15-111, Page 397. ~~5~~4. MPN-Real Time PCR Method for the tlh gene for total V. parahaemolyticus as described in Kinsey et al., 2015. ISSC 2015 Summary of Actions Proposal 15-113, Page 418 ~~6. Direct Plating Procedure in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, and as described in the 'Direct Plating Procedure for the Enumeration of Total and Pathogenic Vibrio parahaemolyticus in Oyster Meats' developed by FDA, Gulf Coast Seafood Laboratory.~~ ~~7~~5. MPN-Real Time PCR Method for the vvh gene for total V. vulnificus as described in Kinsey et al., 2015. The Alkaline Phosphatase Probe Laboratory Evaluation Checklist will also be rescinded in its entirety.
12. Public Health Significance	The critical reagent required for the test method is not produced and is no longer supported by any manufacturer, indicating a lack of ability to perform the method. There are multiple alternative methods in the Program for the detection and confirmation of Vibrios, including BAM and Real-Time PCR Methods. Additionally, the ISSC Constitution, Bylaws, and Procedures states in Procedure XV, Section xi. that a method is subject to recantation when reagents are no longer available. As such, there should be no impact to the Program and the protection of public health and the table indicating Approved Methods for Vibrio Enumeration will reflect the available choices of analyses.
13. Cost Information	No direct cost to rescind the method. However, laboratories required to switch from the probe method due to its lack of availability may incur some additional costs, depending on the method implemented.
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist Modifications - pH Meter Calibration
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to modify checklist line items in multiple NSSP Laboratory Evaluation Checklists related to calibration of laboratory pH meters. The proposed edits are applicable to the following NSSP Laboratory Evaluation Checklists: 1. Microbiology, line item 1.4.5 2. PCR Microbiology, line item 1.4.5 3. Mouse Bioassay and Scotia Rapid Test for Paralytic Shellfish Poisoning (PSP), line item 1.4.6 4. Probe Microbiology, line item 1.4.5 5. Marbionc Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA, line item 1.4.5 6. Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV, line item 1.4.4 7. Paralytic Shellfish Poisoning (PSP PCOX-HPLC), line item 1.4.6 8. Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP), line item 1.4.6 Replacement Text for pH Meter Calibration Requirements: The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. The specific checklists and text are in the accompanying document.
12. Public Health Significance	The proposed replacement language standardizes the language regarding calibration of pH meters for all mentioned checklists. This change reflects current laboratory equipment QC practices for pH meters.
13. Cost Information	None

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
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TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**
C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Multiple Tube Fermentation Technique for Seawater (APHA) [PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	8, 11		1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s):
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory science.
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4	pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	11		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. ~~The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the electrode isopotential point (pH 7) and the second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.~~
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.)*
K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are verified monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**9, 36**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**

K	9		1.4.22 Standards thermometers are verified annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____

C	**9**		**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 32		1.4.24 The accuracy of working thermometers are verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. Results are recorded and records maintained.
O	11		1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing	
O	9		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3 Sample containers are made of glass or some other inert material.
O	9		1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.
K	9		1.5.5 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7 Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8 In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10 With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
K	9		1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2 Routine autoclave maintenance is performed and the records are maintained.
C	**29, 32, 33**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**

K	11		1.6.4	An autoclave standards thermometer has been calibrated using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5	The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination _____
K	1		1.6.6	Working autoclave thermometers are verified against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	32		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.*)
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures ≥160°C.
K	9		1.6.11	A thermometer capable of determining temperatures ≥160°C accurately is used to monitor the operation of the hot-air sterilizing oven.
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11		1.6.13	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14	Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15**	**The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16**	**The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17	Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18	Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
C	**2**		**1.6.19**	**The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20**	**The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21	Hardwood applicator transfer sticks or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization _____

C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks/presterilized loops is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of A-1 medium which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared and sterilized according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.)* Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non- detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination _____**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	9		1.7.9 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10 Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.11 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**40**		**1.7.12 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____**

O	9		1.7.13	Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14	The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II – SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium. (*Circle appropriate one.*)**
C	**9, 34**		**2.2.2**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**

C	**9**		**2.2.3**	**In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.6	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
K	9		2.2.8	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
K	9, 11		2.3.3	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. (*Circle the method of transfer.*)
C	**9**		**2.3.4**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium without salicin. Study records are maintained and are available upon request.**

C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**9, 34**		**2.5.4**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.5**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
C	**2**		**2.5.8**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation*. Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
C	**2,5**		**2.5.9**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.11**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition.
K	7		2.6.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5	Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**

C	**2**		**2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and the record is maintained.**
K	2, 11		2.7.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10 The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11 Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12 Forceps tips are clean.
O	11		2.7.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20 Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar	
K	11		2.8.1 Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2 The phosphate buffered saline is properly sterilized.**
K	23		2.8.3 A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4 Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar	
C	**24**		**2.9.1 mTEC agar is used.**
C	**23, 34**		**2.9.2 The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.3 The membrane is placed grid side up within the sterile filter apparatus.**

C	**23, 25**		**2.9.4 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.5 Sample volumes are filtered under vacuum.**
K	26		2.9.6 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.7 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.8 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.9 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**		**2.9.10 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.11 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ± 0.5°C for two (2) hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.12 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ± 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**

C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
K	9, 11, 21, 35		2.12.8 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.9 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 35**		**2.13.1 The sample is shaken vigorously (25 times in a 12 ”arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 35**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 35**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 35**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 35**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control**
C	**9, 11, 35**		**2.13.10 Inoculated plates are incubated inverted at 35 ± 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 35**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**

PART III – SHELLFISH SAMPLES				
		3.1 Collection and Transportation of Samples		
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination		
K	2,32		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**, 32		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**, 32		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	2, 9		3.2.10	At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9		3.2.11	A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
C	**9**		**3.2.13**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.14	APHA *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA		
C	**9**		**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. (*Circle the medium used.*)**
K	9		3.3.2	Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.

C	**9**		**3.3.3**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.5	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.6**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____Negative Process control _____**
K	9		3.3.7	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
K	9, 11		3.4.2	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. (*Circle the method of transfer.*)
C	**9**		**3.4.3**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	15 to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**

C	**9**		**3.6.5 An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6 Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7 Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8 Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9 Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10 Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11 A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC	
K	9		3.7.1 Colony counts determined in accordance with Pa rt III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
C	**19**		**3.7.2 Colony counts are reported as CFU/grams of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP	
C	**2,3**		**3.8.1 Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2 Double strength modified MacConkey agar is used.
C	**3**		**3.8.3 Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4 Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5 Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6 The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7 The sample homogenate is cultured within two (2) minutes of blending.**
C	**2,3**		**3.8.8 Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9 Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10 The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**3, 13**		**3.8.12 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.13 Plates are stacked no more than three (3) high in the incubator.**

C	**2**		**3.8.14 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive Process Control: _____Negative Process Control:_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
Bacteriological Examination of Shellfish Meats for Male Specific Coliphage (MSC)			
		3.10 MSC Equipment and Supplies	
K	2		3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100mL.
K	2		3.10.2 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		3.10.3 The sterility of each batch/ lot of pre-sterilized or reusable syringes, filters and/or filter units is determined. Results are recorded and records maintained.
C	**2**		**3.10.4 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.5 The temperature of the incubator used is maintained at 36 ± 1°C.**
K	2		3.10.6 The temperature of the freezer is maintained at ≤- 15°C.
C	**1**		**3.10.7 The sterility of disposable 50 mL centrifuge tubes is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28, 31		3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28, 31		3.11.2 Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28, 31		3.11.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	2		3.11.4 Unsterilized soft agar is stored at ≤-15°C for up to three (3) months.
K	27, 28, 31		3.11.5 The soft agar is sterilized for 15 minutes at 121°C before use.
K	28, 31		3.11.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.
K	2, 27, 28, 31		3.11.7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		3.12 Preparation of Host Culture for MSC Analysis	
C	**28, 31**		**3.12.1 *E. coli* Famp ATCC 700891 is the bacterial host strain.**
K	27, 28, 31		3.12.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.

K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28, 31**		**3.12.4 After inoculation, the host cell growth broth culture is not shaken.**
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.13.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.14 MSC Sample Analysis	
C	**28, 31**		**3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.**
C	**27, 28, 31**		**3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.**
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	**27, 28, 31**		**3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.**
C	**27, 28, 31**		**3.14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28, 31**		**3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28, 31**		**3.14.11 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.**
K	27, 28, 31		3.14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive Control: _____
K	27, 28, 31		3.14.13 Room temperature growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.14.15 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28, 31		3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28, 31**		**3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.15 Computation of Results - MSC	
C	**27**		**3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28, 31		3.15.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	2		3.15.4 The MSC count is rounded off conventionally to give a whole number.
		PART IV – MSC WASTEWATER ANALYSIS	
		4.1 Collection and Transportation of Samples	
C	**11**		**4.1.1 Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**39**		**4.1.2 Effluent sample bottles must contain 0.2 mL of 10% sodium thiosulfate solution for every 200 mL of sample to inactivate any residual chlorine.**
K	1		4.1.3 Samples are identified with collector's name, sampling location, time and date of collection.
C	**9**		**4.1.4 Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**

O	1		4.1.5	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**4.1.6**	**Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies		
K	38		4.2.1	Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.
K	9		4.2.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		4.2.3	The sterility of each batch/lot of pre-sterilized or reusable syringes, filters, and/or filter units is determined. Results are recorded and records maintained.
C	**27, 28**		**4.2.4**	**The balance used provides a sensitivity of at least 0.01g.**
C	**27, 28**		**4.2.5**	**The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6	The temperature of the freezer is maintained at ≤ -15°C.
C	**28**		**4.2.7**	**The sterility of the transfer vessel utilized is determined with each lot. Results are recorded and records maintained.**
		4.3 MSC Media Preparation		
K	28		4.3.1	Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2.	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28		4.3.3	Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4	Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
K	27, 28		4.3.5	The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.
K	27, 28		4.3.6	Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.
K	27, 28		4.3.7	Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis		
C	**28**		**4.4.1**	***E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2	Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3	Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**4.4.4**	**After inoculation, the host cell growth broth culture is not shaken.**

		4.5 MSC Sample Analysis		
C	**38**		**4.5.1**	**Wastewater sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10-2) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.**
K	27, 28		4.5.2	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.5.3	Two hundred microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample.
C	**27, 28**		**4.5.4**	**2.5 mL of sample is added to a tube of tempered soft agar.**
C	**27, 28**		**4.5.5**	**The tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.5.6**	**The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			**4.5.7**	**Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.**
K	27, 28		4.5.8	Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28		4.5.9	Room Temperature Growth broth is used as the negative control or blank.
K	27, 28		4.5.10	Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		4.5.11	A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28		4. 5.12	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**4.5.13**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.6 Computation of Results - MSC		
C	**38**		**4.6.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted. The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**38**		**4.6.2**	**When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
K	28		4.6.3	The formula used for determining the density of MSC in PFU/100 mL is: High Range: N * 1000 = PFU/100mL; Low Range: N * 5 = PFU/100mL; Where N is the total number of plaques counted on all plates.
O	9		4.6.4	The MSC count is rounded off conventionally to give a whole number.

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SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is ≥ **4 or**

   b. The total # of Key nonconformities is ≥ **13 or**

   c. The total # of Critical, Key and Other is ≥ **18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is ≥ **1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form *LAB-100 Microbiology,* Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K – Key O – Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15 minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e. pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. ~~A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.~~
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11**	**Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 ± 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5°C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22	All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		

K	5		1.5.1	Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3**	**The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14	Reusable pipets are stored and sterilized in aluminum or stainless steel containers.

K	5		1.6.15	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.
C	**2**		**1.6.16**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18**	**The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19	Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation		
K	13, 14		1.7.1	Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2	Media components are properly stored in a cool dry place.
O	6		1.7.3	Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**6**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**6**		**1.7.6**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8	The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9**	**Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4	Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5	Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6	All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II – SAMPLES				
		2.1 Sample Collection, Transportation and Receipt		

C	**2, 6**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5		2.1.2	Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3	Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 6		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2	Blades of shucking knives are not corroded.
K	5		2.2.3	The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6	Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7	Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9**	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10**	**A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11**	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14**	**Samples are blended for 60 to 120 seconds until homogenous.**
PART III- PCR METHOD FOR VIBRIO VULNIFICUS AND VIBRIO PARAHAEMOLYTICUS DETECTION IN OYSTERS				
		3.1 APW Enrichment		
K	5		3.1.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**3.1.2**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**3.1.3**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution _____**

C	**2, 15**		**3.1.4**	**For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **The process control cultures accompany the samples throughout incubation, isolation, and confirmation. Records are maintained.**
C	**13**		**3.1.5**	**Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**3.1.6**	**Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
		3.2 PCR Reagents		
C	**14, 15**		**3.2.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.2.2	Fluorescent probes are stored in light occluding tubes or containers.
C	**14, 15, 18, 19**		**3.2.3**	**The PCR forward and reverse primers and probes are appropriate for the platform.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE/TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.2.4**	**Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**14, 18**		**3.2.5**	**Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.2.6**	**Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**
C	**21, 22**		**3.2.7**	**Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.2.8**	**PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.3 DNA Extraction		
C	**14, 18**		**3.3.1**	**All microcentrifuge tubes and pipet tips are sterile.**

C	**14, 18**		**3.3.2**	**Pipet tips have aerosol barriers.**
K	14, 18		3.3.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.3.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.3.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.3.6**	**One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.3.7**	**Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.3.8	A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.3.9**	**After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**
K	14, 18		3.3.10	Frozen extracts are analyzed within 6 months of frozen storage.
		3.4 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**3.4.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.4.2**	**For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.4.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.4.4**	**Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.4.5**	**Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.5 PCR		
C	**14, 19**		**3.5.1**	**If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.5.2**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.5.3	Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix for each batch of Master Mix prepared as a no template control.
C	**14, 19**		**3.5.4**	**Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.**
C	**14, 19**		**3.5.5**	**Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix**.
O	14, 19		3.5.6	Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control.
K	14, 19		3.5.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**16**		**3.5.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		3.6 PCR Amplification		
C	**14, 19**		**3.6.1**	**The appropriate instrument platform is used for the protocol.**
K	16		3.6.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.6.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.6.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.

C	**14, 19**		**3.6.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.7 Computation of Results		
K	14, 19		3.7.1	All runs in which the positive control generates a Ct value for the target(s) of interest and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.7.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**3.7.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**3.7.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.7.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.7.6**	**Any reaction in which no Ct value is generated for the target(s) of interest or the internal control is considered invalid and should be re-tested.**
C	**13**		**3.7.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.7.8	For APW enrichment, results are reported as MPN/g of sample.

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PCR MICROBIOLOGY COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-III	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the PCR Microbiology Component:

1. **Does Not Conform Status**: The PCR Microbiological component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥4 or**
   b. The total # of Key nonconformities is **≥13 or**
   c. The total # of Critical, Key and Other is **≥18**
2. **Provisionally Conforms Status**: The PCR microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *6 – PCR Microbiology Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Mouse Bioassay and Scotia Rapid Test for Paralytic Shellfish Poisoning (PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Mouse Bioassay for Paralytic Shellfish Poisoning (MBA PSP)
	Scotia Rapid Test for Paralytic Shellfish Poisoning (SRT PSP)

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	5, 6, 8		1.1.1 Written Plan adequately covers allof the following: (check √ thoseitems which apply)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operatingprocedures (SOPs).
			d. Internal quality control measures for equipment, calibration, maintenance, repair, performanceand rejection criteria established.
			e. Laboratorysafety.
			f. Internal performance assessment.
			g. Externalperformanceassessment.
			h. Animal care.
C	**6**		**1.1.2 The QAplan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/countylaboratories, the analyst(s) meet the state/county educationaland experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisormust have at least a bachelor's degree or equivalent in microbiology, biology, chemistry or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
		1.3 Work Area	
O	5, 6		1.3.1 Adequate for the workload and storage.
O	5		1.3.2 Cleanand welllighted.
O	5		1.3.3 Adequate temperature control.
O	5		1.3.4 All work surfaces arenonporous and easily cleaned.
C	**8**		**1.3.5 Aseparate, quiet area with adequate temperature controlformice acclimation and injection is maintained.**
		1.4 Laboratory Equipment	
O	2		1.4.1 The pH meter has a standard accuracyof 0.1 pH units.
K	9		1.4.2 pH paper in the appropriate range (i.e. 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	7		1.4.3 pH electrodes consist of pHhalf-celland reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).
K	6		1.4.4 pH meter is calibrated daily when in use. Results arerecorded and records are maintained.

K	5		1.4.5	Effect of temperature has been compensated for by an ATC probe; use of a triode or by manual adjustment.
K	5		1.4.6	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. ~~A minimum of two standard buffer solutions is used to calibratethe pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standardbuffer solutions are used once and discarded.~~
K	6, 12		1.4.7	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determinationof slope. (Circle method used).
K	2		1.4.8	The balances beingused provide an appropriate sensitivity at the weights of use. a. To preparereference solution, the balancemust have a sensitivity of at least 0.1 g at a load of 1 g. b. For sample extraction, the balance must have a sensitivity of at least 0.1 g at a load of 100 g. c. For gravimetric extract volume adjustment, the balance must have a sensitivity of at least 0.1 g at a load of 200 g. d. To weigh mice for assay, the balance must have a sensitivity of at least 0.1 g at a load of 20 g.
K	4,5		1.4.9	The balance calibration is checkedmonthly according to the manufacturer's specifications using NIST Class S, ASTM Class 1or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10	Refrigerator temperatureis maintained between 0 and 4˚C.
K	5		1.4.11	Refrigerator temperature is monitored at least oncedaily on workdays. Results are recorded and records are maintained.
K	4		1.4.12	Freezer temperature is maintained within manufacturer's tolerance.
K	5		1.4.13	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records are maintained.
C	**10**		**1.4.14**	**All in-service thermometers are properly calibrated andimmersed. Results are recorded and records are maintained.**
O	6		1.4.15	All glassware is clean.
C	**5**		**1.4.16**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records are maintained.**
C	**9**		**1.4.17**	**An alkaline or acid based detergent is used for washing glassware/labware.**
		1.5 Reagents and Reference Solution Preparation and Storage		
C	**9**		**1.5.1**	**Any residual (unused) STX diHCl standard solution is never stored after the ampule has been opened.**
K	15		1.5.2	PSP reference solution (1 µg/mL) is prepared gravimetrically and diluted with 0.001 M HCl solution.

K	9		1.5.3	Prepared PSP referencesolution is stored under refrigeration in a sealed non-reactive container. Solution may be stored indefinitely as long as there is no detectable evaporation loss as determined by weight. If evaporation is detected, the solution is discarded appropriately. Records are maintained.
C	**14**		**1.5.4**	**All working dilutions from the PSP referencesolutionare prepared gravimetrically using 0.001 M HCl.**

K	9		1.5.5	All workingdilutions prepared from the PSP reference solution are discarded appropriately after use.
C	**5**		**1.5.6**	**Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm – cm resistance(2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 °C. (Circle the appropriate water quality descriptor determined). Results are recorded and records are maintained.**
K	5		1.5.7	Reagentwater is analyzed for residual chlorine monthly and is at a non-detectable level(≤ 0.1 mg/L). Results are recorded and records are maintained. Specify method of determination_____.
K	5		1.5.8	Reagentwater contains < 100 CFU/mL as determinedmonthly using the heterotrophic platecount method. Results are recorded and records are maintained.
		1.6 Collection and Transportation of Samples		
O	2		1.6.1	Shellstockare collected in clean, waterproof, punctureresistantcontainers, loosely sealed.
K	2		1.6.2	Shellstocksamples are labeled with collector's name, type of shellstock, the sourceor harvest area, samplingstation, time, dateand place (if applicable) of collection.
C	**2**		**1.6.3**	**Immediately aftercollection, shellstock samples areplaced in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °Cwith ice or cold packs for transport to the laboratory.**
K	15, 9		1.6.4	Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**14**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – ANALYSIS OF SHELLFISH FOR PSP TOXINS – MOUSE BIOASSAY (MBA)				
		2.1 Preparation of Samples for Analysis – Homogenization		
C	**15, 9**		**2.1.1**	**At least 12 animals (or moreto provide 100 g of shellfish meat) are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	2		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	2		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	2		2.1.4	The inside surfaces of the shells andmeats are rinsed with fresh water to remove sand or other foreign material.
O	2		2.1.5	Shellfish meats areremoved from the shellby separating the adductor muscles and tissue connectingat the hinge.

C	**2**		**2.1.6 Damage to the body of the molluskis minimized in the process of opening.**
O	2		2.1.7 Shucked shellfish are drained on a #10 meshsieve or equivalent without layeringfor 5 minutes.
K	2		2.1.8 Pieces of shell and drainage are discarded.
C	**2**		**2.1. 9 Drained meats or previouslycooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60 - 120 seconds).**
		2.2 Preparation of Samples for Analysis – APHA/AOAC Digestion & Extraction	
K	15, 9		2.2.1 Sample homogenates are extractedas soon as possible (preferably the same day) or stored in the freezer.
K	2		2.2.2 100 grams of homogenized sample is weighed into a beaker.
K	2		2.2.3 The sample homogenateis extracted in a 1:1 weight/volume ratio by adding 0.1 M HCl or 0.18 M HCl (*circle the appropriate choice*).
K	2		2.2.4 Homogenate/acidmixtureis stirred thoroughly before boiling to completely mix the contents.
C	**2**		**2.2.5 To prevent toxintransformation, the pH of the homogenate/acid mixturebefore boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
C	**2**		**2.2.6 The homogenate/acid mixture is promptly brought to its boiling point, then gently boiled at 100 ± 1 ºC for 5 minutes.**
O	9		2.2.7 The homogenate/acid mixture is boiled under adequate ventilation (e.g. fume hood).
O	9		2.2.8 The homogenate/acid mixture is allowed to cool to room temperature.
C	**2**		**2.2.9 The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirringthe mixture.**
K	2		2.2.10 The homogenate/acidmixture is adjusted gravimetrically to thepre-boilingweight using 0.001 M HC1.
K	2		2.2.11 The homogenate/acidmixture is allowed to separate bygravity or by centrifugation (e.g. centrifugedat 3,000 RPMfor 5 minutes).
K	9		2.2.12 If theextracted sample cannot be assayed immediately, then the supernatant is decanted and stored in a sealed container under refrigeration for up to 24 hours or frozen for longer storage.
K	9		2.2.13 Refrigerated extracts areallowedto reachambient temperature before being bioassayed or tested by the SRT for PSP.
		2.3 Mouse Bioassay (MBA) for PSP	
K	2		2.3.1 A 26-gauge hypodermic needle is used for intraperitoneal injections.
C	**2**		**2.3.2 Healthy mice in the weight range of 17.0 -23.0 grams (19 - 21 grams is preferable) from a stock colony are used for routineassays. Previously injected mice are never re-used for a bioassay.** **Stock strain:_____ Source:_____**
C	**9**		**2.3.3 Mice are allowed to acclimate at least 24 hours prior to injection. In some cases, 48 hours may be required.**

C	**9**		**2.3.4**	**A conversion factor (CF) for the lab has been appropriately determined.** **Lab CF:_____Date CF established:_____**
C	**2**		**2.3.5**	**The CF value is checkedweeklyif assays are done onone or several days during the week or once each day that assays are performed if they are performed less than once per week.** **Date of current CF check:_____CF verified: yes/no (*circle choice*)**
C	**2**		**2.3.6**	**If the lab CF is not verified during a check, the lab follows the appropriate procedure for establishing a temporary CF to use for the day/week.**
C	**2, 9**		**2.3.7**	**If the lab CF fails to be verified, the cause is investigated and the situation is corrected. If the cause cannot be determined with reasonable certainty and the lab CF fails to be verified > three times in a year, the lab CF is recalculated through a restandardization procedure.**
K	9		2.3.8	Mice are weighed to the nearest 0.1 g.
C	**2**		**2.3.9**	**Mice are injected intraperitoneallywith 1 mL of extracted sample.**
K	2		2.3.10	For CF checks, five mice are injected.
K	9		2.3.11	For routine assays, three mice (two when both survive) are injected per sample.
C	**2**		**2.3.12**	**Elapsed time post-injection is accurately determinedand recorded.**
C	**2**		**2.3.13**	**When death occurs, the time of death to the nearest second is noted at the last gasping breath and recorded.**
C	**9, 2**		**2.3.14**	**Mice arecontinually observed for up to 20 minutes after injection, then periodically observed for a total time of up to 60 minutes after injection.**
C	**2**		**2.3.15**	**If the median corrected mouse unit is greater than 1.92 (5 minutes), then the sample is diluted with0.001 M HCl as appropriate to achieve a median corrected mouse unit, MCMU of 1.39-1.92 (a death time of 5-7 minutes).**
		2.4 Calculation of toxicity for MBA		
C	**2**		**2.4.1**	**The death time for each mouseis converted to mouseunits (MU) using Sommer's Tableand recorded. Any mice survivingbeyond 60 minutes are recorded as < 0.875 MU.**
C	**2**		**2.4.2**	**The weight for each mouse is corrected to mouse units using the table of weights in Recommended Procedures (Table 7) and interpolated for weights not listed.**
C	**2**		**2.4.3**	**The Corrected Mouse Unit (CMU) for each mouse injected is calculated as follows:** **Deathtime in MU x Weight correction in MU = CMU**
C	**2**		**2.4.4**	**The Median Corrected Mouse Unit (MCMU) for each sample is calculated and used in the final toxicity calculation for that sample.**

C	**2**		**2.4.5**	**The toxicity of each sample is calculated as follows:** **µg STX eq/100 g of sample= MCMU x CF x DF x 200 except when less than 100 grams of sample is used for analysis.** **In this case an adjustment for sample weight must be made such that the formula for calculating sample toxicity becomes:** **µg STX eq/100 grams of sample = MCMU x CF x DF x 200/Adjusted weight of the acidified sample x 200.** **Where:** **MCMU=Median Corrected Mouse Unit for the sample** **CF=Laboratory Conversion Factor** **DF=Dilution Factor (e.g. 1:1 dilution, DF=2)**
C	**11**		**2.4.6**	**Any value equal to or greater than 80 µg STX eq/l00 g of sample is actionable.**
PART III – ANALYSIS FOR PSP TOXINS – SCOTIA RAPID TEST (SRT)				
		3.1 Screening by Scotia Rapid Test (SRT)		
K	9		3.1.1	Before beginning any screening, the following items are recorded for the SRT kit in use. a. Date received. b. Batch/lot numbers for all kit components (test strip and PSP AOAC buffer). c. Expiration dates for all kit components. d. Date opened and/or used.
K	13		3.1.2	When placed into service, all kit components are within the accepted expiration dates.
C	**13**		**3.1.3**	**The desiccant pouch inside the test strip wrapping is blue in color, indicating suitability for use. Any test strip wrapping containing a pink desiccant pouch is discarded.**
K	13		3.1.4	All kit components are stored according to the manufacturer's recommendations.
C	**9**		**3.1.5**	**A positive control of 80 µg STX eq/100 g of sample is used to test new kit lots and buffers. Results are recorded and records maintained.**
C	**9**		**3.1.6**	**Micropipettes with appropriate ranges for the volumes being measured are used.**
K	9		3.1.7	All micropipettes are maintained and calibrated according to manufacturer's instructions. Results are recorded and records maintained.
C	**13**		**3.1.8**	**400 µL of buffer solution is accurately transferred to a small tube.**
C	**13**		**3.1.9**	**100 µL of sample extract is accurately added to the buffer.**
K	13		3.1.10	The buffer/sample mixture is carefully mixed by inserting the tip of the micropipette into the mixture and pipetting up and down at least three times.
C	**13**		**3.1.11**	**100 µL of the thoroughly mixed solution is added to the test strip sample well.**
K	9		3.1.12	Micropipette tips are not reused.
K	13		3.1.13	Inoculated test strips are allowed to react with the sample mixture for the period of time recommended by the manufacturer.

C	**13**		**3.1.14**	**The test strip result is interpreted according to the instruction card provided by the manufacturer, whichis specific to each batch/lot of test strips. Results are recorded and records are maintained.**
K	13		3.1.15	If a test result is interpreted as invalid; the pH of thesample extract is checked and adjusted as needed to fall between pH 2.0 – 4.0. Fresh PSP AOAC buffer is used to re-test the sample on a newtest strip.
C	**13**		**3.1.16**	**If the same sample is interpreted as invalid on two different test strips, then the sample is assumed to contain interferingsubstances, and an alternative test method is used.**
C	**11**		**3.1.17**	**Any positive result on a SRT is actionable.**

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4. Consult freezer product literature.
5. APHA/WEF/AWWA. 1992. *Standard Methodsfor the Examinationof Waterand Wastewater*, 18th Edition. APHA, Washington, D.C.
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10. U.S. Departmentof Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
11. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2013. *NSSP Guide to the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
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13. Consult SRT manufacturer instruction manual/ literature
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LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PARALYTIC SHELLFISH POISON COMPONENT: PARTS I, II, III	
A. Results Total# of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total# of Critical, Key and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the PSP, MBA and/or SRT Component

1. **Conforms Status:** The PSP, MBA and/or SRT componentof this Laboratory is in conformity with NSSP requirements if all of the following apply.

   a. No Critical nonconformities.
   b. and <6 Key nonconformities.
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The PSP, MBA and/or SRT component of this Laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.

   a. the number of Critical nonconformities is ≥ 1 but < 4,
   b. and <6 Key nonconformities.
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status:** The PSP, MBA and/or SRT component of this Laboratory is not in conformity with NSSP requirements when any of the following apply.

   a. The total# of Critical nonconformities is ≥4.
   b. or total # of Key nonconformities is ≥ 6.
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____Date:_____

LEOSignature: _____Date:_____

NSSP Form *2 – Mouse Bioassay and Scotia Rapid Test for PSP Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Alkaline Phosphatase Probe

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating [PART III]
	Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation [PART III]
	Alkaline Phosphatase Probe Hybridization [PART III] - **Note: Temperature ranges for hybridization can be expanded with an appropriate study as described in the method.**

PART I – QUALITY ASSURANCE				
CODE	**REF**			**ITEM**
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (check those items which apply).
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a Vibrio proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well-lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of at least 0.1 pH units.
K	9		1.4.2	The pH electrodes being used consist of a pH half-cell and reference half-cell or equivalent combination electrode free from Ag/AgCl or contains an ion exchange barrier preventing passage of Ag ions into the solution which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment.
K	4		1.4.4	The pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	6		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. ~~A minimum of two (2) standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.~~
K	4, 17		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope *(Circle the method used).*
K	5, 15		1.4.7	The balances used provide a sensitivity of at least 0.01 g at the weights of use for direct plating and 0.1 g for MPN.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance calibrations is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12, 15**		**1.4.10**	**Refrigerator temperatures in which AP-probes are stored are maintained between 2 and 8 ºC.**
K	1		1.4.11	The temperature of general purpose refrigerators, those not containing AP-probes, are maintained between 0 and 4 ºC.
C	**2**		**1.4.12**	**Freezer temperatures are maintained at -15 ºC or below.**
K	6		1.4.13	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12**		**1.4.14**	**The temperature of the incubator is maintained at 35 ± 2.0 ºC.**
C	**6**		**1.4.15**	**Working thermometers used in the air incubators are graduated in at least 0.5 ºC increments.**
K	5, 8		1.4.16	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
C	**6**		**1.4.17**	**Temperature of the water bath is maintained appropriately under all loading conditions.**
C	**5**		**1.4.18**	**Working thermometers used in the water bath are graduated in at least 0.1 ºC increments.**
K	4, 6		1.4.19	Air incubator/water bath temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.20**	**All working thermometers are appropriately immersed.**
C	**5**		**1.4.21**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**5, 6**		**1.4.22**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35, 42, 54 and/or 55 ºC (54 ºC for *Vibrio parahaemolyticus* and 55 ºC for *Vibrio vulnificus*). These calibration records (certificates of calibration) are maintained.**

K	3		1.4.23 Standards thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____

C	**5**		**1.4.24 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with at least an accuracy of ±0.05 ºC are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.25 The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	8		1.4.26 Appropriate pipet aids are available and used to inoculate samples.
K	7		1.4.27 Micropipettors are calibrated annually and checked for accuracy quarterly at volumes of use. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing	
K	5		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and sample.
O	5		1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method of preparation is used to ensure the appropriate volumes of diluent.
C	**5**		**1.5.5 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1 mL aliquots; nor, are pipettes larger than 1.1 mL used to deliver 0.1 mL aliquots.**
K	5		1.5.6 In washing reusable pipets, glassware and labware, a succession of at least three (3) fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**8**		**1.5.7 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.8 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded, and records maintained.**
		1.6 Sterilization and Decontamination	
K	5		1.6.1 The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2 Routine autoclave maintenance is performed, and the records are maintained.

C	**19, 20, 21**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer, or an appropriate working temperature monitoring device.**

K	2, 5, 6		1.6.4 An autoclave standards thermometer (or data logger) has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. If in-house checks for accuracy of the standards thermometer will be conducted at the steam point, calibration of the autoclave standards thermometer at 100 °C is also recommended, but not required.
K	2, 10, 18		1.6.5 The autoclave standards thermometer (or data logger) is checked every five (5) years for accuracy at either 121 °C by a qualified calibration laboratory; or, is checked in-house at the steam point (100 °C) if it has been previously calibrated at both 100 °C and 121 °C. Any change in temperature at the steam point changes the calibrated temperature at 121 °C by the same magnitude. Date of most recent determination: _____
K	2, 8		1.6.6 Working autoclave thermometers (or data loggers) are checked against the autoclave standards thermometer at 121 ºC yearly. Date of last check:_____Method:_____
K	6		1.6.7 Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded, and the records maintained.
O	6		1.6.8 Heat sensitive tape is used with each autoclave batch.
K	6, 8		1.6.9 Autoclave sterilization records including the length of sterilization cycle, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle the appropriate type or types*)
K	5, 8		1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 ºC.
K	8		1.6.11 Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	8		1.6.12 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded, and records maintained.
K	5		1.6.13 Reusable pipets are stored and sterilized in aluminum or stainless-steel containers.
K	5		1.6.14 Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 ºC for two (2) hours.
C	**2**		**1.6.15 The sterility of reusable pipets is determined with each load sterilized. Results are recorded, and records maintained.**

C	**2**		**1.6.16 The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded, and records maintained.**
C	**2**		**1.6.17 The sterility of pre-sterilized disposable pipettes, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded, and records maintained.**
K	8		1.6.18 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.

		1.7 Media and Reagent Preparation	
C	**12, 15**		**1.7.1 Media and reagents are prepared from the individual components and pH adjusted appropriately, except in the case of TCBS, which is commercially dehydrated.**
K	1, 5, 8		1.7.2 Dehydrated media, and media and reagent components are properly stored in a cool, clean, dry place.
K	1		1.7.3 Media and components are labeled with the analyst's initials, date of receipt, date opened or date of preparation, if applicable (dye solutions).
C	**1, 2, 7**		**1.7.4 Caked or expired media or components are discarded.**
C	**6**		**1.7.5 Reagent water is distilled or deionized (*circle appropriate choice*), tested monthly and exceeds 0.5 megohms-cm resistivity (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 ºC. (*Circle the appropriate water quality descriptor determined*). Results are recorded and the records maintained.**
C	**6**		**1.7.6 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 mg/L). Results are recorded, and records maintained.** **Specify method of determination: _____**
K	6		1.7.7 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded, and records maintained.
K	12		1.7.8 The volume and concentration of media (APW) in the tube is suitable for the amount of sample inoculated.
C	**2**		**1.7.9 The total time of exposure of the sugar containing agar VVA to autoclave temperatures does not exceed 45 minutes. Total exposure time of APW and T1N3 agar does not exceed 60 minutes. TCBS, CC and mCPC are not autoclaved.**
C	**1**		**1.7.10 Media and diluent sterility is determined for each load sterilized. Results are recorded, and records maintained.**
C	**1**		**1.7.11 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Positive *Vibrio parahaemolyticus* productivity control_____** **Negative *Vibrio parahaemolyticus* productivity control_____** **Positive *Vibrio vulnificus* productivity control_____** **Negative *Vibrio vulnificus* productivity control_____**

C	**6, 12**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded, and records are maintained.**
		1.8 Storage of Prepared Culture Media and Reagents		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	2		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	2		1.8.3	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	6		1.8.4	Storage under refrigeration of prepared agar plates in sealed plastic bags shall not exceed two (2) weeks.
K	6		1.8.5	Storage under refrigeration of prepared broth media with loose fitting closures shall not exceed one (1) month.
K	6		1.8.6	Storage under refrigeration of prepared broth media and diluent with screw-cap closures shall not exceed three (3) months.
K	12, 15		1.8.7	Refrigerated prepared plates are dried inverted before use to permit the sample to be completely absorbed into the medium to prevent colony spreading, for direct plating.
K	2, 6		1.8.8	All prepared broth media and diluent stored under refrigeration are warmed to room temperature prior to use, at temperatures that do not exceed the medium's incubation temperature.
K	15		1.8.9	Storage at room temperature of Lysis Solution, Ammonium Acetate Buffer, 20XSSC, 1XSSC/SDS, and 3XSSC/SDS for the hybridization procedure shall not exceed three (3) months.
K	15		1.8.10	Storage under refrigeration of Hybridization Buffer for the hybridization procedure shall not exceed one (1) week.
C	**15**		**1.8.11**	**NBT/BCIP solution and 1XSSC for the hybridization procedure should be made fresh the day of use.**
		PART II – SHELLFISH SAMPLES		
		2.1 Sample Handling and Receipt		
C	**1, 5, 12, 15**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5, 15		2.1.2	Shellfish samples are received in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	1, 5		2.1.3	Samples are received labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5, 12, 15**		**2.1.4**	**Immediately after collection, samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 ºC with ice or cold packs for transport to the laboratory or rejected. Direct contact of the shellfish with ice in the transport container should be avoided. Once received, the samples are placed under refrigeration unless processed immediately.**
K	5, 15		2.1.5	If ice is used in sample transport, samples are rejected if melt water has come in contact with the samples.

C	**15**		**2.1.6**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 hours. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 hours once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 11		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2, 11		2.2.2	Blades of shucking knives are not corroded.
K	5, 11		2.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 11		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5, 11		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5, 11		2.2.6	Shellfish are allowed to drain in a clean container or on clean towels prior to opening.
K	2, 5, 11		2.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol, or gloves are donned. The gloves, if worn, are latex, nitrile and/or stainless-steel mesh to protect analyst's hands from injury.
C	**5, 11**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5, 11, 12, 15**		**2.2.9**	**The contents of the shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**12, 15**		**2.2.10**	**A representative sample of 10 to 14 shellfish is used for analysis.**
C	**2, 11**		**2.2.11**	**The quantity of meat and liquor is sufficient to cover the blender blades or additional shellfish are used in order to ensure sample homogeneity.**
K	5, 12, 13, 15		2.2.12	Either a 1:1 dilution is made, or the sample is homogenized without dilution. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	12, 14, 15		2.2.13	Sterile phosphate buffered saline (pH 7.4) or alkaline peptone water (APW) is used as the sample diluent. If APW is used, sample analysis is conducted immediately.
C	**12, 15**		**2.2.14**	**Samples are blended for 90-120 seconds until homogenous.**
PART III – ALKALINE PHOSPHATASE PROBE METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN SHELLFISH				
		3.1 Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating		
C	**2, 12, 15**		**3.1.1**	**For oyster samples, two tenths (0.20) of a gram of the initial 1:1 diluted homogenate (or 0.10 g of undiluted homogenate) and/or appropriate dilutions are used as inoculum. Dilutions are made in sterile PBS or APW. If APW is used, time from initial dilution until plating does not exceed 30 minutes.** **For samples other than oysters, 100 µl of the 1:10 dilution and/or subsequent dilutions should be used as inoculum.**

K	12, 15		3.1.2	For analysis of total *V. parahaemolyticus*, at least one (1) T1N3 plate is inoculated to be probed for the *tlh* gene. For pathogenic *V. parahaemolyticus*, at least two (2) T1N3 plates are inoculated to be probed for the *tdh* gene. For analysis of *V. vulnificus*, at least one (1) VVA plate is inoculated to be probed for the *vvhA* gene.
K	12, 15		3.1.3	Sterile cell spreaders are used to spread each inoculum evenly onto the dry T1N3 and/or VVA agar plates.

C	**2**		**3.1.4**	**For *V. parahaemolyticus* analysis, a *tdh*+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**		**3.1.5**	**The process control cultures accompany the samples throughout incubation and hybridization and color development phases of the method. Results are recorded, and records are maintained.**
C	**12, 15**		**3.1.6**	**Inoculated plates are incubated 16-24 hours at 35 ± 2 ºC. All plates are used for colony lifts and hybridization, except for those with confluent growth.**
		3.2 Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation		
K	11, 12		3.2.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**12**		**3.2.2**	**The 1:10 dilution is prepared gravimetrically with sterile PBS. All successive dilutions are prepared volumetrically.**
C	**12, 16**		**3.2.3**	**Appropriate sample dilutions are inoculated into sterile APW.** **Specify dilution(s) used:_____** **Specify number of tubes per dilution:_____**
C	**2**		**3.2.4**	**For *V. parahaemolyticus* analysis, a tdh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**		**3.2.5**	**The process control cultures accompany the samples throughout incubation, isolation and confirmation. Results are recorded, and records are maintained.**
C	**12**		**3.2.6**	**Inoculated APW enrichment tubes are incubated at 35 ± 2.0 ºC.**
C	**12**		**3.2.7**	**Tubes are read after 18-24 hours of incubation. Clear tubes are negative. Turbid tubes are positive. Positive tubes are confirmed as *Vibrio parahaemolyticus* or *Vibrio vulnificus* as appropriate.**
K	12		3.2.8	A loopful from the top one (1) cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation.
C	**12**		**3.2.9**	**TCBS plates are incubated at 35 ± 2 ºC and mCPC or CC plates are incubated at 35-40 ºC for 18-24 hours.**

C	**12**		**3.2.10**	**Presumptive colonies are selected meeting these phenotypic characteristics:** ***V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, two (2) to three (3) mm in diameter. Interfering large, opaque and yellow colonies are avoided.** ***V. vulnificus* appear on mCPC or CC agar as round, flat, opaque, yellow colonies, one (1) to two (2) mm in diameter. Typical positives have "fried egg" appearance. Purple/blue colonies are avoided.**
C	**12**		**3.2.11**	**A sterile 96-well microtiter plate is filled with 100 µl/well of APW. Presumptive vibrios are picked from a selective agar plate using a sterile toothpick or wood transfer stick to individual wells. The plate is incubated 3-5 hours or overnight at 35 ± 2 °C. A 48-prong replicator is used to replicate/transfer isolates in the wells to an agar plate (T1N3 for *V. parahaemolyticus* and VVA for *V. vulnificus*).**
C	**12**		**3.2.12**	**Plates are incubated at 35 ± 2 ºC for 18-24 hours.**
		3.3 Alkaline Phosphatase Probe Hybridization: Filter Preparation		
C	**12, 15**		**3.3.1**	**VVA/T1N3 plates are overlaid with labeled (sample number, dilution) #541 Whatman filters for one (1) to 30 minutes.**
K	12, 15		3.3.2	Filters are transferred with colony side up to a plastic or glass Petri dish lid containing one (1) ml of lysis solution to wet the filter.
C	**12, 15**		**3.3.3**	**Filters are microwaved to dryness, but not brown. Microwave for 15-30 seconds/filter, depending on the wattage of the microwave. Additional heating cycles may be required.**
K	12, 15		3.3.4	Filters are neutralized for five (5) minutes in an appropriate vessel or container with ammonium acetate (4 ml/filter) on a shaker at room temperature.
C	**12, 15**		**3.3.5**	**#541 Whatman filters are rinsed two (2) times in 1X SSC buffer (10 ml/filter) for 1-2 minutes. Filters may be air dried and stored at this point.**
C	**12, 15**		**3.3.6**	**Up to 30 filters are incubated in proteinase K solution (10 ml/filter) for 30 minutes at 42 °C with shaking (~50 rpm).**
K	12, 15		3.3.7	Filters are rinsed three (3) times in 1X SSC (10 ml/filter) for 10 minutes at room temperature with shaking at 50-125 rpm. Filters may be air dried and stored at this point.
		3.4 Alkaline Phosphatase Probe Hybridization: Hybridization		
C	**12, 15**		**3.4.1**	**For total *V. parahaemolyticus* (*tlh*), the 5'AP-labeled probe 5'aa agc gga tta tgc aga agc act g 3' is used.** **For pathogenic *V. parahaemolyticus* (*tdh*), the 5'AP-labeled probe 5'gg ttc tat tcc aag taa aat gta ttt g 3' is used.** **For *V. vulnificus* (*vvhA*), the 5'AP-labelled probe 5'ga gct gtc acg gca gtt gga acc a 3' is used.**
C	**12, 15**		**3.4.2**	**Probes are stored in the refrigerator and are not frozen.**
K	12, 15		3.4.3	A maximum of five (5) filters to be hybridized with the same probe are added to a plastic bag.

C	**12, 15**		**3.4.4**	**Filters are presoaked in 10-15 ml of hybridization buffer for 30 minutes at 54-± 0.1 ºC for *V. parahaemolyticus* (*tlh* and *tdh*) or 55 ± 0.1 °C for *V. vulnificus* with shaking.**
C	**12, 15**		**3.4.5**	**Used buffer is discarded and 10 ml of fresh pre-warmed buffer per bag is added. Probe (final concentration of 0.5 pmol/ml) is quickly added to each bag and incubated for 1 hour at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**
K	15		3.4.6	Filters are removed from the bag(s) and transferred to an appropriate vessel or container. Up to 30 filters hybridized with the same probe can be combined.
C	**12, 15**		**3.4.7**	**Filters are rinsed two (2) times for 10 minutes each in 1X SSC – 1% SDS (for tlh and *Vibrio vulnificus*) or 3X SSC – 1% SDS (for tdh) (10 ml/filter) at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**
K	12, 15		3.4.8	Filters are rinsed five (5) times for five (5) minutes each in 1X SSC (10 ml/filter) at room temperature with shaking.
		3.5 Alkaline Phosphatase Probe Hybridization: Color development		
C	**12, 15**		**3.5.1**	**In a petri dish containing 20 ml of NBT/BCIP solution, filters (5 or fewer) are added and incubated with gentle shaking at room temperature, or at 35 ºC for faster results. The petri dish is kept covered to omit light.**
K	12, 15		3.5.2	Color development of the positive control is checked every 30 minutes. Reaction time varies.
K	12, 15		3.5.3	Filters are rinsed in tap or deionized/distilled water (10 ml/filter) three (3) times for 10 minutes each to stop color development.
C	**12, 15**		**3.5.4**	**Reactions of test sample colonies are compared to the positive and negative process control cultures. Positive reactions appear as purple or brown spots, yellow spots are considered negative reactions. Filters are stored in the dark.**
		3.6 Alkaline Phosphatase Probe Hybridization: Computation of Results		
C	**12, 15**		**3.6.1**	**For direct plating, probe-positive colonies are counted and multiplied by the plated dilution factor of the sample to determine the concentration. Note that filter colonies must correspond to colonies visible on the agar plate.**
K	15		3.6.2	For direct plating, results are reported as CFU/g of sample.
C	**12**		**3.6.3**	**For APW enrichment, upon identification of probe-positive colonies refer to the original positive APW dilutions and record MPN value as derived in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	12, 16		3.6.4	For APW enrichments, results are reported as MPN/g of sample or pass/fail in the case of PHP samples.

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LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
ALKALINE PHOSPHATASE PROBE MICROBIOLOGICAL COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-III	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Alkaline Phosphatase Probe Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The Alkaline Phosphatase Probe Microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform Provisionally Conforms Conforms

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form *9 – Alkaline Phosphatase Probe Checklist*, Rev June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
MARBIONC Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C – Critical K - Key O - Other N/A - Not Applicable

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	3, 6		1.1.1	Written Plan adequately covers all the following: (check √ those that apply)
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**3**		**1.1.2**	**QA Plan is implemented.**
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	3, 6		1.3.1	Adequate for workload and storage.
O	6		1.3.2	Clean and well lighted.
O	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment		
O	4		1.4.1	The pH meter has a standard accuracy of 0.1 unit.
K	4		1.4.2	pH paper in the appropriate range (i.e., 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	3		1.4.3	The pH meter is ca libra ted daily when in use. Results are recorded, and records are maintained.
K	6		1.4.4	Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	6		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. ~~The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions (pH 7 and 10) is used to calibrate the pH meter. Standard buffer solutions are used once and discarded.~~
K	3, 7		1.4.6	Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.

K	2, 4		1.4.7	The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	6		1.4.8	The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded, and records are maintained.

K	1		1.4.9	Refrigerator temperature is maintained between 0 and 4 ºC.
K	6		1.4.10	Refrigerator temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
K	11		1.4.11	Freezer temperature is maintained at -10 ºC or below.
K	6		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**9**		**1.4.13**	**All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.14	All glassware is clean.
C	**11**		**1.4.15**	**Absorbance Microplate reader equipped with filter for measurement at 450 nm is used.**
O	2		1.4.16	Absorbance Microplate reader performance is evaluated at least annually using manufacturer instructions or a check standard microplate at the appropriate wavelength (450) to assess alignment, accuracy, reproducibility, and linearity. Method used:_____
K	2		1.4.17	Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded, and records are maintained.
O	11		1.4.18	A centrifuge capable of holding 15 mL or 50 mL polypropylene tubes is used.
		1.5 Reagents and Reference Solution Preparation and Storage		
C	**11**		**1.5.1**	**All solvents and reagents used are ACS grade materials or better.**
O	6		1.5.2	Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded, and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	6		1.5.3	Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**11**		**1.5.4**	**Brevetoxin-3 (BTX-3 or PbTx-3) provided with the MARBIONC ELISA kit is used as the reference standard.**
C	**11**		**1.5.5**	**Stock standard solution is made by diluting brevetoxin-3 reference standard to 1 µg/ml in 100% methanol in a volumetric flask.**
C	**11**		**1.5.6**	**Working standard solution (100 ng/ml) is made by diluting 1 ml of stock solution to 10 ml in a volumetric flask using 100% methanol.**
K	11		1.5.7	Extraction solvent (80% methanol) is made by adding 800 ml of methanol to a 1 L graduated cylinder and bringing the total volume to 1 L with water.
K	11		1.5.8	Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are used within 1 week of preparation. pH of prepared media is determined to ensure it is consistent with manufacturers requirements. Results are recorded, and records are maintained.
K	11		1.5.9	Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are stored in refrigerator for no longer than 1 week and brought to room temperature before use.

K	11		1.5.10 Gelatin stock solution is prepared by dissolving 5 g gelatin in 100 ml water and stirring the solution over gentle heat on a stir plate until clear. Gela tin stock solution is aliquoted into smaller volumes (e.g. 15 ml centrifuge tubes) and refrigerated.
K	11		1.5.11 Blocking buffer is prepared by dissolving 1 pouch in 200 ml water. Blocking buffer solution is aliquoted into 50-ml centrifuge tubes and refrigerated.
K	11		1.5.12 PGT (PBS, 0.05% Tween, 0.5% gelatin) is made fresh daily by measuring 5 ml liquified gelatin stock solution into a 50-ml centrifuge tube and filling to 50 ml with PBS-Tween.

C	**11**		**1.5.13 Stock and working standard solutions are stored -10 ºC or below.**
C	**5**		**1.5.14 All standards used are within expiration date (or 1 year from open date if not provided).**
		1.6 Collection and Transportation of Samples	
O	4, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	4, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**4, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	2, 10		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
		PART II – ASSAY OF SHELLFISH FOR NSP TOXINS	
		2.1 Preparation of Sample	
C	**4**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	4		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	4		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	4		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	4		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**4**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	4		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.

K	4		2.1.8	Pieces of shell and drainage are discarded.
C	**2, 4**		**2.1.9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction		
K	4		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**11**		**2.2.2**	**One (1) gram of homogenized sample is weighed into a 15 ml or 50 ml polypropylene centrifuge tube and subsequently extracted.**
C	**11**		**2.2.3**	**The sample homogenate is extracted by adding 9 ml extraction solvent (80% aqueous methanol) and vortexing at highest speed for 2 minutes.**
C	**11**		**2.2.4**	**The homogenate/methanol mixture is centrifuged at a minimum of 3000*xg* for 10 minutes.**
C	**11**		**2.2.5**	**The supernatant is transferred to a clean, labeled graduated 15-ml centrifuge tube and brought to a final volume of 10 ml with extraction solvent.**
K	11		2.2.6	Crude extracts are sealed tightly in glass vials and stored at -10 ºC or below until analyzed.
		2.3 Analysis		
C	**11**		**2.3.1**	**Only high-binding flat-bottom plates no older than 1 year are used (e.g. Nunc Maxisorp Immunoplates).**
C	**11**		**2.3.2**	**When pipetting kit reagents that are pre-diluted in glycerol (A, C, and D):** **a. the pipet tip is not pre-rinsed,** **b. only the very tip of the pipet tip is inserted into the vial to withdraw the required amount,** **c. the tip is submerged into the buffer when dispensing and rinsed several times with buffer to ensure complete transfer**
K	2		2.3.3	The working standard solution and crude sample extracts are brought to room temperature and thoroughly mixed before use. TMB is brought to room temperature in the dark prior to Assay Step 7.
C	**11**		**2.3.4**	**Assay Step 1: Reagent A is diluted by 300 (or as specified in kit instructions) in PBS, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS (no Tween for this wash step).**
C	**11**		**2.3.5**	**Assay Step 2: Each well is filled with 250 µl of blocking buffer. The plate is incubated on a plate shaker for 30 minutes. After 30 minutes, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**
C	**11**		**2.3.6**	**Assay Step 3a: Crude sample extracts are diluted with PGT for analysis. The minimum dilution for shellfish extracts is 1:40 (25 µl + 975 µl PGT) (resulting in a sample dilution of 1:400). Serial dilutions (n=7) of each crude sample extract are prepared in PGT.**
C	**11**		**2.3.7**	**Assay Step 3b: A standard calibration curve of seven concentrations (0.078-5.0 ng PbTx-3/ml) is prepared in PGT and is included on each plate.**
C	**11**		**2.3.8**	**Assay Step 4: 100 µl of each sample or standard dilution is loaded on to the microplate as well as two reference wells (containing PGT only) adjacent to each set of sample dilutions. Each dilution of standard or sample is added to duplicate wells. Plate layout identifying locations of samples and standards on the plate is documented.**

C	**11**		**2.3.9 Assay Step 5: Reagent C is diluted by 300 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate (which contains samples or standards), and the plate is incubated on a plate shaker for 90 minutes. After 90 minutes, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**
C	**11**		**2.3.10 Assay Step 6: Reagent D is diluted by 800 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, all wells are rinsed 3 times with 300 µl PBS-Tween, and one final time with 300 µl PBS only to ensure no Tween remains on the plate.**
C	**11**		**2.3.11 Assay Step 7: Each well is filled with 100 µl of room temperature TMB (3,3'5,5'-Tetramethylbenzidine) and incubated until a blue color develops in the reference wells. The reaction is stopped by adding 100 µl of 0.5M sulfuric acid solution to each well, and the absorbance in the wells at 450 nm is measured in a microplate reader.**
K	11		2.3.12 Plates are covered with microplate sealing film during all incubation steps (Steps 1-6 above).
C	**11**		**2.3.13 Plates are protected from light by covering with aluminum foil during color development (Step 7 above).**
K	11		2.3.14 The timing of the final step is reassessed with each new lot of kit reagents and each new lot of TMB to ensure that reference well absorbance values fall into an appropriate range.
		2.4 Quality Control	
C	**11**		**2.4.1 Acceptance of assay (plate) results is dependent on meeting the following criteria:** **a. Absorbance of standard reference wells (Amax) must be ≥ 0.6.** **b. CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (30-70% inhibition) must be < 20%.**
C	**11**		**2.4.2 Acceptance of individual sample results is dependent on meeting the following criteria:** **a. CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (30-70% inhibition) must be < 20%.** **b. CV of calculated concentrations of different sample dilutions within the linear range of the assay (30-70% inhibition) must be < 20%.**
		2.5 Calculation of Sample Toxin Concentration	
C	**11**		**2.5.1 Absorbance values are converted to % color inhibition:** **a. % inhibition = [1-(Avg of duplicate A/Amax)] x 100%where Amax is the average absorbance of the reference wells oriented below the sample or standard dilutions**
C	**11**		**2.5.2 Using the 4-parameter logistic (4PL) curve in an appropriate software program, a curve is fit to the positive control with ng toxin/ml on the x-axis (log scale) and % inhibition on the y-axis (linear scale).**
C	**11**		**2.5.3 The concentrations for sample dilutions falling within 30%-70% inhibition are interpolated from the standard curve.**
C	**11**		**2.5.4 Sample toxin concentration is calculated by multiplying the interpolated concentration by the sample dilution factor and dividing by 1000 to obtain PbTx-3 eq. results in ppm.**
C	**11**		**2.5.5 If more than one dilution of a sample falls within 30%-70%, the mean of the two calculated concentrations is used.**

C	**8**		**2.5.6**	**A result of ≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters is considered negative and can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.** **A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.8 ppm in oysters) requires additional testing by an Approved Method to support management actions.**
O	8		2.5.7	Laboratory reports to the Shellfish Management Authority detail sample date, location, species (matrix), date tested, analyst name, result of ELISA in ppm, and the actionable ELISA threshold for the species tested.

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
4. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
5. Consult reference standard product literature.
6. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
7. Consult pH electrode product literature.
8. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
9. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
10. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.
11. MARBIONC Enzyme-linked Immunosorbent Assay (ELISA) for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish. (ISSC proposal 17-107, supporting documents Appendix A)

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
NEUROTOXIC SHELLFISH POISON (NSP or Brevetoxin) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the brevetoxin (NSP) ELISA Component

1. **Conforms Status:** The NSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities.
   b. and <6 Key nonconformities.
   c. and <12 Total nonconformities**.**

2. **Provisionally Conforms Status:** The NSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. the number of critical nonconformities is **≥ 1** but **< 4**.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The NSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is **≥ 4**.
   b. or the total # of Key nonconformities is **≥ 6.**
   c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Paralytic Shellfish Poisoning (PSP HPLC-PCOX)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	5, 8		1.1.1	Written Plan adequately covers all the following: (check √ those that apply)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**5**		**1.1.2**	**QA Plan is implemented.**
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	**State's Human Resources Department**		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	**USDA Microbiology & EELAP**		1.2.4	In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	**5**		**1.2.5**	**LC-Operator must be competent in the operation and maintenance of a basic liquid chromatography system.**
		1.3 Work Area		
O	5, 8		1.3.1	Adequate for workload and storage.
O	8		1.3.2	Clean and well lighted.
O	8		1.3.3	Adequate temperature control.
O	8		1.3.4	All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment.		
O	6		1.4.1	The pH meter has a standard accuracy of 0.1 unit.
K	6		1.4.2	pH paper in the appropriate range (i.e. 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	10		1.4.3	pH electrodes consist of pH half-cell and reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).
K	5		1.4.4	pH meter is calibrated daily when in use. Results are recorded and records are maintained.
K	8		1.4.5	Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	8		1.4.6	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded.

			~~A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.~~

K	5, 11		1.4.7 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	6		1.4.8 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8, 9		1.4.9 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.11 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	1		1.4.12 Freezer temperature is maintained at -20 ºC or below.
K	8		1.4.13 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**13**		**1.4.14 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.15 All glassware is clean.
K	3		1.4.16 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. binary mobile phase system delivering a pulse-free flow of 0.5-2.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 5-30 µL injections, d. temperature controlled column compartment capable of controlling temperature between 10 – 50 ºC, and e. fluorescence detector able to achieve the required sensitivity at an excitation wavelength (λ) of 330 nm and emission of 390 nm.
K	3, 4		1.4.17 The post-column reaction system used is equipped with the following: a. reactor module capable of maintaining 85 ºC, b. dual reagent pumps capable of delivering accurate flows of 0.4 mL/min, and c. if applicable, a reaction coil (knitted or equivalent) having a total volume of 1 mL and a length of 5 m x 0.5 mm.
K	6		1.4.18 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.19 A boiling water bath with sufficient volume to cover the sample/acid mixture is used for extraction.
O	3		1.4.20 Centrifuge capable of holding 50 mL polypropylene tubes.
K	3		1.4.21 Microcentrifuge capable of holding 1.5 mL microcentrifuge tubes and generating a minimum of 16000 g or equivalent is used.
		1.5	**Reagents and Reference Solution Preparation and Storage**
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**

C	**8**		**1.5.2 Water is glass distilled or deionized and exceeds 0.5 megaohm resistance or is less than 2 µSiemens/cm conductivity at 25 ºC to be tested and recorded monthly for resistance or conductivity and the results are recorded.**
K	8		1.5.3 Water is analyzed for residual chlorine monthly and is at a nondetectable level (≤0.1 ppm) Results are recorded and records are maintained.
K	8		1.5.4 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained.
K	8		1.5.5 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.7 The binary mobile phase system used to analyze the GTX and STX toxins consists of:** **1.5.7.1 Mobile Phase A, which contains 11 mM heptane sulfonate and 5.5 mM phosphoric acid (H_3PO_4), pH 7.1.** **1.5.7.2 Mobile Phase B, which contains 11 mM heptane sulfonate, 16.5 mM H_3PO_4 and11.5% acetonitrile (MeCN), pH 7.1.**
C	**3**		**1.5.8 The binary mobile phase system used to analyze the C toxins consists of:** **1.5.8.1 Mobile Phase A, which contains 2 mM tetrabutyl ammonium phosphate, pH 5.8.** **1.5.8.2 Mobile Phase B, which contains 2 mM tetrabutyl ammonium phosphate in 4% acetonitrile, pH 5.8.**
C	**3**		**1.5.8 The post-column oxidant consists of 100 mM H_3PO_4 and 5 mM periodic acid (H_5IO_6), pH 7.8.**
C	**3**		**1.5.9 The post-column acid used is 0.75 M nitric acid (HNO_3).**
C	**3**		**1.5.10 The heptane sulphonate used in mobile phase A and mobile phase B to analyze for GTX and STX toxins is prepared the day of use or refrigerated for up to one week.**
C	**3**		**1.5.11 The pH of mobile phases and the post-column oxidant are adjusted as follows:** **a. Mobile phase A and mobile phase B for the GTX and STX toxins are adjusted to 7.1 with ammonium hydroxide (NH_4OH),** **b. Mobile phase A and mobile phase B for the C toxins are adjusted to 5.8 in one direction only with 10% acetic acid (HOAc) if too basic or 1% NH_4OH if too acidic, and** **c. The post-column oxidant is adjusted to 7.8 with 5 M sodium hydroxide (NaOH).** **d. Mobile phases and post-column reagents are filtered before use if the HPLC does not have a degreaser.**
C	**3,7**		**1.5.12 Only certified reference materials are used for standard solutions. Source of the reference standard:**
C	**7**		**1.5.13 NRC Zero-Mus or a negative control matched matrix is used as a matrix blank as appropriate. Source of the negative matrix:_____**
C	**7**		**1.5.14 All primary standards are stored appropriately as per supplier recommendations.**

C	**7**		**1.5.15**	**All standards used are within expiration date.**
C	**3**		**1.5.16**	**All standards are prepared gravimetrically.**
K	3		1.5.17	Intermediate mixes of primary standards are made up in 0.003 M HCl for the GTX/STX toxins or pH 5 glass distilled/deionized water for the C toxins labeled with the date of preparation and the expiration date and stored appropriately. The pH of the glass distilled/deionized water is adjusted when necessary by the dropwise addition of 10% acetic acid (HOAc).

C	**3**		**1.5.18**	**Working standards are made up from primary standard or intermediate mixes by dilution with toxin-free, deproteinated, matrix matched extracts.**
C	**7**		**1.5.19**	**Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.20**	**Quality Control shellfish tissues are stored frozen.**
C	**7**		**1.5.21**	**Working standards are labeled with the date of preparation, stored appropriately and used within 3 months of preparation.**
		1.6	**Collection and Transportation of Samples**	
O	6		1.6.1	Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6		1.6.2	Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**6**		**1.6.3**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	14		1.6.4	Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**6**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR PSP TOXINS				
		2.1	**Preparation of Sample**	
C	**6**		**2.1.1**	**At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	6		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4	The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.

C	**6**		**2.1.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8	Pieces of shell and drainage are discarded.
C	**6**		**2.1.9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**

		2.2 Digestion of Sample		
K	6		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**3**		**2.2.2**	**Five (5) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
K	3		2.2.3	The sample homogenate is extracted in a 1:1 w/v ratio with 0.1 M HCl.
K	3		2.2.4	Homogenate/acid mixture is vortexed thoroughly before boiling to completely mix the contents.
C	**3**		**2.2.5**	**To prevent toxin transformation, the pH of the homogenate/acid mixture before boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH.**
C	**3**		**2.2.6**	**Samples in capped 50 mL polypropylene centrifuge tubes are extracted in a boiling water bath for 5 minutes.**
K	3		2.2.7	The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of 5 M HCl. Any sample with a pH of less than 2.0 is discarded and extracted again.
K	3		2.2.8	The homogenate/acid mixture is allowed to separate by gravity or by centrifugation.
		2.3 Deproteination		
C	**3**		**2.3.1**	**500 µL of sample extract is deproteinated with 25 µL of 30% trichloroacetic acid, vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
C	**3**		**2.3.2**	**The pH of the deproteinated extract is adjusted with 35 µL of 1.0 M NaOH vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
K	3		2.3.3	An aliquot of the deproteinated supernatant is filtered through a 0.2 µm filter.
		2.4 Analysis		
C	**2**		**2.4.1**	**A standard calibration curve (of at least six concentrations) is performed upon initial instrument set up, following any major hardware maintenance activity, or when the continuing calibration verification (CCV) indicates significant drift (> 30% for individual toxin) from the calibration. Results are recorded and records are maintained.**
K	3		2.4.2	10 µL is injected for GTX/STX toxins and 5 µL is injected for C-toxins.
K	3		2.4.3	Samples are stored in the sample compartment of the autosampler at 4 °C during analysis. Otherwise samples must be analyzed within 20 hours if the autosampler is held at room temperature.
K	3		2.4.4	A column heater that is capable of maintaining 30-40 °C for the GTX/STX toxins and 10-20 °C for the C toxins is used in the analysis.

C	**3**		**2.4.5 The appropriate analytical column is used.** **a. GTX/STX Toxins: Agilent Zorbax Bonus-RP column, 4.6 mm x 150 mm, 3.5 µm or equivalent.** **b. C Toxins: Thermo BetaBasic 8, 4.6 mm x 250 mm, 5 µm or equivalent.**
		2.5	**System Suitability**
K	2		2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for each individual toxin.

C	**3**		**2.5.2 The resolution and retention time criteria that must be met are:** **a. For GTX and STX toxins, the matrix peak must be at least 70% baseline resolved between GTX3 and GTX2.** **b. For GTX and STX toxins, GTX5 must be at least 40% baseline resolved between dcGTX3 and dcGTX2.** **c. For GTX and STX toxins, dcSTX and STX must be at least 70% baseline resolved.** **d. For GTX and STX toxins, the retention time of GTX4 must be between 5 and 7 minutes.** **e. For the C toxins, C2 must be at least 70% baseline resolved between C1 and C2.** **f. For the C toxins, the retention time of C1 must be between 4 and 7 minutes.**
C	**2**		**2.5.3 Daily injection schedules must include the adequate frequency of injection standards based on an assessment of individual standard toxin variability. Variability in peak response must be less than 10% for calculation of toxicity in samples.**
		2.6	**Calculation of Toxicity**
C	**4**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** $\mu gSTXdiHCleq/100g = \mu MM \times \frac{333333.33}{111111111111} \times \frac{FFFFFFFF}{EEEEEE.FFFFFF} \times \frac{WWEE+VVFFFF}{WWEE} \times RRRRRREE \times 111111$ **Where:** **µM = Concentration of toxin in the extract, in µM;** **Fvol = Final volume of the deproteinized extract (e.g. 560 µL);** **Ext.vol = Volume of crude extract used (e.g. 500 µL);** **Wt = Weight of sample used;** **Vol = Volume of acid extractant used (e.g. 5 mL); and** **ReTx = Relative toxicity of toxin vs. Saxitoxin.**
C	**3**		**2.6.2 The individual toxicities for each toxin are summed to obtain the overall sample toxicity in µg STX equivalents/100 g (µg/100 g).**

Toxin	ReTx	Toxin	ReTx
GTX1	0.9940	NEO	0.9243
GTX2	0.3592	STX	1.0000
GTX3	0.6379	dcSTX	0.5131
GTX4	0.7261	C1	0.0060
GTX5	0.0644	C2	0.0963
dcGTX2	0.1538	C3	0.0133
dcGTX3	0.3766	C4	0.0576

C	**12**		**2.6.3 Any value at or above 80 µg STX equivalents /100 g of meat is actionable.**

REFERENCES:

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. AOAC Official Methods of Analysis (2011). AOAC Official Method 2011.02 Paralytic Shellfish Toxins in Mussels, Clams, Oysters, and Scallops Post-Column Oxidation (PCOX) Method.
4. Oshima, Y. 1995. J. AOAC Int. 78: 528-532.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16: 54-60.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2011. *NSSP Guide to the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PARALYTIC SHELLFISH POISON (PSP), PCOX COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the PSP, PCOX Component

1. **Conforms Status:** The PSP, PCOX component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

2. **Provisionally Conforms Status:** The PSP, PCOX component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. the number of critical nonconformities is **≥ 1** but **< 4**.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The PSP, PCOX component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is **≥ 4**.
   b. or the total # of Key nonconformities is **≥ 6.**
   c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____Date:_____

LEO Signature:_____Date:_____

NSSP Form *4 – PSP HPLC PCOX Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3		1.1.1 Written Plan (Check √ those items which apply).
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			f. Internal performance assessment.
			g. External performance assessment.
C	**2**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
C	**6**		**1.2.5 Training regarding radiation laboratory safety, handling and disposal practices is documented and records are maintained.**
C	**15**		**1.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitoxin in this assay. Alternatively, the laboratory uses less than 50 µCi per year and adheres to the American Radiolabeled Chemical (ARC) exemption status.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**

		1.4 Laboratory Equipment	
C	**4**		**1.4.1 Any lab equipment that may come into contact with [^{3}H]-STX at any point in the preparation or assay procedures must be specially labelled and must remain in the work area designated for low-level radiation work.**
O	5		1.4.2 The pH meter has a standard accuracy of 0.1 pH units.
K	7		1.4.3 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8		1.4.4 The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1		1.4.5 The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.
K	1		1.4.6 The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. ~~The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.~~
O	9		1.4.7 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.
K	6		1.4.8 pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6		1.4.9 The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3		1.4.10 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
			1.4.11 Balances must be calibrated by an external service at least once per year. Results are recorded and records maintained.
K	2		1.4.12 Refrigerator temperatures are maintained between 0 and 4 °C. Freezer securityfor ^{3}HSTX and cold STX must meet state and federal requirements for these materials.

K	1		1.4.13 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**4, 6, 10**		**1.4.14 Freezer temperature used to store [^{3}H] STX standard, rat brain membrane tissue preparation, interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -80 °C or below. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.**
K	6, 10		1.4.15 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.16 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
O	8		1.4.17 All glassware is clean.
C	**3**		**1.4.18 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.19 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
C	**6**		**1.4.20 Micropipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.**
C	**11**		**1.4.21 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.22 Minimum radiation safety equipment and protocols include the following: A wipe-test is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
		1.5 Reference Solution Reagent Storage, Preparation and Security	
C	**12**		**1.5.1 [^{3}H] STX standard is stored in a freezer at -80 °C or below.**
C	**10**		**1.5.2 Concentration of [^{3}H] STX standard is calculated from the lot information provided by the supplier with each batch.**
K	6		1.5.3 Unopened diHCl STX standard may be stored at room temperature or refrigerated.
C	**10**		**1.5.4 Preparation of MOPS assay buffer includes the following:** **a. 100 mM MOPS/L.** **b. 100 mM choline chloride/L.** **c. pH adjustment to 7.4 with NaOH.** **e. refrigerated storage at 4 °C.** **d. Maintained ice cold while in use.**
C	**10**		**1.5.6 Bulk standard curve dilutions are st[illegible] (1) month.**

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No criticality code or reference determined in LC

K	1		1.5.7 Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice).* b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____ c. Water contains <100 CFU/mL using the heterotrophic plate count method.

			1.6 Rat Brain Membrane Tissue Preparation and Storage
C	**10**		**1.6.1 MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to MOPS/choline chloride fresh on the day of use.**
C	**10**		**1.6.2 The cerebral cortex of 6-week old Sprague-Dawley rats is used in membrane tissue preparations, placed in iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL) and homogenized with no visible chunks remaining in the homogenate. This procedure is repeated until twenty (20) rat brains have been processed.**
C	**10**		**1.6.3 The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**
K	10		1.6.4 The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).
K	10		1.6.5 The resuspended rat brain tissue preparations are pooled and the centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue**.**
K	10		1.6.6 The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.
K	10		1.6.7 The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed for 20 seconds to obtain a homogeneous membrane tissue preparation.
C	**10**		**1.6.8 Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months.**
			1.7 Rat Brain Membrane Tissue Protein Receptor Determination
C	**10**		**1.7.1 The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube method) or equivalent.**

C	**10**		**1.7.2 The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined.**
K	10		1.7.3 Dilutions of the protein/receptor concentration of the rat brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous.
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS - RBA			
		2.1 Collection and Transportation of Samples	
C	**5**		**2.1.1 A representative sample of shellfish is collected.**
K	5		2.1.2 Shellfish samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	5		2.1.3 Shellfish samples are labeled with the collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**5**		**2.1.4 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	6, 13		2.1.5 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
		2.2 Preparation of Samples for Analysis – Homogenization	
C	**5, 6**		**2.2.1 At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8 Pieces of shell and drainage are discarded.

C	**5, 6**		**2.2.4 Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender <u>with all freeze-thaw liquid</u> forhomogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender <u>with all freeze-thaw liquid</u> for homogenization.**
K	6, 13		2.2.5 Homogenates should be extracted immediately. If homogenates must be stored, they should be frozen.
		2.3 Preparation of Samples for Analysis – Extraction	
K	5, 10		2.3.1 0.1 M HCl is used for extractions.
K	5, 10		2.3.2 At least five (5) grams of tissue +/- 0.1g is extracted using a 1:1 mass to volume ratio of 0.1 M HCl.
C	**10**		**2.3.3 The pH of the sample is checked and adjusted as necessary to between 3.0– 4.0.**
C	**10**		**2.3.4 Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**

C	**6**		**2.3.5 The sample is promptly brought to a boil at 99.0 +/- 1.0 °C and gently boiled for 5 minutes.**
O	6		2.3.6 The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.7 The sample is allowed to cool to room temperature.
C	**10**		**2.3.8 The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	5, 10		2.3.9 The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water).
K	10		2.3.10 The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; then b. an aliquot of the sample is centrifuged at 3000 x g for 10 minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.11 The sample extract is analyzed immediately, refrigerated at 4 °C in a sealed container for up to 24 hours, or frozen at -20 °C.
		2.4 Sample Assay	
K	6		2.4.1 One analyst performs the entire plate set-up for the assay.
K	6		2.4.2 Microtubes containing dilutions and samples are vortexed immediately before dispensing.
K	10		2.4.3 The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).
C	**10**		**2.4.4 The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension.**

K	10		2.4.5 Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.
C	**10**		**2.4.6 The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.7 Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane.
K	10		2.4.8 All wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, in order to ensure even pressure and filtration across the plate.
C	**10**		**2.4.9 Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate).**
K	10		2.4.10 [^{3}H] STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.
C	10		**2.4.11 An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).**
K	10		2.4.12 Standard curve fitting is calculated using appropriate software program.
C	**10**		**2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.15 The IC_{50} is in acceptable range (2.0 nM +/- 30%). If the IC_{50} is outside this range, the assay results are rejected and the assay must be repeated**
C	**10**		**2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.**
K	4		2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.
		2.5 Calculation of Sample Toxicity	
C	**10**		**2.5.1 When more than one dilution falls within B/B_o of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**

C	**10**		**2.5.2 Sample toxicity is calculated as follows:** **(nM STX equiv.) x (sample dilution) x (210 µL total volume/35 µL sample = mM STX equivalent in extract** **(nM STX diHCl equiv. in extract) x 1L/1000 mL x 372 ng/nmol x1 µg/1000 ng =µg STX diHCl equiv./mL** **µg STX diHCl equiv./mL x mL extract/g shellfish x 1000 g/kg =µg STX diHCl equiv./kg**
C	**14**		**2.5.3 Any value equal to or greater than 80 µg STX diHCl equiv./100 g) of sample is actionable.**
C	**14**		**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:**

REFERENCES

1. American Public Health Association (APHA). 1992. *Standard Methods for Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
2. American Public Health Association (APHA). 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
3. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition. APHA, Washington, D.C.
4. Appendix C: Radiation Safety Requirements, ISSC Proposal 13-114 Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination.
5. American Public Health Association (APHA). 1970. Recommended Procedures for the Examination of Sea Water and Shellfish, Fourth Edition. APHA, Washington, D.C.
6. Good Laboratory Practice.
7. Fisher J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.
9. Consult pH electrode product literature.
10. Association of Official Analytical Chemists (AOAC). 2016. Official Method 2011.27 Paralytic Shellfish Toxins (PSTs) in Shellfish Receptor Binding Assay.
11. Consult instrument manufacturer instructions.
12. Technical Data Sheet, American Radiolabeled Chemicals, Inc. 101 Arc Drive, St. Louis, MO 63146.
13. Wilt, d. s. (ed). 1974. Proceedings of the 8th National Shellfish Sanitation Workshop. U. S. Food and Drug Administration, Washington, D.C.
14. U. S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. NSSP *Guide for the Control of Molluscan Shellfish*. FDA/ISSC, Washington D.C. and Columbia, S.C.
15. U. S. Nuclear Regulatory Commission Materials, Section 30.18, 10 CFR Part 30, and American Radiolabeled Chemicals Licenses.

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
RECEPTOR BINDING ASSAY FOR PSP COMPONENT: (Part I-II)	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical (C), Key (K), and Other (O) Nonconformities	____ ____ ____

B. Criteria for Determining Laboratory Status of the RBA for PSP:

1. **Conforms Status:** The RBA for PSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a.
   b.
   c.

2. **Provisionally Conforms Status:** The RBA for PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply:
   a.
   b.
   c.

3. **Does Not Conform Status**: The RBA for PSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a.
   b
   c.

Olson, Gina
2024-05-01 18:53:00

Criteria not included in checklist proposal and not determined in LC

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *11 – RBA PSP Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check √ those that apply)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
C	**5**		**1.1.2 QA Plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	**5**		**1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.**
		1.3 Work Area	
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment	
K	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit. [Only applicable if using the sample cleanup procedure]
K	5		1.4.2 The pH meter is calibrated daily when in use. Results are recorded and records are maintained. [Only applicable if using the sample cleanup procedure]
K	8		1.4.3 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment. [Only applicable if using the sample cleanup procedure]
K	8		1.4.4 The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions are used to calibrate the pH meter. The calibration must include the pH 7 buffer and a second buffer near the expected sample pH (i.e., pH 4 or pH 10) in order to bracket the measurement range. Standard buffer solutions are used once and discarded. ~~The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions is used to calibrate the pH~~

			~~meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e., pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.~~ [Only applicable if using the sample cleanup procedure]

K	5, 11		1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure. [Only applicable if using the sample cleanup procedure]
K	6, 2		1.4.6 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8		1.4.7 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.8 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	4,15		1.4.10 Freezer temperature is maintained at -10 ºC or below.
K	8		1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**13**		**1.4.12 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.13 All glassware is clean.
K	4		1.4.14 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 1.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 20 µL injections, d. temperature controlled column compartment capable of controlling temperature at 40 ºC, e. ultraviolet detector/diode array detector able to achieve the required sensitivity at a wavelength (A) of 242 nm, and f. a data collection system (e.g., computer, integrator).
K	2		1.4.15 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	4		1.4.16 A solid phase extraction (SPE) vacuum manifold capable of holding 3 mL cartridges is used. [Only applicable if using the sample cleanup procedure]
O	4		1.4.17 A centrifuge capable of holding 50 mL polypropylene tubes is used.
		1.5 Reagents and Reference Solution Preparation and Storage	
C	**4, 15**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	8		1.5.2 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)

K	8		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**4, 15**		**1.5.4 The mobile phase system used to analyze domoic acid consists of: 10% aqueous acetonitrile (v/v) and 0.1% trifluoroacetic acid (TFA).**
O	4		1.5.5 Mobile phase is filtered before use if the HPLC does not have a degasser.

C	**7**		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
K	4, 15		1.5.7 A cartridge wash solution is made up of 1 volume acetonitrile to 9 volumes of water (i.e., 10% aqueous acetonitrile). [Only applicable if using the sample cleanup procedure]
K	4		1.5.8 Citrate buffer (0.5 M, pH 3.2) is made up by dissolving 40.4 g citric acid monohydrate and 14 g triammonium citrate in 400 mL water, then adding 50 mL acetonitrile and diluting the total to 500 mL with water [or equivalent buffer]. [Only applicable if using the sample cleanup procedure]
C	**7**		**1.5.9 NRC CRM Zero-Mus or a negative control is used as a blank to ensure that there is no carry over between samples/standards. Source of the negative control: _____**
C	**7**		**1.5.10 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.11 All standards used are within expiration date.**
C	**2**		**1.5.12 All standards are prepared either gravimetrically or using positive displacement pipettes.**
C	**4, 15**		**1.5.13 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 50% aqueous methanol). Dilution with toxin-free, cartridge wash solution (aqueous acetonitrile) is allowed if using the diluted crude sample or the sample cleanup procedure.**
C	**7**		**1.5.14 Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.15 Quality Control shellfish tissues are stored frozen.**
		1.6 Collection and Transportation of Samples	
O	6, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**6, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**

K	14, 2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.

C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR ASP TOXINS			
		2.1 Preparation of Sample	
C	**6, 1**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., 3 geoduck gut balls).**
O	6		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**6**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8 Pieces of shell and drainage are discarded.
C	**2, 6**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meat s and their drip loss liquid or thawed homogenates with their freeze- thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction	
K	4,6		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**4**		**2.2.2 Four (4) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**4**		**2.2.3 The sample homogenate is extracted with 16 mL extraction solvent (1:1 methanol:water [also referred to as 50% aqueous methanol]).**
K	4, 15		2.2.4 Homogenate/extract mixture is centrifuged and filtered before analysis.
K	4, 15		2.2.5 The filtered extract is injected into the HPLC or loaded into the autosampler immediately.

K	4		2.2.6 When crude samples are diluted, dilutions are made by diluting 1 mL of filtered sample supernatant into a 5 mL volumetric flask and diluted with water to 5 mL.
K	4, 15		2.2.7 Crude extracts are sealed tightly and stored at -10 ºC or below.
		2.3 Sample Cleanup (Optional)	
O	4, 15		2.3.1 Three (3) mL SAX cartridges (500 mg silica derivatized with quaternary ammonium silane) are used for cleanup.
K	4		2.3.2 The SAX cartridge is conditioned with 6 mL methanol, followed by 3 mL water, followed by 3 mL extraction solvent (1:1 methanol:water).
C	**4, 15**		**2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.**
K	4, 15		2.3.4 Five (5) mL of filtered extract is loaded onto the cartridge and flowed slowly (~1 drop/s) until sample meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.5 Five (5) mL of wash solution (1:9 acetonitrile:water) is loaded to the cartridge and washed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4		2.3.6 0.5 mL of citrate buffer (0.5 M, pH 3.2) is loaded to the cartridge and flowed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.7 A 2 mL volumetric tube is placed under the cartridge and any domoic acid is eluted into the tube by loading and flowing as much citrate buffer as needed slowly (~ 1 drop/s) until the 2 mL mark is reached on the tube.
C	**4, 15**		**2.3.8 The solution is thoroughly mixed before withdrawing an aliquot for analysis.**
K	4, 15		2.3.9 The cleaned up extract is injected into the HPLC or loaded into the autosampler immediately.
		2.4 Analysis	
C	**2**		**2.4.1 A standard calibration curve (of at least six concentrations) is performed daily. Results are recorded and records are maintained.**
K	4, 15		2.4.2 Twenty (20) µL of extract is injected for analysis.
K	2		2.4.3 Samples are stored in the sample compartment of the autosampler at 4°C during analysis. Otherwise samples must be analyzed within 9 hours if the autosampler is held at room temperature.
K	4, 15		2.4.4 A column heater is used and the temperature is maintained at 40 °C during the analysis.
C	**4**		**2.4.5 The appropriate analytical column is used: 25 cm x 4.6 mm id packed with 5 µm Vydac 201TP octadecylsilica or equivalent.**
K	2		2.4.6 The column is stored following the manufacturer's instructions when not in use.
O	2		2.4.7 If a precolumn in-line filter and/or a compatible guard column (e.g., 201GCC54T) are/is used, rejection criteria are established to determine when to change the filter/guard column.
C	**2**		**2.4.8 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.**
		2.5 System Suitability	

C	**2**		**2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for domoic acid.**
C	**3**		**2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of L-tryptophan and domoic acid.**
K	2		2.5.3 Peak asymmetry is routinely monitored to evaluate the performance of the column. Results are recorded and records maintained.
C	**2**		**2.5.4 The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3.**
C	**2,4**		**2.5.5 Daily injection schedules must include the adequate frequency of injection standards and extraction blanks based on an assessment of individual standard toxin variability and lack of carry over.**
C	**2**		**2.5.6 Repeated injections of calibrated standards/samples agree within ±5% (as determined through the use of the coefficient of variation).**

			2.6 Calculation of Sample Toxicity
C	**4, 15**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** **µg/g domoic acid (DA) = DA injected × $\frac{VV}{WW}$ × (F)** **where:** **DA injected = the concentration in µg/ml of the extract injected;** **V = total volume of homogenate and extraction solvent (mL);** **W = weight (g) of tissue homogenate extracted (e.g., 4 g); and** **F = dilution factor (e.g., if SAX cleanup or crude sample dilution are performed).** **The concentration of DA injected may be determined using the nearest standard or the equation of the day's standard curve.**
C	**4, 15**		**2.6.2 Calculated domoic acid concentrations include the sum of domoic acid and epimer peaks, when the epimer represents 5% or more of the peak area.**
C	**12**		**2.6.3 Any value at or above 20 ppm (mg/kg or µg/g) domoic acid is actionable.**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice. 21 CFR 58.
3. AOAC Official Method 991.26 Domoic Acid in Mussels. Liquid Chromatography Method. First Action 1991. Final Action 1999.
4. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1995. J. AOAC Int. 78(2): 543-554.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 192. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16: 54-60.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.
15. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1991. A Rapid Extraction and Clean-up Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Bioscience, Technical Report #64, National Research Council Canada #33001.

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist Modification - Quality Assurance Technical Requirement
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The proposal is to modify text for 1.1.2 checklist line items in the following NSSP Laboratory Evaluation Checklists: 1. Microbiology 2. PCR Microbiology 3. Probe Microbiology 4. RBA for PSP 5. PSP MBA and Scotia Rapid Test 6. Domoic Acid HPLC-UV 7. PSP PCOX HPLC 8. NSP MBA 9. DSP LC-MS/MS Specific edits are reflected in the attached supporting documentation.
12. Public Health Significance	The proposed modification is to improve efficiency in the current NSSP Laboratory Evaluation checklists.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
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CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Mouse Bioassay and Scotia Rapid Test for Paralytic Shellfish Poisoning (PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Mouse Bioassay for Paralytic Shellfish Poisoning (MBA PSP)
	Scotia Rapid Test for Paralytic Shellfish Poisoning (SRT PSP)

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	5, 6, 8		1.1.1	Written Plan adequately covers all of the following: (check √ those items which apply)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
				h. Animal care.
C	**6**		**1.1.2**	**~~The QA plan is implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experiencerequirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meet the state/county educational and experiencerequirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial/private laboratories, the analyst(s) meets the state/county educational and experiencerequirements for processing samples in a public health laboratory.
		1.3 Work Area		
O	5, 6		1.3.1	Adequate for the workload and storage.
O	5		1.3.2	Clean and well lighted.
O	5		1.3.3	Adequate temperature control.
O	5		1.3.4	All work surfaces are nonporous and easily cleaned.
C	**8**		**1.3.5**	**A separate, quiet area with adequate temperature control for mice acclimation and injection is maintained.**
		1.4 Laboratory Equipment		
O	2		1.4.1	The pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH paper in the appropriate range (i.e. 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	7		1.4.3	pH electrodes consist of pH half-cell and reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).

K	6		1.4.4	pH meter is calibrated daily when in use. Results are recorded and records are maintained.
K	5		1.4.5	Effect of temperature has been compensated for by an ATC probe; use of a triode or by manual adjustment.
K	5		1.4.6	A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.
K	6, 12		1.4.7	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of slope. (Circle method used).
K	2		1.4.8	The balances being used provide an appropriate sensitivity at the weights of use. a. To prepare reference solution, the balance must have a sensitivity of at least 0.1 g at a load of 1 g. b. For sample extraction, the balance must have a sensitivity of at least 0.1 g at a load of 100 g. c. For gravimetric extract volume adjustment, the balance must have a sensitivity of at least 0.1 g at a load of 200 g. d. To weigh mice for assay, the balance must have a sensitivity of at least 0.1 g at a load of 20 g.
K	4,5		1.4.9	The balance calibration is checked monthly according to the manufacturer's specifications using NIST Class S, ASTM Class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10	Refrigerator temperature is maintained between 0 and 4˚C.
K	5		1.4.11	Refrigerator temperature is monitored at least once daily on workdays. Results are recorded and records are maintained.
K	4		1.4.12	Freezer temperature is maintained within manufacturer's tolerance.
K	5		1.4.13	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records are maintained.
C	**10**		**1.4.14**	**All in-service thermometers are properly calibrated and immersed. Results are recorded and records are maintained.**
O	6		1.4.15	All glassware is clean.
C	**5**		**1.4.16**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records are maintained.**
C	**9**		**1.4.17**	**An alkaline or acid based detergent is used for washing glassware/labware.**
		1.5 Reagents and Reference Solution Preparation and Storage		
C	**9**		**1.5.1**	**Any residual (unused) STX diHCl standard solution is never stored after the ampule has been opened.**
K	15		1.5.2	PSP reference solution (1 µg/mL) is prepared gravimetrically and diluted with 0.001 M HCl solution.

K	9		1.5.3	Prepared PSP referencesolution is stored under refrigeration in a sealed non-reactive container. Solution may be stored indefinitely as long as there is no detectable evaporation loss as determined by weight. If evaporation is detected, the solution is discarded appropriately. Records are maintained.
C	**14**		**1.5.4**	**All working dilutions from the PSP reference solution are prepared gravimetrically using 0.001 M HCl.**
K	9		1.5.5	All working dilutions prepared from the PSP reference solution are discarded appropriately after use.
C	**5**		**1.5.6**	**Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm – cm resistance(2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 °C. (Circle the appropriate water quality descriptor determined). Results are recorded and records are maintained.**
K	5		1.5.7	Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (≤ 0.1 mg/L). Results are recorded and records are maintained. Specify method of determination_____.
K	5		1.5.8	Reagent water contains < 100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained.
		1.6 Collection and Transportation of Samples		
O	2		1.6.1	Shellstock are collected in clean, waterproof, puncture resistant containers, loosely sealed.
K	2		1.6.2	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**2**		**1.6.3**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	15, 9		1.6.4	Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**14**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – ANALYSIS OF SHELLFISH FOR PSP TOXINS – MOUSE BIOASSAY (MBA)				
		2.1 Preparation of Samples for Analysis – Homogenization		

C	**15, 9**		**2.1.1**	**At least 12 animals (or more to provide 100 g of shellfish meat) are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	2		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	2		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	2		2.1.4	The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	2		2.1.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**2**		**2.1.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	2		2.1.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	2		2.1.8	Pieces of shell and drainage are discarded.
C	**2**		**2.1. 9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60 - 120 seconds).**
		2.2	**Preparation of Samples for Analysis – APHA/AOAC Digestion & Extraction**	
K	15, 9		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
K	2		2.2.2	100 grams of homogenized sample is weighed into a beaker.
K	2		2.2.3	The sample homogenate is extracted in a 1:1 weight/volume ratio by adding 0.1 M HCl or 0.18 M HCl (*circlethe appropriate choice*).
K	2		2.2.4	Homogenate/acid mixture is stirred thoroughly before boiling to completely mix the contents.
C	**2**		**2.2.5**	**To prevent toxin transformation, the pH of the homogenate/acid mixture before boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
C	**2**		**2.2.6**	**The homogenate/acid mixture is promptly brought to its boiling point, then gently boiled at 100 ± 1 ºC for 5 minutes.**
O	9		2.2.7	The homogenate/acid mixture is boiled under adequate ventilation (e.g. fume hood).
O	9		2.2.8	The homogenate/acid mixture is allowed to cool to room temperature.
C	**2**		**2.2.9**	**The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	2		2.2.10	The homogenate/acid mixture is adjusted gravimetrically to the pre-boiling weight using 0.001 M HC1.
K	2		2.2.11	The homogenate/acid mixture is allowed to separate by gravity or by centrifugation (e.g. centrifuged at 3,000 RPM for 5 minutes).
K	9		2.2.12	If the extracted sample cannot be assayed immediately, then the supernatant is decanted and stored in a sealed container under refrigeration for up to 24 hours or frozen for longer storage.
K	9		2.2.13	Refrigerated extracts areallowed to reach ambient temperature before being bioassayed or tested by the SRT for PSP.

		2.3 Mouse Bioassay (MBA) for PSP		
K	2		2.3.1	A 26-gauge hypodermic needle is used for intraperitoneal injections.
C	**2**		**2.3.2**	**Healthy mice in the weight range of 17.0 -23.0 grams (19 - 21 grams is preferable) from a stock colony are used forroutine assays. Previouslyinjected mice are never re-used for a bioassay.** **Stock strain:_____ Source:_____**
C	**9**		**2.3.3**	**Mice are allowed to acclimate at least 24 hours prior to injection. In some cases, 48 hours may be required.**
C	**9**		**2.3.4**	**A conversion factor (CF) for the lab has been appropriately determined.** **Lab CF:_____Date CF established:_____**
C	**2**		**2.3.5**	**The CF value is checkedweekly if assays are done on one or several days during the week or once each day that assays are performed if they are performed less than once per week.** **Date of current CF check:_____ CF verified: yes/no (*circle choice*)**
C	**2**		**2.3.6**	**If the lab CF is not verified during a check, the lab follows the appropriate procedure for establishing a temporary CF to use for the day/week.**
C	**2, 9**		**2.3.7**	**If the lab CF fails to be verified, the cause is investigated and the situation is corrected. If the cause cannot be determined with reasonable certainty and the lab CF fails to be verified > three times in a year, the lab CF is recalculated through a restandardization procedure.**
K	9		2.3.8	Mice are weighed to the nearest 0.1 g.
C	**2**		**2.3.9**	**Mice are injected intraperitoneally with 1 mL of extracted sample.**
K	2		2.3.10	For CF checks, five mice are injected.
K	9		2.3.11	For routine assays, three mice (two when both survive) are injected per sample.
C	**2**		**2.3.12**	**Elapsed time post-injection is accurately determined and recorded.**
C	**2**		**2.3.13**	**When death occurs, the time of death to the nearest second is noted at the last gasping breath and recorded.**
C	**9, 2**		**2.3.14**	**Mice are continually observed for up to 20 minutes after injection, then periodically observed for a total time of up to 60 minutes after injection.**
C	**2**		**2.3.15**	**If the median corrected mouse unit is greater than 1.92 (5 minutes), then the sample is diluted with0.001 M HCl as appropriate to achieve a median corrected mouse unit, MCMU of 1.39-1.92 (a death time of 5-7 minutes).**
		2.4 Calculation of toxicity for MBA		
C	**2**		**2.4.1**	**The death time for each mouse is converted to mouse units (MU) using Sommer's Table and recorded. Any mice surviving beyond 60 minutes are recorded as < 0.875 MU.**
C	**2**		**2.4.2**	**The weight for each mouse is corrected to mouse units using the table of weights in Recommended Procedures (Table 7) and interpolated for weights not listed.**

C	**2**		**2.4.3**	**The Corrected Mouse Unit (CMU) for each mouse injected is calculated as follows:** **Death time in MU x Weight correction in MU = CMU**
C	**2**		**2.4.4**	**The Median Corrected Mouse Unit (MCMU) for each sample is calculated and used in the final toxicity calculation for that sample.**

C	**2**		**2.4.5**	**The toxicity of each sample is calculated as follows:** **µg STX eq/100 g of sample = MCMU x CF x DF x 200 except when less than 100 grams of sample is used for analysis.** **In this case an adjustment for sample weight must be made such that the formula for calculating sample toxicity becomes:** **µg STX eq/100 grams of sample = MCMU x CF x DF x 200/Adjusted weight of the acidified sample x 200.** **Where:** **MCMU=Median Corrected Mouse Unit for the sample** **CF=Laboratory Conversion Factor** **DF=Dilution Factor (e.g. 1:1 dilution, DF=2)**
C	**11**		**2.4.6**	**Any value equal to or greater than 80 µg STX eq/l00 g of sample is actionable.**
PART III – ANALYSIS FOR PSP TOXINS – SCOTIA RAPID TEST (SRT)				
		3.1 Screening by Scotia Rapid Test (SRT)		
K	9		3.1.1	Before beginning any screening, the following items are recorded for the SRT kit in use. a. Date received. b. Batch/lot numbers for all kit components (test strip and PSP AOAC buffer). c. Expiration dates for all kit components. d. Date opened and/or used.
K	13		3.1.2	When placed into service, all kit components are within the accepted expiration dates.
C	**13**		**3.1.3**	**The desiccant pouch inside the test strip wrapping is blue in color, indicating suitability for use. Any test strip wrapping containing a pink desiccant pouch is discarded.**
K	13		3.1.4	All kit components are stored according to the manufacturer's recommendations.
C	**9**		**3.1.5**	**A positive control of 80 µg STX eq/100 g of sample is used to test new kit lots and buffers. Results are recorded and records maintained.**
C	**9**		**3.1.6**	**Micropipettes with appropriate ranges for the volumes being measured are used.**
K	9		3.1.7	All micropipettes are maintained and calibrated according to manufacturer's instructions. Results are recorded and records maintained.
C	**13**		**3.1.8**	**400 µL of buffer solution is accurately transferred to a small tube.**
C	**13**		**3.1.9**	**100 µL of sample extract is accurately added to the buffer.**

K	13		3.1.10 The buffer/sample mixture is carefully mixed by inserting the tip of the micropipette into the mixture and pipetting up and down at least three times.
C	**13**		**3.1.11 100 µL of the thoroughly mixed solution is added to the test strip sample well.**
K	9		3.1.12 Micropipette tips are not reused.
K	13		3.1.13 Inoculated test strips are allowed to react with the sample mixture for the period of time recommended by the manufacturer.

C	**13**		**3.1.14 The test strip result is interpreted according to the instruction card provided by the manufacturer, which is specific to each batch/lot of test strips. Results are recorded and records are maintained.**
K	13		3.1.15 If a test result is interpreted as invalid; the pH of the sample extract is checked and adjusted as needed to fall between pH 2.0 – 4.0. Fresh PSP AOAC buffer is used to re-test the sample on a new test strip.
C	**13**		**3.1.16 If the same sample is interpreted as invalid on two different test strips, then the sample is assumed to contain interfering substances, and an alternative test method is used.**
C	**11**		**3.1.17 Any positive result on a SRT is actionable.**

REFERENCES:

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13. Consult SRT manufacturer instruction manual / literature
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LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PARALYTIC SHELLFISH POISON COMPONENT: PARTS I, II, III	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the PSP, MBA and/or SRT Component

1. **Conforms Status:** The PSP, MBA and/or SRT component of this Laboratory is in conformity with NSSP requirements if all of the following apply.

   a. No Critical nonconformities.
   b. and <6 Key nonconformities.
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The PSP, MBA and/or SRT component of this Laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.

   a. the number of Critical nonconformities is ≥ 1 but < 4,
   b. and <6 Key nonconformities.
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status:** The PSP, MBA and/or SRT component of this Laboratory is not in conformity with NSSP requirements when any of the following apply.

   a. The total # of Critical nonconformities is ≥4.
   b. or total # of Key nonconformities is ≥ 6.
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

NSSP Form *2 – Mouse Bioassay and Scotia Rapid Test for PSP Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Multiple Tube Fermentation Technique for Seawater (APHA) [PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	8, 11		1.1.1 Written Plan (Check those items which apply.)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**8**		**1.1.2 ~~QA Plan Implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
K	11		1.1.3 The Laboratory participates in a proficiency testing program annually. Specify Program(s): _____
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory science.
		1.3 Work Area	
O	8,11		1.3.1 Adequate for workload and storage.
K	11		1.3.2 Clean, well-lighted.
K	11		1.3.3 Adequate temperature control.
O	11		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5 Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment	
O	9		1.4.1 To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2 pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3 The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.

K	8		1.4.4	pH meter is calibrated daily or with each use. Results are recorded and records maintained.
K	11		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the electrode isopotential point (pH 7) and the second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.)*
K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are verified monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**9, 36**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are verified annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____

C	**9**		**1.4.23**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 32		1.4.24	The accuracy of working thermometers are verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. Results are recorded and records maintained.
O	11		1.4.25	Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing		
O	9		1.5.1	Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2	Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3	Sample containers are made of glass or some other inert material.
O	9		1.5.4	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.
K	9		1.5.5	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6**	**Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7	Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8	In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10**	**With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	9		1.6.1	Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2	Routine autoclave maintenance is performed and the records are maintained.
C	**29, 32, 33**		**1.6.3**	**The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**

K	11		1.6.4	An autoclave standards thermometer has been calibrated using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5	The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination _____
K	1		1.6.6	Working autoclave thermometers are verified against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	32		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.*)
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures ≥160°C.
K	9		1.6.11	A thermometer capable of determining temperatures ≥160°C accurately is used to monitor the operation of the hot-air sterilizing oven.
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11		1.6.13	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14	Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15**	**The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16**	**The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17	Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18	Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
C	**2**		**1.6.19**	**The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20**	**The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21	Hardwood applicator transfer sticks or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization _____

C	**2**		**1.6.22**	**The sterility of the hardwood applicator transfer sticks/presterilized loops is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23	Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation		
K	3, 5		1.7.1	Media is commercially dehydrated except in the case of A-1 medium which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2	Media is prepared and sterilized according to manufacturer's instructions.
O	11		1.7.3	Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**11**		**1.7.6**	**Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.)* Results are recorded and the records maintained.**
C	**11**		**1.7.7**	**Reagent water is analyzed for residual chlorine monthly and is at a non- detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination _____**
K	11		1.7.8	Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	9		1.7.9	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10**	**Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.11**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**40**		**1.7.12**	**Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____**

O	9		1.7.13	Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14	The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II – SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium. (*Circle appropriate one.*)**
C	**9, 34**		**2.2.2**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**

C	**9**		**2.2.3**	**In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.6	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.8	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
K	9, 11		2.3.3	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. (*Circle the method of transfer.*)
C	**9**		**2.3.4**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium without salicin. Study records are maintained and are available upon request.**

C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**9, 34**		**2.5.4**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.5**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
C	**2**		**2.5.8**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation*. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**		**2.5.9**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.11**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition.
K	7		2.6.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5	Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**

C	**2**		**2.7.7**	**Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8**	**When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and the record is maintained.**
K	2, 11		2.7.9	New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10**	**The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11	Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12	Forceps tips are clean.
O	11		2.7.13	Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14	Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15	If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16	Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17**	**Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18	A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19	The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20	Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar		
K	11		2.8.1	Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2**	**The phosphate buffered saline is properly sterilized.**
K	23		2.8.3	A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4	Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar		
C	**24**		**2.9.1**	**mTEC agar is used.**
C	**23, 34**		**2.9.2**	**The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.3**	**The membrane is placed grid side up within the sterile filter apparatus.**

C	**23, 25**		**2.9.4**	**Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.5**	**Sample volumes are filtered under vacuum.**
K	26		2.9.6	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.7**	**The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.8**	**The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.9**	**Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**		**2.9.10**	**Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.11**	**Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ± 0.5°C for two (2) hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.12**	**After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ± 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar		
C	**23**		**2.10.1**	**All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2**	**Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3**	**When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4**	**The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5**	**Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment		
C	**9, 11, 21**		**2.11.1**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**

C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
K	9, 11, 21, 35		2.12.8 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.9 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 35**		**2.13.1 The sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 35**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 35**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 35**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 35**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control _____**
C	**9, 11, 35**		**2.13.10 Inoculated plates are incubated inverted at 35 ± 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 35**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**

PART III – SHELLFISH SAMPLES				
		3.1 Collection and Transportation of Samples		
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination		
K	2,32		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**, 32		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**, 32		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	2, 9		3.2.10	At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9		3.2.11	A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
C	**9**		**3.2.13**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.14	APHA *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA		
C	**9**		**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. (*Circle the medium used.*)**
K	9		3.3.2	Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.

C	**9**		**3.3.3**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.5	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.6**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control _____**
K	9		3.3.7	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
K	9, 11		3.4.2	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. (*Circle the method of transfer.*)
C	**9**		**3.4.3**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	15 to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**

C	**9**		**3.6.5**	**An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6	Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7**	**Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8	Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9	Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10	Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11	A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC		
K	9		3.7.1	Colony counts determined in accordance with Pa rt III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
C	**19**		**3.7.2**	**Colony counts are reported as CFU/grams of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP		
C	**2,3**		**3.8.1**	**Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2	Double strength modified MacConkey agar is used.
C	**3**		**3.8.3**	**Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4	Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5	Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6**	**The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7**	**The sample homogenate is cultured within two (2) minutes of blending.**
C	**2,3**		**3.8.8**	**Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9	Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10	The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**3, 13**		**3.8.12**	**When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.13**	**Plates are stacked no more than three (3) high in the incubator.**

C	**2**		**3.8.14**	**Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive Process Control: _____ Negative Process Control:_____**
		3.9 Computation of Results - ETCP		
K	11		3.9.1	Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2	A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3**	**All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4**	**Results are reported as CFU/100 grams of sample.**
Bacteriological Examination of Shellfish Meats for Male Specific Coliphage (MSC)				
		3.10 MSC Equipment and Supplies		
K	2		3.10.1	Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100mL.
K	2		3.10.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		3.10.3	The sterility of each batch/ lot of pre-sterilized or reusable syringes, filters and/or filter units is determined. Results are recorded and records maintained.
C	**2**		**3.10.4**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.5**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
K	2		3.10.6	The temperature of the freezer is maintained at ≤-15°C.
C	**1**		**3.10.7**	**The sterility of disposable 50 mL centrifuge tubes is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28, 31		3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28, 31		3.11.2	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28, 31		3.11.3	Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	2		3.11.4	Unsterilized soft agar is stored at ≤-15°C for up to three (3) months.
K	27, 28, 31		3.11.5	The soft agar is sterilized for 15 minutes at 121°C before use.
K	28, 31		3.11.6	Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.
K	2, 27, 28, 31		3.11.7	Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		3.12 Preparation of Host Culture for MSC Analysis		
C	**28, 31**		**3.12.1**	***E. coli* Famp ATCC 700891 is the bacterial host strain.**
K	27, 28, 31		3.12.2	Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.

K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28, 31**		**3.12.4 After inoculation, the host cell growth broth culture is not shaken.**
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.13.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.14 MSC Sample Analysis	
C	**28, 31**		**3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.**
C	**27, 28, 31**		**3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.**
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	**27, 28, 31**		**3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.**
C	**27, 28, 31**		**3.14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28, 31**		**3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28, 31**		**3.14.11 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.**
K	27, 28, 31		3.14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive Control: _____
K	27, 28, 31		3.14.13 Room temperature growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.14.15 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28, 31		3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28, 31**		**3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.15 Computation of Results - MSC	
C	**27**		**3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28, 31		3.15.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	2		3.15.4 The MSC count is rounded off conventionally to give a whole number.
		PART IV – MSC WASTEWATER ANALYSIS	
		4.1 Collection and Transportation of Samples	
C	**11**		**4.1.1 Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**39**		**4.1.2 Effluent sample bottles must contain 0.2 mL of 10% sodium thiosulfate solution for every 200 mL of sample to inactivate any residual chlorine.**
K	1		4.1.3 Samples are identified with collector's name, sampling location, time and date of collection.
C	**9**		**4.1.4 Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**

O	1		4.1.5	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**4.1.6**	**Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies		
K	38		4.2.1	Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.
K	9		4.2.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		4.2.3	The sterility of each batch/lot of pre-sterilized or reusable syringes, filters, and/or filter units is determined. Results are recorded and records maintained.
C	**27, 28**		**4.2.4**	**The balance used provides a sensitivity of at least 0.01g.**
C	**27, 28**		**4.2.5**	**The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6	The temperature of the freezer is maintained at ≤ -15°C.
C	**28**		**4.2.7**	**The sterility of the transfer vessel utilized is determined with each lot. Results are recorded and records maintained.**
		4.3 MSC Media Preparation		
K	28		4.3.1	Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2.	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28		4.3.3	Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4	Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
K	27, 28		4.3.5	The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.
K	27, 28		4.3.6	Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.
K	27, 28		4.3.7	Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis		
C	**28**		**4.4.1**	***E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2	Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3	Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**4.4.4**	**After inoculation, the host cell growth broth culture is not shaken.**

		4.5 MSC Sample Analysis		
C	**38**		**4.5.1**	**Wastewater sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10-2) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.**
K	27, 28		4.5.2	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.5.3	Two hundred microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample.
C	**27, 28**		**4.5.4**	**2.5 mL of sample is added to a tube of tempered soft agar.**
C	**27, 28**		**4.5.5**	**The tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.5.6**	**The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			**4.5.7**	**Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.**
K	27, 28		4.5.8	Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28		4.5.9	Room Temperature Growth broth is used as the negative control or blank.
K	27, 28		4.5.10	Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		4.5.11	A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28		4. 5.12	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**4.5.13**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.6 Computation of Results - MSC		
C	**38**		**4.6.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted. The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**38**		**4.6.2**	**When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
K	28		4.6.3	The formula used for determining the density of MSC in PFU/100 mL is: High Range: N * 1000 = PFU/100mL; Low Range: N * 5 = PFU/100mL; Where N is the total number of plaques counted on all plates.
O	9		4.6.4	The MSC count is rounded off conventionally to give a whole number.

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37. American Public Health Association. 2001. *Compendium of Methods for the Microbiological Examination of Foods*. APHA. Washington, D.C.
38. Male-Specific Coliphage Quantification from Wastewater SLV, Proposal 15-114.
39. "9060 SAMPLES (2017)", *Standard Methods for the Examination of Water and Wastewater* DOI: 10.2105/SMWW.2882.184
40. American Public Health Association (APHA). 2017. *Standard Methods for the Examination of Water and Wastewater*, 23rd Edition, APHA/AWWA/WEF. p. 9-5, 9-16 through 9-19.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

NSSP Form *LAB-100 Microbiology,* Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Paralytic Shellfish Poisoning (PSP HPLC-PCOX)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	5, 8		1.1.1	Written Plan adequately covers all the following: (check √ those that apply)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**5**		**1.1.2**	~~**QA Plan is implemented.**~~ **The laboratory follows all internal policies and procedures while implementing the documented quality system.**
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	**State's Human Resources Department**		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	**USDA Microbiology & EELAP**		1.2.4	In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	**5**		**1.2.5**	**LC-Operator must be competent in the operation and maintenance of a basic liquid chromatography system.**
		1.3 Work Area		
O	5, 8		1.3.1	Adequate for workload and storage.
O	8		1.3.2	Clean and well lighted.
O	8		1.3.3	Adequate temperature control.
O	8		1.3.4	All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment.		
O	6		1.4.1	The pH meter has a standard accuracy of 0.1 unit.
K	6		1.4.2	pH paper in the appropriate range (i.e. 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	10		1.4.3	pH electrodes consist of pH half-cell and reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).
K	5		1.4.4	pH meter is calibrated daily when in use. Results are recorded and records are maintained.
K	8		1.4.5	Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	8		1.4.6	A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.

K	5, 11		1.4.7 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	6		1.4.8 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8, 9		1.4.9 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.11 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	1		1.4.12 Freezer temperature is maintained at -20 ºC or below.
K	8		1.4.13 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**13**		**1.4.14 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.15 All glassware is clean.
K	3		1.4.16 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. binary mobile phase system delivering a pulse-free flow of 0.5-2.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 5-30 µL injections, d. temperature controlled column compartment capable of controlling temperature between 10 – 50 ºC, and e. fluorescence detector able to achieve the required sensitivity at an excitation wavelength (λ) of 330 nm and emission of 390 nm.
K	3, 4		1.4.17 The post-column reaction system used is equipped with the following: a. reactor module capable of maintaining 85 ºC, b. dual reagent pumps capable of delivering accurate flows of 0.4 mL/min, and c. if applicable, a reaction coil (knitted or equivalent) having a total volume of 1 mL and a length of 5 m x 0.5 mm.
K	6		1.4.18 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.19 A boiling water bath with sufficient volume to cover the sample/acid mixture is used for extraction.
O	3		1.4.20 Centrifuge capable of holding 50 mL polypropylene tubes.
K	3		1.4.21 Microcentrifuge capable of holding 1.5 mL microcentrifuge tubes and generating a minimum of 16000 g or equivalent is used.
		1.5 Reagents and Reference Solution Preparation and Storage	
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
C	**8**		**1.5.2 Water is glass distilled or deionized and exceeds 0.5 megaohm resistance or is less than 2 µSiemens/cm conductivity at 25 ºC to be tested and recorded monthly for resistance or conductivity and the results are recorded.**
K	8		1.5.3 Water is analyzed for residual chlorine monthly and is at a nondetectable level (≤0.1 ppm) Results are recorded and records are maintained.

K	8		1.5.4 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained.
K	8		1.5.5 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.7 The binary mobile phase system used to analyze the GTX and STX toxins consists of:** **1.5.7.1 Mobile Phase A, which contains 11 mM heptane sulfonate and 5.5 mM phosphoric acid (H_3PO_4), pH 7.1.** **1.5.7.2 Mobile Phase B, which contains 11 mM heptane sulfonate, 16.5 mM H_3PO_4 and11.5% acetonitrile (MeCN), pH 7.1.**
C	**3**		**1.5.8 The binary mobile phase system used to analyze the C toxins consists of:** **1.5.8.1 Mobile Phase A, which contains 2 mM tetrabutyl ammonium phosphate, pH 5.8.** **1.5.8.2 Mobile Phase B, which contains 2 mM tetrabutyl ammonium phosphate in 4% acetonitrile, pH 5.8.**
C	**3**		**1.5.8 The post-column oxidant consists of 100 mM H_3PO_4 and 5 mM periodic acid (H_5IO_6), pH 7.8.**
C	**3**		**1.5.9 The post-column acid used is 0.75 M nitric acid (HNO_3).**
C	**3**		**1.5.10 The heptane sulphonate used in mobile phase A and mobile phase B to analyze for GTX and STX toxins is prepared the day of use or refrigerated for up to one week.**
C	**3**		**1.5.11 The pH of mobile phases and the post-column oxidant are adjusted as follows:** **a. Mobile phase A and mobile phase B for the GTX and STX toxins are adjusted to 7.1 with ammonium hydroxide (NH_4OH),** **b. Mobile phase A and mobile phase B for the C toxins are adjusted to 5.8 in one direction only with 10% acetic acid (HOAc) if too basic or 1% NH_4OH if too acidic, and** **c. The post-column oxidant is adjusted to 7.8 with 5 M sodium hydroxide (NaOH).** **d. Mobile phases and post-column reagents are filtered before use if the HPLC does not have a degreaser.**
C	**3,7**		**1.5.12 Only certified reference materials are used for standard solutions. Source of the reference standard:**
C	**7**		**1.5.13 NRC Zero-Mus or a negative control matched matrix is used as a matrix blank as appropriate. Source of the negative matrix:_____**
C	**7**		**1.5.14 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.15 All standards used are within expiration date.**
C	**3**		**1.5.16 All standards are prepared gravimetrically.**
K	3		1.5.17 Intermediate mixes of primary standards are made up in 0.003 M HCl for the GTX/STX toxins or pH 5 glass distilled/deionized water for the C toxins labeled with the date of preparation and the expiration date and stored appropriately. The pH of the glass distilled/deionized water is adjusted when necessary by the dropwise addition of 10% acetic acid (HOAc).

C	**3**		**1.5.18**	**Working standards are made up from primary standard or intermediate mixes by dilution with toxin-free, deproteinated, matrix matched extracts.**
C	**7**		**1.5.19**	**Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.20**	**Quality Control shellfish tissues are stored frozen.**
C	**7**		**1.5.21**	**Working standards are labeled with the date of preparation, stored appropriately and used within 3 months of preparation.**
		1.6	**Collection and Transportation of Samples**	
O	6		1.6.1	Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6		1.6.2	Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**6**		**1.6.3**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	14		1.6.4	Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**6**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR PSP TOXINS				
		2.1	**Preparation of Sample**	
C	**6**		**2.1.1**	**At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	6		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4	The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**6**		**2.1.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8	Pieces of shell and drainage are discarded.
C	**6**		**2.1.9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**

		2.2 Digestion of Sample		
K	6		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**3**		**2.2.2**	**Five (5) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
K	3		2.2.3	The sample homogenate is extracted in a 1:1 w/v ratio with 0.1 M HCl.
K	3		2.2.4	Homogenate/acid mixture is vortexed thoroughly before boiling to completely mix the contents.
C	**3**		**2.2.5**	**To prevent toxin transformation, the pH of the homogenate/acid mixture before boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH.**
C	**3**		**2.2.6**	**Samples in capped 50 mL polypropylene centrifuge tubes are extracted in a boiling water bath for 5 minutes.**
K	3		2.2.7	The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of 5 M HCl. Any sample with a pH of less than 2.0 is discarded and extracted again.
K	3		2.2.8	The homogenate/acid mixture is allowed to separate by gravity or by centrifugation.
		2.3 Deproteination		
C	**3**		**2.3.1**	**500 µL of sample extract is deproteinated with 25 µL of 30% trichloroacetic acid, vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
C	**3**		**2.3.2**	**The pH of the deproteinated extract is adjusted with 35 µL of 1.0 M NaOH vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
K	3		2.3.3	An aliquot of the deproteinated supernatant is filtered through a 0.2 µm filter.
		2.4 Analysis		
C	**2**		**2.4.1**	**A standard calibration curve (of at least six concentrations) is performed upon initial instrument set up, following any major hardware maintenance activity, or when the continuing calibration verification (CCV) indicates significant drift (> 30% for individual toxin) from the calibration. Results are recorded and records are maintained.**
K	3		2.4.2	10 µL is injected for GTX/STX toxins and 5 µL is injected for C-toxins.
K	3		2.4.3	Samples are stored in the sample compartment of the autosampler at 4 °C during analysis. Otherwise samples must be analyzed within 20 hours if the autosampler is held at room temperature.
K	3		2.4.4	A column heater that is capable of maintaining 30-40 °C for the GTX/STX toxins and 10-20 °C for the C toxins is used in the analysis.
C	**3**		**2.4.5**	**The appropriate analytical column is used.** **a. GTX/STX Toxins: Agilent Zorbax Bonus-RP column, 4.6 mm x 150 mm, 3.5 µm or equivalent.** **b. C Toxins: Thermo BetaBasic 8, 4.6 mm x 250 mm, 5 µm or equivalent.**
		2.5 System Suitability		
K	2		2.5.1	The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for each individual toxin.

C	**3**		**2.5.2 The resolution and retention time criteria that must be met are:** **a. For GTX and STX toxins, the matrix peak must be at least 70% baseline resolved between GTX3 and GTX2.** **b. For GTX and STX toxins, GTX5 must be at least 40% baseline resolved between dcGTX3 and dcGTX2.** **c. For GTX and STX toxins, dcSTX and STX must be at least 70% baseline resolved.** **d. For GTX and STX toxins, the retention time of GTX4 must be between 5 and 7 minutes.** **e. For the C toxins, C2 must be at least 70% baseline resolved between C1 and C2.** **f. For the C toxins, the retention time of C1 must be between 4 and 7 minutes.**
C	**2**		**2.5.3 Daily injection schedules must include the adequate frequency of injection standards based on an assessment of individual standard toxin variability. Variability in peak response must be less than 10% for calculation of toxicity in samples.**
		2.6 Calculation of Toxicity	
C	**4**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** $\mu gSTXdiHCleq/100g = \mu MM \times \frac{333333.33}{111111111111} \times \frac{FFFFFFFF}{EEEEEE.FFFFFF} \times \frac{WWEE+VVFFFF}{WWEE} \times RRRRRREE \times 111111$ **Where:** **µM = Concentration of toxin in the extract, in µM;** **Fvol = Final volume of the deproteinized extract (e.g. 560 µL);** **Ext.vol = Volume of crude extract used (e.g. 500 µL);** **Wt = Weight of sample used;** **Vol = Volume of acid extractant used (e.g. 5 mL); and** **ReTx = Relative toxicity of toxin vs. Saxitoxin.**
C	**3**		**2.6.2 The individual toxicities for each toxin are summed to obtain the overall sample toxicity in µg STX equivalents/100 g (µg/100 g).**
C	**12**		**2.6.3 Any value at or above 80 µg STX equivalents /100 g of meat is actionable.**

Toxin	ReTx	Toxin	ReTx
GTX1	0.9940	NEO	0.9243
GTX2	0.3592	STX	1.0000
GTX3	0.6379	dcSTX	0.5131
GTX4	0.7261	C1	0.0060
GTX5	0.0644	C2	0.0963
dcGTX2	0.1538	C3	0.0133
dcGTX3	0.3766	C4	0.0576

REFERENCES:

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. AOAC Official Methods of Analysis (2011). AOAC Official Method 2011.02 Paralytic Shellfish Toxins in Mussels, Clams, Oysters, and Scallops Post-Column Oxidation (PCOX) Method.
4. Oshima, Y. 1995. J. AOAC Int. 78: 528-532.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16: 54-60.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2011. *NSSP Guide to the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.

LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PARALYTIC SHELLFISH POISON (PSP), PCOX COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the PSP, PCOX Component

1. **Conforms Status:** The PSP, PCOX component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

2. **Provisionally Conforms Status:** The PSP, PCOX component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. the number of critical nonconformities is **≥ 1** but **< 4**.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The PSP, PCOX component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is **≥ 4**.
   b. or the total # of Key nonconformities is **≥ 6.**
   c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____Date:_____

LEO Signature:_____Date:_____

NSSP Form *4 – PSP HPLC PCOX Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check √ those that apply)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
C	**5**		**1.1.2 ~~QA Plan is implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	**5**		**1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.**
		1.3 Work Area	
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment	
K	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit. [Only applicable if using the sample cleanup procedure]
K	5		1.4.2 The pH meter is calibrated daily when in use. Results are recorded and records are maintained. [Only applicable if using the sample cleanup procedure]
K	8		1.4.3 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment. [Only applicable if using the sample cleanup procedure]

K	8		1.4.4 The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e., pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded. [Only applicable if using the sample cleanup procedure]
K	5, 11		1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure. [Only applicable if using the sample cleanup procedure]
K	6, 2		1.4.6 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8		1.4.7 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.8 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	4,15		1.4.10 Freezer temperature is maintained at -10 ºC or below.
K	8		1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**13**		**1.4.12 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.13 All glassware is clean.
K	4		1.4.14 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 1.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 20 µL injections, d. temperature controlled column compartment capable of controlling temperature at 40 ºC, e. ultraviolet detector/diode array detector able to achieve the required sensitivity at a wavelength (A) of 242 nm, and f. a data collection system (e.g., computer, integrator).
K	2		1.4.15 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	4		1.4.16 A solid phase extraction (SPE) vacuum manifold capable of holding 3 mL cartridges is used. [Only applicable if using the sample cleanup procedure]
O	4		1.4.17 A centrifuge capable of holding 50 mL polypropylene tubes is used.
		1.5 Reagents and Reference Solution Preparation and Storage	
C	**4, 15**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	8		1.5.2 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)

K	8		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**4, 15**		**1.5.4 The mobile phase system used to analyze domoic acid consists of: 10% aqueous acetonitrile (v/v) and 0.1% trifluoroacetic acid (TFA).**
O	4		1.5.5 Mobile phase is filtered before use if the HPLC does not have a degasser.

C	**7**		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
K	4, 15		1.5.7 A cartridge wash solution is made up of 1 volume acetonitrile to 9 volumes of water (i.e., 10% aqueous acetonitrile). [Only applicable if using the sample cleanup procedure]
K	4		1.5.8 Citrate buffer (0.5 M, pH 3.2) is made up by dissolving 40.4 g citric acid monohydrate and 14 g triammonium citrate in 400 mL water, then adding 50 mL acetonitrile and diluting the total to 500 mL with water [or equivalent buffer]. [Only applicable if using the sample cleanup procedure]
C	**7**		**1.5.9 NRC CRM Zero-Mus or a negative control is used as a blank to ensure that there is no carry over between samples/standards. Source of the negative control: _____**
C	**7**		**1.5.10 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.11 All standards used are within expiration date.**
C	**2**		**1.5.12 All standards are prepared either gravimetrically or using positive displacement pipettes.**
C	**4, 15**		**1.5.13 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 50% aqueous methanol). Dilution with toxin-free, cartridge wash solution (aqueous acetonitrile) is allowed if using the diluted crude sample or the sample cleanup procedure.**
C	**7**		**1.5.14 Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.15 Quality Control shellfish tissues are stored frozen.**
		1.6 Collection and Transportation of Samples	
O	6, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**6, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**

K	14, 2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.

C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR ASP TOXINS			
		2.1 Preparation of Sample	
C	**6, 1**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., 3 geoduck gut balls).**
O	6		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**6**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8 Pieces of shell and drainage are discarded.
C	**2, 6**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meat s and their drip loss liquid or thawed homogenates with their freeze- thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction	
K	4,6		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**4**		**2.2.2 Four (4) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**4**		**2.2.3 The sample homogenate is extracted with 16 mL extraction solvent (1:1 methanol:water [also referred to as 50% aqueous methanol]).**
K	4, 15		2.2.4 Homogenate/extract mixture is centrifuged and filtered before analysis.
K	4, 15		2.2.5 The filtered extract is injected into the HPLC or loaded into the autosampler immediately.

K	4		2.2.6 When crude samples are diluted, dilutions are made by diluting 1 mL of filtered sample supernatant into a 5 mL volumetric flask and diluted with water to 5 mL.
K	4, 15		2.2.7 Crude extracts are sealed tightly and stored at -10 ºC or below.
		2.3 Sample Cleanup (Optional)	
O	4, 15		2.3.1 Three (3) mL SAX cartridges (500 mg silica derivatized with quaternary ammonium silane) are used for cleanup.
K	4		2.3.2 The SAX cartridge is conditioned with 6 mL methanol, followed by 3 mL water, followed by 3 mL extraction solvent (1:1 methanol:water).
C	**4, 15**		**2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.**
K	4, 15		2.3.4 Five (5) mL of filtered extract is loaded onto the cartridge and flowed slowly (~1 drop/s) until sample meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.5 Five (5) mL of wash solution (1:9 acetonitrile:water) is loaded to the cartridge and washed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4		2.3.6 0.5 mL of citrate buffer (0.5 M, pH 3.2) is loaded to the cartridge and flowed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.7 A 2 mL volumetric tube is placed under the cartridge and any domoic acid is eluted into the tube by loading and flowing as much citrate buffer as needed slowly (~ 1 drop/s) until the 2 mL mark is reached on the tube.
C	**4, 15**		**2.3.8 The solution is thoroughly mixed before withdrawing an aliquot for analysis.**
K	4, 15		2.3.9 The cleaned up extract is injected into the HPLC or loaded into the autosampler immediately.
		2.4 Analysis	
C	**2**		**2.4.1 A standard calibration curve (of at least six concentrations) is performed daily. Results are recorded and records are maintained.**
K	4, 15		2.4.2 Twenty (20) µL of extract is injected for analysis.
K	2		2.4.3 Samples are stored in the sample compartment of the autosampler at 4°C during analysis. Otherwise samples must be analyzed within 9 hours if the autosampler is held at room temperature.
K	4, 15		2.4.4 A column heater is used and the temperature is maintained at 40 °C during the analysis.
C	**4**		**2.4.5 The appropriate analytical column is used: 25 cm x 4.6 mm id packed with 5 µm Vydac 201TP octadecylsilica or equivalent.**
K	2		2.4.6 The column is stored following the manufacturer's instructions when not in use.
O	2		2.4.7 If a precolumn in-line filter and/or a compatible guard column (e.g., 201GCC54T) are/is used, rejection criteria are established to determine when to change the filter/guard column.
C	**2**		**2.4.8 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.**
		2.5 System Suitability	

C	**2**		**2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for domoic acid.**
C	**3**		**2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of L-tryptophan and domoic acid.**
K	2		2.5.3 Peak asymmetry is routinely monitored to evaluate the performance of the column. Results are recorded and records maintained.
C	**2**		**2.5.4 The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3.**
C	**2,4**		**2.5.5 Daily injection schedules must include the adequate frequency of injection standards and extraction blanks based on an assessment of individual standard toxin variability and lack of carry over.**
C	**2**		**2.5.6 Repeated injections of calibrated standards/samples agree within ±5% (as determined through the use of the coefficient of variation).**

			2.6 Calculation of Sample Toxicity
C	**4, 15**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** **µg/g domoic acid (DA) = DA injected × $\frac{VV}{WW}$ × (F)** **where:** **DA injected = the concentration in µg/ml of the extract injected;** **V = total volume of homogenate and extraction solvent (mL);** **W = weight (g) of tissue homogenate extracted (e.g., 4 g); and** **F = dilution factor (e.g., if SAX cleanup or crude sample dilution are performed).** **The concentration of DA injected may be determined using the nearest standard or the equation of the day's standard curve.**
C	**4, 15**		**2.6.2 Calculated domoic acid concentrations include the sum of domoic acid and epimer peaks, when the epimer represents 5% or more of the peak area.**
C	**12**		**2.6.3 Any value at or above 20 ppm (mg/kg or µg/g) domoic acid is actionable.**

REFERENCES

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6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
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12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
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LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
AMNESIC SHELLFISH POISON (ASP or domoic acid) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the ASP (domoic acid) Component

1. **Conforms Status: The ASP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The ASP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The ASP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥ 4**.
   - b. or the total # of Key nonconformities is **≥ 6.**
   - c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____Date:_____

LEO Signature:_____Date:_____

NSSP Form *5 – Domoic Acid HPLC-UV Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* detection in Oysters [PART IV] QuantStudio DX

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	~~**The QA plan is implemented.**~~ **The laboratory follows all internal policies and procedures while implementing the documented quality system.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15-minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11**	**Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22	All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.

K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3**	**The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14	Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.

C	**2**		**1.6.16**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18**	**The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19	Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation		
K	13, 14		1.7.1	Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2	Media components are properly stored in a cool dry place.
O	6		1.7.3	Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**6**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**6**		**1.7.6**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (<0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8	The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9**	**Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4	Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5	Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6	All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II – SAMPLES AND PROCESSING				
		2.1 Sample Collection, Transportation and Receipt		
C	**2, 6**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**

K	5		2.1.2	Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3	Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 6		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2	Blades of shucking knives are not corroded.
K	5		2.2.3	The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6	Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7	Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9**	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10**	**A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11**	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14**	**Samples are blended for 60 to 120 seconds until homogenous.**
		2.3 APW Enrichment		
K	5		2.3.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**2.3.2**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**2.3.3**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution_____**

C	**2, 15, 23**		**2.3.4 For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **An uninoculated APW blank will serve as the uninoculated process control.** **The process control cultures accompany the samples throughout the method. Records are maintained.**
C	**13**		**2.3.5 Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**2.3.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
PART III- PCR METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN OYSTERS SMARTCYCLER II AND AB 7500 FAST			
		3.1 PCR Reagents	
C	**14, 15**		**3.1.1 Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.1.2 Fluorescent probes are stored in light occluding tubes or containers.
C	**14, 15, 18, 19**		**3.1.3 The PCR forward and reverse primers and probes are appropriate for the platform.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE/TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.1.4 Lyophilized forward and reverse primers, and probes, are reconstituted with TE buffer to produce a 0.1 mM stock solution.**
C	**14, 18**		**3.1.5 Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.1.6 Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**

C	**21, 22**		**3.1.7**	**Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.1.8**	**PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.2 DNA Extraction		
C	**14, 18**		**3.2.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.2.2**	**Pipet tips have aerosol barriers.**
K	14, 18		3.2.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.2.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.2.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.2.6**	**One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.2.7**	**Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.2.8	A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.2.9**	**After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**
K	14, 18		3.2.10	Frozen extracts are analyzed within 6 months of frozen storage.
		3.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**3.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.3.2**	**For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.3.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.3.4**	**Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.3.5**	**Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.4 PCR		
C	**14, 19**		**3.4.1**	**If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.4.2**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.4.3	Two (2) µL of APW blank extracted from the uninoculated process control is added to a reaction tube or plate well containing 23µL of Master Mix.
K	14, 19		3.4.4	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
K	14, 19		3.4.5	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
C	**14, 19**		**3.4.6**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix as a no template control. A no template control is included with each PCR run.**

C	**14, 19**		**3.4.7**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control. A positive PCR control is included with each PCR run.**
K	14, 19		3.4.8	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.4.9**	**After centrifugation, tubes or plates are inserted into the instrument.**
		3.5 PCR Amplification		
C	**14, 19**		**3.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		3.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.5.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.6 Computation of Results		
K	14, 19		3.6.1	All runs in which the positive PCR control generates a Ct value for the target(s) of interest and the no template control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**3.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**3.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control is considered invalid and should be re-tested.**
C	**13**		**3.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from either the calculator or MPN table in conjunction with dilution selection tool in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.6.8	For APW enrichment, results are reported as MPN/g of sample.

PART IV- PCR METHOD FOR *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* DETECTION IN OYSTERS QUANTSTUDIO DX			
		4.1 PCR Reagents	
C	**14, 15**	**4.1.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15	4.1.2	Fluorescent probes are stored in light occluding tubes or containers.
C	**26**	**4.1.3**	**The PCR forward and reverse primers, and probes target.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3' Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3' Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3' WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3' WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3' Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3' Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3' Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3' Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3' TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3' TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3' Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ **<u>For Vv Real-time PCR Method</u>** vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3' vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3' vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGBNFQ
C	**14, 18**	**4.1.4**	**Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**26**	**4.1.5**	**Storage of thawed working stocks of primers and probes are stored between 2-8°C, not to exceed 2 weeks.**
C	**26**	**4.1.6**	**Storage of aliquoted working stocks of primers and probes stored in -20°C manual defrost freezer does not exceed 1 year.**
C	**26**	**4.1.7**	**Taqman Environmental Mastermix 2.0 is stored in -20°C manual defrost freezer until first use. After first use, it is stored between 2-8°C.**
C	**26**	**4.1.8**	**Internal control (IC) is stored in -20°C manual defrost freezer until first use. After first use, they are stored between 2-8°C.**
		4.2 DNA Extraction	
C	**14, 18**	**4.2.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**	**4.2.2**	**Pipet tips have aerosol barriers.**
K	14, 18	4.2.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18	4.2.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**	**4.2.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**

C	**26**		**4.2.6**	**Two-hundred (200) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**26**		**4.2.7**	**For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.**
K	26		4.2.8	Extracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen extracts are analyzed within 1 month of frozen storage.
C	**26**		**4.2.9**	**A tlh+ trh+ tdh+ *V. parahaemolyticus* (WA4647 or equivalent), a tlh+ tdh+ Orf8+ *V. parahaemolyticus* (BAA-240 or equivalent), and vvha+ *V. vulnificus* (ATCC 27562 or equivalent) cultures are extracted and combined to serve as the positive PCR (amplification) control.**
		4.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**4.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**26**		**4.3.2**	**For each reaction, add the specified amount of water, appropriate primer and probe concentrations, and Taqman Environmental Master Mix.**
K	14, 16, 18		4.3.3	The Master Mix is vortexed to mix constituents and then briefly spun immediately prior to dispensing aliquots to reaction tubes or plates.
C	**14, 16, 18, 26**		**4.3.4**	**Eighteen (18) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**4.3.5**	**Master Mix must be used on the day of preparation or stored at –20°C until time of use.**
		4.4 PCR		
C	**14, 19, 26**		**4.4.1**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 18µL of Master Mix for a total PCR reaction volume of 20µL.**
K	14, 19, 26		4.4.2	Two (2) µL of extracted blank APW from the uninoculated process control is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.3	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.4	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
C	**14, 19, 26**		**4.4.5**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as a no template control.**
C	**14, 19, 26**		**4.4.6**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as the positive PCR (amplification) control.**
K	14, 19, 26		4.4.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 30 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**16**		**4.4.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		4.5 PCR Amplification		
C	**14, 19**		**4.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		4.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**4.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		4.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**4.5.5**	**The analysis settings are adjusted as specified in the protocol.**

		4.6 Computation of Results		
K	14, 19		4.6.1	All runs in which the NTC has no amplification; positive control generates a Ct value for the target(s) of interest; and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	2		**4.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**4.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**4.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		4.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**4.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control (except NTC) is considered invalid and should be re-tested.**
C	**13**		**4.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		4.6.8	For APW enrichment, results are reported as MPN/g of sample.

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PCR MICROBIOLOGY COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the PCR Microbiology Component:

1. **Does Not Conform Status**: The PCR Microbiology component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥4 or**
   b. The total # of Key nonconformities is **≥13 or**
   c. The total # of Critical, Key and Other is **≥18**
2. **Provisionally Conforms Status**: The PCR Microbiology component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *6 – PCR Microbiology Checklist*, Rev. December 2025

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3		1.1.1 Written Plan (Check √ those items which apply).
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			f. Internal performance assessment.
			g. External performance assessment.
C	**2**		**1.1.2 ~~The QA plan is implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
C	**6**		**1.2.5 Training regarding radiation laboratory safety, handling and disposal practices is documented and records are maintained.**
C	**15**		**1.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitoxin in this assay. Alternatively, the laboratory uses less than 50 µCi per year and adheres to the American Radiolabeled Chemical (ARC) exemption status.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**

		1.4 Laboratory Equipment	
C	**4**	**1.4.1**	**Any lab equipment that may come into contact with [^{3}H]-STX at any point in the preparation or assay procedures must be specially labelled and must remain in the work area designated for low-level radiation work.**
O	5	1.4.2	The pH meter has a standard accuracy of 0.1 pH units.
K	7	1.4.3	The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8	1.4.4	The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1	1.4.5	The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.
K	1	1.4.6	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	9	1.4.7	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.
K	6	1.4.8	pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6	1.4.9	The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3	1.4.10	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
K	2	1.4.11	Refrigerator temperatures are maintained between 0 and 4 °C. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.
K	1	1.4.12	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**4, 6, 10**	**1.4.13**	**Freezer temperature used to store [^{3}H] STX standard, rat brain membrane tissue preparation, interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -80 °C or below. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.**

K	6, 10		1.4.14 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.15 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
O	8		1.4.16 All glassware is clean.
C	**3**		**1.4.17 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.18 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
C	**6**		**1.4.19 Micropipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.**
C	**11**		**1.4.20 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.21 Minimum radiation safety equipment and protocols include the following: A wipe-test is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
		1.5 Reference Solution Reagent Storage, Preparation and Security	
C	**12**		**1.5.1 [^{3}H] STX standard is stored in a freezer at -80 °C or below.**
C	**10**		**1.5.2 Concentration of [^{3}H] STX standard is calculated from the lot information provided by the supplier with each batch.**
K	6		1.5.3 Unopened diHCl STX standard may be stored at room temperature or refrigerated.
C	**10**		**1.5.4 Preparation of MOPS assay buffer includes the following:** **a. 100 mM MOPS/L.** **b. 100 mM choline chloride/L.** **c. pH adjustment to 7.4 with NaOH.** **e. refrigerated storage at 4 °C.** **d. Maintained ice cold while in use.**
C	**10**		**1.5.6 Bulk standard curve dilutions are stored at 4 °C for up to one (1) month.**
K	1		1.5.7 Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice).* b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____ c. Water contains <100 CFU/mL using the heterotrophic plate count method.

		1.6 Rat Brain Membrane Tissue Preparation and Storage		
C	**10**		**1.6.1**	**MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to MOPS/choline chloride fresh on the day of use.**
C	**10**		**1.6.2**	**The cerebral cortex of 6-week old Sprague-Dawley rats is used in membrane tissue preparations, placed in iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL) and homogenized with no visible chunks remaining in the homogenate. This procedure is repeated until twenty (20) rat brains have been processed.**
C	**10**		**1.6.3**	**The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**
K	10		1.6.4	The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).
K	10		1.6.5	The resuspended rat brain tissue preparations are pooled and the centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue**.**
K	10		1.6.6	The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.
K	10		1.6.7	The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed for 20 seconds to obtain a homogeneous membrane tissue preparation.
C	**10**		**1.6.8**	**Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months.**
		1.7 Rat Brain Membrane Tissue Protein Receptor Determination		
C	**10**		**1.7.1**	**The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube method) or equivalent.**
C	**10**		**1.7.2**	**The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined.**
K	10		1.7.3	Dilutions of the protein/receptor concentration of the rat brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous.
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS - RBA				
		2.1 Collection and Transportation of Samples		
C	**5**		**2.1.1**	**A representative sample of shellfish is collected.**
K	5		2.1.2	Shellfish samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	5		2.1.3	Shellfish samples are labeled with the collector’s name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.

C	**5**		**2.1.4 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	6, 13		2.1.5 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
		2.2 Preparation of Samples for Analysis – Homogenization	
C	**5, 6**		**2.2.1 At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8 Pieces of shell and drainage are discarded.
C	**5, 6**		**2.2.4 Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender <u>with all freeze-thaw liquid</u> for homogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender <u>with all freeze-thaw liquid</u> for homogenization.**
K	6, 13		2.2.5 Homogenates should be extracted immediately. If homogenates must be stored, they should be frozen.
		2.3 Preparation of Samples for Analysis – Extraction	
K	5, 10		2.3.1 0.1 M HCl is used for extractions.
K	5, 10		2.3.2 At least five (5) grams of tissue +/- 0.1g is extracted using a 1:1 mass to volume ratio of 0.1 M HCl.

C	**10**		**2.3.3 The pH of the sample is checked and adjusted as necessary to between 3.0– 4.0.**

C	**10**		**2.3.4 Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**
C	**6**		**2.3.5 The sample is promptly brought to a boil at 99.0 +/- 1.0 °C and gently boiled for 5 minutes.**
O	6		2.3.6 The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.7 The sample is allowed to cool to room temperature.
C	**10**		**2.3.8 The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	5, 10		2.3.9 The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water).
K	10		2.3.10 The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; then b. an aliquot of the sample is centrifuged at 3000 x g for 10 minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.11 The sample extract is analyzed immediately, refrigerated at 4 °C in a sealed container for up to 24 hours, or frozen at -20 °C.
		2.4 Sample Assay	
K	6		2.4.1 One analyst performs the entire plate set-up for the assay.
K	6		2.4.2 Microtubes containing dilutions and samples are vortexed immediately before dispensing.
K	10		2.4.3 The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).
C	**10**		**2.4.4 The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension.**
K	10		2.4.5 Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.
C	**10**		**2.4.6 The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.7 Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane.
K	10		2.4.8 All wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, in order to ensure even pressure and filtration across the plate.
C	**10**		**2.4.9 Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate).**
K	10		2.4.10 [^{3}H] STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.
C	10		**2.4.11 An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).**

K	10		2.4.12 Standard curve fitting is calculated using appropriate software program.
C	**10**		**2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.15 The IC_{50} is in acceptable range (2.0 nM +/- 30%). If the IC_{50} is outside this range, the assay results are rejected and the assay must be repeated**
C	**10**		**2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.**
K	4		2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.
		2.5 Calculation of Sample Toxicity	
C	**10**		**2.5.1 When more than one dilution falls within B/B_0 of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**
C	**10**		**2.5.2 Sample toxicity is calculated as follows:** **(nM STX equiv.) x (sample dilution) x (210 µL total volume/35 µL sample = mM STX equivalent in extract** **(nM STX diHCl equiv. in extract) x 1L/1000 mL x 372 ng/nmol x1 µg/1000 ng =µg STX diHCl equiv./mL** **µg STX diHCl equiv./mL x mL extract/g shellfish x 1000 g/kg =µg STX diHCl equiv./kg**
C	**14**		**2.5.3 Any value equal to or greater than 80 µg STX diHCl equiv./100 g) of sample is actionable.**
C	**14**		**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:**

REFERENCES

1. American Public Health Association (APHA). 1992. *Standard Methods for Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
2. American Public Health Association (APHA). 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
3. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition. APHA, Washington, D.C.
4. Appendix C: Radiation Safety Requirements, ISSC Proposal 13-114 Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination.
5. American Public Health Association (APHA). 1970. Recommended Procedures for the Examination of Sea Water and Shellfish, Fourth Edition. APHA, Washington, D.C.
6. Good Laboratory Practice.
7. Fisher J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.
9. Consult pH electrode product literature.
10. Association of Official Analytical Chemists (AOAC). 2016. Official Method 2011.27 Paralytic Shellfish Toxins (PSTs) in Shellfish Receptor Binding Assay.
11. Consult instrument manufacturer instructions.
12. Technical Data Sheet, American Radiolabeled Chemicals, Inc. 101 Arc Drive, St. Louis, MO 63146.
13. Wilt, d. s. (ed). 1974. Proceedings of the 8th National Shellfish Sanitation Workshop. U. S. Food and Drug Administration, Washington, D.C.
14. U. S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. NSSP *Guide for the Control of Molluscan Shellfish*. FDA/ISSC, Washington D.C. and Columbia, S.C.
15. U. S. Nuclear Regulatory Commission Materials, Section 30.18, 10 CFR Part 30, and American Radiolabeled Chemicals Licenses.

LABORATORY:

DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
RECEPTOR BINDING ASSAY FOR PSP COMPONENT: (Part I-II)	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical (C), Key (K), and Other (O) Nonconformities	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the RBA for PSP:

1. **Conforms Status:** The RBA for PSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The RBA for PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. The number of Critical nonconformities is ≥ 1 but < 4;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status**: The RBA for PSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is ≥4;
   b. or total # of Key nonconformities is ≥ 6;
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *11 – RBA PSP Checklist*, Rev. September 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
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SHELLFISH LABORATORY EVALUATION CHECKLIST
MARBIONC Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C – Critical K - Key O - Other N/A - Not Applicable

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	3, 6		1.1.1	Written Plan adequately covers all the following: (check √ those that apply)
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**3**		**1.1.2**	**~~QA Plan is implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	3, 6		1.3.1	Adequate for workload and storage.
O	6		1.3.2	Clean and well lighted.
O	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment		
O	4		1.4.1	The pH meter has a standard accuracy of 0.1 unit.
K	4		1.4.2	pH paper in the appropriate range (i.e., 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	3		1.4.3	The pH meter is ca libra ted daily when in use. Results are recorded, and records are maintained.
K	6		1.4.4	Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	6		1.4.5	The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions (pH 7 and 10) is used to calibrate the pH meter. Standard buffer solutions are used once and discarded.
K	3, 7		1.4.6	Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	2, 4		1.4.7	The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.

K	6		1.4.8	The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded, and records are maintained.

K	1		1.4.9	Refrigerator temperature is maintained between 0 and 4 ºC.
K	6		1.4.10	Refrigerator temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
K	11		1.4.11	Freezer temperature is maintained at -10 ºC or below.
K	6		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**9**		**1.4.13**	**All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.14	All glassware is clean.
C	**11**		**1.4.15**	**Absorbance Microplate reader equipped with filter for measurement at 450 nm is used.**
O	2		1.4.16	Absorbance Microplate reader performance is evaluated at least annually using manufacturer instructions or a check standard microplate at the appropriate wavelength (450) to assess alignment, accuracy, reproducibility, and linearity. Method used:_____
K	2		1.4.17	Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded, and records are maintained.
O	11		1.4.18	A centrifuge capable of holding 15 mL or 50 mL polypropylene tubes is used.
		1.5 Reagents and Reference Solution Preparation and Storage		
C	**11**		**1.5.1**	**All solvents and reagents used are ACS grade materials or better.**
O	6		1.5.2	Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded, and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	6		1.5.3	Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**11**		**1.5.4**	**Brevetoxin-3 (BTX-3 or PbTx-3) provided with the MARBIONC ELISA kit is used as the reference standard.**
C	**11**		**1.5.5**	**Stock standard solution is made by diluting brevetoxin-3 reference standard to 1 µg/ml in 100% methanol in a volumetric flask.**
C	**11**		**1.5.6**	**Working standard solution (100 ng/ml) is made by diluting 1 ml of stock solution to 10 ml in a volumetric flask using 100% methanol.**
K	11		1.5.7	Extraction solvent (80% methanol) is made by adding 800 ml of methanol to a 1 L graduated cylinder and bringing the total volume to 1 L with water.
K	11		1.5.8	Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are used within 1 week of preparation. pH of prepared media is determined to ensure it is consistent with manufacturers requirements. Results are recorded, and records are maintained.
K	11		1.5.9	Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are stored in refrigerator for no longer than 1 week and brought to room temperature before use.
K	11		1.5.10	Gelatin stock solution is prepared by dissolving 5 g gelatin in 100 ml water and stirring the solution over gentle heat on a stir plate until clear. Gela tin stock solution is aliquoted into smaller volumes (e.g. 15 ml centrifuge tubes) and refrigerated.

K	11		1.5.11	Blocking buffer is prepared by dissolving 1 pouch in 200 ml water. Blocking buffer solution is aliquoted into 50-ml centrifuge tubes and refrigerated.
K	11		1.5.12	PGT (PBS, 0.05% Tween, 0.5% gelatin) is made fresh daily by measuring 5 ml liquified gelatin stock solution into a 50-ml centrifuge tube and filling to 50 ml with PBS-Tween.

C	**11**		**1.5.13**	**Stock and working standard solutions are stored -10 ºC or below.**
C	**5**		**1.5.14**	**All standards used are within expiration date (or 1 year from open date if not provided).**
		1.6 Collection and Transportation of Samples		
O	4, 1		1.6.1	Shellstock are collected in clean, waterproof, puncture resistant containers.
K	4, 1		1.6.2	Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**4, 1**		**1.6.3**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	2, 10		1.6.4	Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – ASSAY OF SHELLFISH FOR NSP TOXINS				
		2.1 Preparation of Sample		
C	**4**		**2.1.1**	**At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	4		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	4		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	4		2.1.4	The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	4		2.1.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**4**		**2.1.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	4		2.1.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	4		2.1.8	Pieces of shell and drainage are discarded.
C	**2, 4**		**2.1.9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**

		2.2 Sample Extraction		
K	4		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**11**		**2.2.2**	**One (1) gram of homogenized sample is weighed into a 15 ml or 50 ml polypropylene centrifuge tube and subsequently extracted.**
C	**11**		**2.2.3**	**The sample homogenate is extracted by adding 9 ml extraction solvent (80% aqueous methanol) and vortexing at highest speed for 2 minutes.**
C	**11**		**2.2.4**	**The homogenate/methanol mixture is centrifuged at a minimum of 3000*xg* for 10 minutes.**
C	**11**		**2.2.5**	**The supernatant is transferred to a clean, labeled graduated 15-ml centrifuge tube and brought to a final volume of 10 ml with extraction solvent.**
K	11		2.2.6	Crude extracts are sealed tightly in glass vials and stored at -10 ºC or below until analyzed.
		2.3 Analysis		
C	**11**		**2.3.1**	**Only high-binding flat-bottom plates no older than 1 year are used (e.g. Nunc Maxisorp Immunoplates).**
C	**11**		**2.3.2**	**When pipetting kit reagents that are pre-diluted in glycerol (A, C, and D):** **a. the pipet tip is not pre-rinsed,** **b. only the very tip of the pipet tip is inserted into the vial to withdraw the required amount,** **c. the tip is submerged into the buffer when dispensing and rinsed several times with buffer to ensure complete transfer**
K	2		2.3.3	The working standard solution and crude sample extracts are brought to room temperature and thoroughly mixed before use. TMB is brought to room temperature in the dark prior to Assay Step 7.
C	**11**		**2.3.4**	**Assay Step 1: Reagent A is diluted by 300 (or as specified in kit instructions) in PBS, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS (no Tween for this wash step).**
C	**11**		**2.3.5**	**Assay Step 2: Each well is filled with 250 µl of blocking buffer. The plate is incubated on a plate shaker for 30 minutes. After 30 minutes, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**
C	**11**		**2.3.6**	**Assay Step 3a: Crude sample extracts are diluted with PGT for analysis. The minimum dilution for shellfish extracts is 1:40 (25 µl + 975 µl PGT) (resulting in a sample dilution of 1:400). Serial dilutions (n=7) of each crude sample extract are prepared in PGT.**
C	**11**		**2.3.7**	**Assay Step 3b: A standard calibration curve of seven concentrations (0.078-5.0 ng PbTx-3/ml) is prepared in PGT and is included on each plate.**
C	**11**		**2.3.8**	**Assay Step 4: 100 µl of each sample or standard dilution is loaded on to the microplate as well as two reference wells (containing PGT only) adjacent to each set of sample dilutions. Each dilution of standard or sample is added to duplicate wells. Plate layout identifying locations of samples and standards on the plate is documented.**
C	**11**		**2.3.9**	**Assay Step 5: Reagent C is diluted by 300 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate (which contains samples or standards), and the plate is incubated on a plate shaker for 90 minutes. After 90 minutes, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**

C	**11**		**2.3.10 Assay Step 6: Reagent D is diluted by 800 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, all wells are rinsed 3 times with 300 µl PBS-Tween, and one final time with 300 µl PBS only to ensure no Tween remains on the plate.**

C	**11**		**2.3.11 Assay Step 7: Each well is filled with 100 µl of room temperature TMB (3,3'5,5'-Tetramethylbenzidine) and incubated until a blue color develops in the reference wells. The reaction is stopped by adding 100 µl of 0.5M sulfuric acid solution to each well, and the absorbance in the wells at 450 nm is measured in a microplate reader.**
K	11		2.3.12 Plates are covered with microplate sealing film during all incubation steps (Steps 1-6 above).
C	**11**		**2.3.13 Plates are protected from light by covering with aluminum foil during color development (Step 7 above).**
K	11		2.3.14 The timing of the final step is reassessed with each new lot of kit reagents and each new lot of TMB to ensure that reference well absorbance values fall into an appropriate range.
		2.4 Quality Control	
C	**11**		**2.4.1 Acceptance of assay (plate) results is dependent on meeting the following criteria:** **a. Absorbance of standard reference wells (Amax) must be ≥ 0.6.** **b. CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (30-70% inhibition) must be < 20%.**
C	**11**		**2.4.2 Acceptance of individual sample results is dependent on meeting the following criteria:** **a. CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (30-70% inhibition) must be < 20%.** **b. CV of calculated concentrations of different sample dilutions within the linear range of the assay (30-70% inhibition) must be < 20%.**
		2.5 Calculation of Sample Toxin Concentration	
C	**11**		**2.5.1 Absorbance values are converted to % color inhibition:** **a. % inhibition = [1-(Avg of duplicate A/Amax)] x 100%where Amax is the average absorbance of the reference wells oriented below the sample or standard dilutions**
C	**11**		**2.5.2 Using the 4-parameter logistic (4PL) curve in an appropriate software program, a curve is fit to the positive control with ng toxin/ml on the x-axis (log scale) and % inhibition on the y-axis (linear scale).**
C	**11**		**2.5.3 The concentrations for sample dilutions falling within 30%-70% inhibition are interpolated from the standard curve.**
C	**11**		**2.5.4 Sample toxin concentration is calculated by multiplying the interpolated concentration by the sample dilution factor and dividing by 1000 to obtain PbTx-3 eq. results in ppm.**
C	**11**		**2.5.5 If more than one dilution of a sample falls within 30%-70%, the mean of the two calculated concentrations is used.**

C	**8**		**2.5.6**	**A result of ≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters is considered negative and can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.** **A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.8 ppm in oysters) requires additional testing by an Approved Method to support management actions.**
O	8		2.5.7	Laboratory reports to the Shellfish Management Authority detail sample date, location, species (matrix), date tested, analyst name, result of ELISA in ppm, and the actionable ELISA threshold for the species tested.

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
4. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
5. Consult reference standard product literature.
6. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
7. Consult pH electrode product literature.
8. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
9. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
10. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.
11. MARBIONC Enzyme-linked Immunosorbent Assay (ELISA) for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish. (ISSC proposal 17-107, supporting documents Appendix A)

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
NEUROTOXIC SHELLFISH POISON (NSP or Brevetoxin) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the brevetoxin (NSP) ELISA Component

1. **Conforms Status:** The NSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities.
   b. and <6 Key nonconformities.
   c. and <12 Total nonconformities**.**

2. **Provisionally Conforms Status:** The NSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. the number of critical nonconformities is **≥ 1** but **< 4**.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The NSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is **≥ 4**.
   b. or the total # of Key nonconformities is **≥ 6.**
   c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

NSSP Form *8 – MARBIONC Brevetoxin ELISA Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Diarrhetic Shellfish Poisoning Toxins (DSP) LC-MS/MS

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "✓"**

C – Critical K - Key O - Other NA - Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 7, 8		1.1.1 Written Plan adequately covers all the following: (check those that apply)
			a. Organization of the laboratory
			b. Staff training requirements
			c. Standard operating procedures
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established
			e. Laboratory safety
			f. Internal performance assessment
			g. External performance assessment
C	**5**		**1.1.2 ~~The QA plan is implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
C	**3**		**1.2.5 LC-MS Operator must be trained in the operation and maintenance of the specific liquid chromatography-mass spectrometry system used.**
		1.3 Work Area	
O	1		1.3.1 Adequate for workload and storage.
O	1		1.3.2 Clean and well lighted.
O	1		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment	
C	**3**		**1.4.1 A heat block or water bath capable of heating samples to 76 ± 2 °C.**
K	2		1.4.2 Balances provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	7, 8		1.4.3 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.4 Refrigerator temperature is maintained between 0 and 4 ºC.
K	7		1.4.5 Refrigerator temperature is monitored at least once daily on working days. Results are recorded and records maintained.
K	2		1.4.6 Freezer temperature is maintained at -10 ºC or below.

K	7		1.4.7 Freezer temperature is monitored at least once daily on working days. Results are recorded and records maintained.
C	**10**		**1.4.8 All in-service thermometers are properly calibrated and immersed.**
K	4		1.4.9 All glassware is clean.
K	3, 12		1.4.10 An ultra-performance liquid chromatography system (UPLC) equipped with the following is used: a. mobile phase system capable of delivering a pulse-free flow of 0.12 mL/min b. solvent degasser (optional) c. autosampler (refrigerated preferred) with loop suitable for five (5) µL injections d. column compartment capable of controlling temperature at 40 ºC e. a data collection system (e.g., computer, integrator)
C	**3**		**1.4.11 A mass spectrometer equipped with the following is used:** **a. an electrospray ionization source operating in negative ion mode and** **b. multiple reaction monitoring scan mode capability.** **c. if a divert valve is used to divert LC flow at the beginning and end of each chromatographic run, the switching time should be at least one minute before the first peak elution and at least one minute after the last peak elution.**
K	2		1.4.12 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.13 A centrifuge capable of generating 2000 x g and holding 15 mL and 50 mL polypropylene tubes is used.
		1.5	**Reagents and Reference Solution Preparation and Storage**
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	7		1.5.2 Water contains < 100 CFU/ml determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	7		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.4 The mobile phase system used to analyze DSP toxins consists of: A: 2 mM ammonium formate and 50 mM formic acid in water B: 2 mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water**
O	2		1.5.5 Mobile phase is degassed manually before use if the UPLC does not have a degasser or if the degasser is not in use.
C	**3**		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
C	**6**		**1.5.7 All primary standards are stored appropriately as per supplier recommendations.**
C	**6**		**1.5.8 All standards used are within their expiration date.**

C	**2, 3**		**1.5.9 All standards are prepared either gravimetrically or using appropriate positive displacement pipettes or syringes.**
C	**3**		**1.5.10 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 100% methanol).**
		1.6 Collection and Transportation of Samples	
O	5, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	5, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**5, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**
K	2		1.6.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle the samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
		PART II – EXAMINATION OF SHELLFISH FOR DSP TOXINS	
		2.1 Preparation of Sample	
C	**2**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., three (3) geoduck gut balls).**
O	5		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	5		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for five (5) minutes.
K	5		2.1.8 Pieces of shell and drainage are discarded.

C	**2, 5**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction	
K	2		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**3**		**2.2.2 Two (2.00) ± 0.05 g of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**3**		**2.2.3 The sample homogenate is extracted with 9 mL of 100% methanol and vortexed to mix.**
K	3		2.2.4 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant decanted into a clean container (e.g. new polypropylene tube or glass vial).
C	**3**		**2.2.5 The tissue pellet is reextracted with nine (9) mL of 100% methanol and homogenized to mix.**
K	3		2.2.6 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant combined with the supernatant in 2.2.4.
K	3		2.2.7 The total extract volume is adjusted to 20 mL with 100% methanol.
K	3		2.2.8 The crude extract is hydrolyzed or stored in the freezer at < -10 °C.
		2.3 Sample Hydrolysis and Cleanup	
K	3		2.3.1 A two (2) mL aliquot of the sample extract is transferred to an appropriately size* glass tube with a phenolic PTFE lined screw cap using a positive displacement pipette or syringe. *Note: A 16 x 100 mm tube will have sufficient volume to perform the hydrolysis and hexane wash steps and fit in a standard 15 mL centrifuge tube adaptor.
K	3		2.3.2 The sample extract is hydrolyzed by adding 250 µL of 2.5 M NaOH and the sample is homogenized with a vortex mixer for 30 seconds.
C	**3**		**2.3.3 Sample tube caps are securely fastened to prevent extract loss, and the weight of the sample tube is recorded. The sample tube is heated at 76 °C for 40 minutes, then allowed to cool to room temperature, dried, and re-weighed. If the weight has dropped by more than 0.1 g, lost volume is replaced using 100% MeOH.**
K	3		2.3.4 Samples are neutralized with 250 µL of 2.5 M HCL and vortexed to mix.
O	3		2.3.5 Nonpolar lipids are removed by adding seven (7) mL of hexanes to the hydrolyzed sample extract and vortexing to mix (2.3.5 – 2.3.7 Preferred).
K	3		2.3.6 The sample extract/hexane mixture is partitioned by centrifuging for 10 minutes at 2000 x g (2.3.5 – 2.3.7 Preferred).
K	3		2.3.7 The hexane layer is removed with a glass pipette and one (1) mL of the hydrolyzed methanolic extract is removed and filtered into an LC-MS glass autosampler vial using a 0.2 µm PTFE syringe tip filter (2.3.5 – 2.3.7 Preferred).

<table>
<tr><td>K</td><td>2, 3</td><td></td><td>2.3.8 The cleaned-up extract is loaded into the autosampler immediately for analysis.</td></tr>
<tr><td colspan="2"></td><td colspan="2">2.4 Analysis</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.1 Analytes are detected in standards and samples using the mass transitions in the table (negative ion mode).
<table>
<tr><th>Compound</th><th>Q1 (m/z)</th><th>Q3 (m/z)</th></tr>
<tr><td>OA</td><td>-803.5</td><td>-255.2</td></tr>
<tr><td>OA</td><td>-803.5</td><td>-113.1</td></tr>
<tr><td>OA</td><td>-803.5</td><td>-151.1</td></tr>
<tr><td>DTX2</td><td>-803.5</td><td>-255.2</td></tr>
<tr><td>DTX2</td><td>-803.5</td><td>-113.1</td></tr>
<tr><td>DTX2</td><td>-803.5</td><td>-151.1</td></tr>
<tr><td>DTX1</td><td>-817.5</td><td>-255.2</td></tr>
<tr><td>DTX1</td><td>-817.5</td><td>-113.1</td></tr>
<tr><td>DTX1</td><td>-817.5</td><td>-151.1</td></tr>
</table></td></tr>
<tr><td>K</td><td>3</td><td></td><td>2.4.2 Other system parameters such as collision energy are optimized for the specific system using standards before analysis.</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.3 A standard calibration curve of at least six (6) concentrations is performed at the beginning of each run. An additional curve is required if a run lasts longer than 24 hours.</td></tr>
<tr><td>K</td><td>3</td><td></td><td>2.4.4 Five (5) µL of extract is injected for analysis.</td></tr>
<tr><td>K</td><td>2, 12</td><td></td><td>2.4.5 Samples are stored in the sample compartment of the autosampler at ≤ 10°C during analysis. Otherwise the samples must be analyzed within 24 hours if the autosampler is held at room temperature.</td></tr>
<tr><td>K</td><td>3</td><td></td><td>2.4.6 A column heater is used and the temperature is maintained at 40 °C, with a tolerance as specified by the manufacturer, during the analysis.</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.7 An Acquity UPLC BEH C18 1.0 × 150 mm, 1.7 µm particle size (or equivalent) analytical column is used for analyte separation</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.8 Analytes are separated on the LC column using gradient elution.</td></tr>
<tr><td>K</td><td>2</td><td></td><td>2.4.9 The column is stored following the manufacturer’s instructions when not in use.</td></tr>
<tr><td>K</td><td>2</td><td></td><td>2.4.10 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.4.11 A matrix or procedural blank (i.e. NRC CRM Zero-Mus or equivalent negative matrix, or methanol carried through sample preparation process at the same time as the samples) should be included in each analytical batch.</td></tr>
<tr><td colspan="2"></td><td colspan="2">2.5 System Suitability</td></tr>
<tr><td>C</td><td>3</td><td></td><td>2.5.1 The retention time of analytes in all samples are within 3% that of one of the intermediate toxin standards, measured from the apex of the peak.</td></tr>
</table>

C	**3**		**2.5.2 Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (Rs) of OA/DTX2 should ≥ 1 when calculated using the equation below (RT is retention time and W is peak width at baseline; Peak 1 is OA and Peak 2 is DTX2).** $$Rs = 2 \times (RT2 - RT1)/(W1 \mp W2)$$
K	2, 3		2.5.3 Each chromatographic peak must be defined by at least 10 data points.
C	**3**		**2.5.4 Reagent blanks (methanol) are analyzed after the high calibration standard, periodically (as determined by the laboratory's internal verification), and after fortified samples to ensure that analyte carryover is not occurring. Analyte carryover is defined as a confirmed peak > LOD.**
C	**3**		**2.5.5 To confirm the presence of each DST, two (2) mass transitions must be observed above the limit of detection (LOD).** **The transition yielding the highest signal-to-noise ratio (S/N) is used for quantitation (i.e. 817.5 → 255.2 for DTX-1, 803.5 → 255.2 for OA and DTX-2). The transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation is ≥ 3.**
C	**3**		**2.5.6 The ratio of the abundance of the confirmation ion transition to the quantitation ion transition is calculated for each toxin. These ion ratios must be within ± 20% of that of the toxin standards in order to confirm toxin identity.**
C	**3**		**2.5.7 When ≥ 10 samples are analyzed, analysts must show that the calibration has not significantly drifted using an option provided below:**
		OPTION 1 – USE OF BRACKETING CALIBRATION CURVES	
C	**3**		**2.5.7.1 A second standard curve is analyzed at the end of the analytical sequence and the averaged peak areas are used for the linear regression.**
C	**3**		**2.5.7.2 The linear regression of the averaged calibration curves must yield an $R^2 \geq 0.98$. Results are recorded and records are maintained.**
C	**3**		**2.5.7.3 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The average of bracketing calibration curves yields an $R^2 < 0.98$.** **b) The difference in the slope between bracketing calibration curves exceeds 25%.** **c) The difference in retention times of the standards in the bracketing standard curves exceeds 3%.**

		OPTION 2 – USE OF A CONTINUING CALIBRATION VERIFICATION (CCV) STANDARD	
C	**3**		**2.5.7.4 The linear regression of the single calibration curve must yield an R^2 ≥ 0.99.**
C	**3**		**2.5.7.5 A continuing calibration verification (CCV) standard, matching on of the intermediate standards from the calibration curve, is analyzed after every 10 samples and at the end of a run.**
C	**3**		**2.5.7.6 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The calibration curve yields an R^2 < 0.99.** **b) The retention time of a CCV sample exceeds 3% of the corresponding standard.*** **c) The peak area of a CCV sample exceeds ± 15% compared to the corresponding standard in the calibration curve.*** *** Samples immediately preceding and post the failed CCV shall be reanalyzed with a new standard curve.**
C	**3**		**2.5.8 Repeated injections of calibration or control samples at a concentration near the action level agree within ± 10% (as determined through the use of the coefficient of variation).**
		2.6 Calculation of Sample Toxicity	
C	**3, 11**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** $\frac{ug}{g} toxin = C \times \frac{V}{W}$ **X Hyd X ReTx** **where:** **C = the concentration in µg/ml of the extract injected, determined using the standard curve** **V = total volume of homogenate and extraction solvent (mL)** **W = weight (g) of tissue homogenate extracted** **Hyd = dilution factor for hydrolysis (1.25)** **ReTx = relative toxicity of toxin vs. Okadaic Acid** **Relative Toxicity Values**

Toxin	ReTx
OA	1
DTX1	1
DTX2	0.6

			The individual toxicities for each toxin are summed to obtain the overall sample toxicity in ppm OA equivalents.
C	**9**		**2.6.2 Any value at or above 0.16 ppm OA equivalents (mg/kg or µg/g) of the sum of any analytes present is actionable. Shellfish Program Management is made aware of positive result. Laboratory action is_____ .**

REFERENCES

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4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
5. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
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   D.C. and Columbia, S.C.
10. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
11. The EFSA Journal. 2009. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. 1306, 1-23.
12. Deeds, J.R. and M.D. Celiz, Personal Communication, Addendum to proposal 19-136 DSP LC MS/MS Additional Ruggedness Testing; effect of the sample storage temperature during analysis (24 hrs), Email Received by ISSC Checklist Subcommittee 2/18/2021.

LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
DIARRHETIC SHELLFISH POISON (DSP) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the DSP Component

1. **Conforms Status: The DSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The DSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The DSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥4**.
   - b. or the total # of Key nonconformities is **≥6.**
   - c. or the total # of Critical, Key, or Other is **≥12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *10 – DSP LC-MS/MS Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Analysis for NSP (Mouse Bioassay)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART 1 - QUALITY ASSURANCE			
CODE	**ITEM**		
	1.1 Quality Assurance (QA) Plan		
C		**1.1.1**	**Written Plan adequately covers the following (check those that apply):**
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, calibration, maintenance, repair and performance.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C		**1.1.2**	**~~QA Plan is implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
	1.2 Work Area		
O		1.2.1	Adequate for workload and storage.
O		1.2.2	Clean and well lighted.
O		1.2.3	All work surfaces are nonporous and easily cleaned.
C		**1.2.4**	**A separate, quiet area with adequate temperature control is maintained for acclimation and injection of mice.**
	1.3 Laboratory Equipment		
K		1.3.1	The differing sensitivities in weight measurements required by various steps in the extraction procedure as well as the bioassay are met by the balances being used. a. To determine sample weight, a sensitivity of at least 0.1 g at load of 100 g is required. b. To determine the weight of the lipid extract and its subsequent volume adjustment, a sensitivity of at least 10 mg at loads of 1 and 10 g is required. c. To determine the weight of the mice used in the bioassay, a sensitivity of 0.1 g at a load of 20 g is required.
O		1.3.2	The calibrations of the balances are checked monthly using NIST Class S or ASTM Class 1 or 2 weights or equivalent. Records are maintained.
K		1.3.3	The temperature maintained by the refrigerator is between 0 and 5°C.
O		1.3.4	Refrigerator temperature is monitored at least once daily. Temperatures are recorded and records are maintained.
	1.4 Reagents		
K		1.4.1	Concentrated (12N) HCl is used to acidify the homogenate.
O		1.4.2	Reagent grade NaCl is used in the extraction procedure.
C		**1.4.3**	**Diethyl ether purified for lipid extraction is used for extracting lipids from the shellfish homogenates.**

C		**1.4.4**	**Cottonseed oil (0.917 g/ml) or a solvent with a similar density (0.915 to 0.927 g/ml) is used as the toxin delivery system. Name of the solvent if substituted for cottonseed oil.** **Name:_____ Specify density:_____**

	1.5 Collection and Transportation of Samples		
O		1.5.1	Shellstock are collected in clean, waterproof, puncture resistant containers.
K		1.5.2	Samples are appropriately labeled with the collector's name, the harvest area and the time and date of collection.
K		1.5.3	Immediately after collection, shellstock samples are placed in dry storage between 0 and 10°C until analyzed.
K		1.5.4	Shellstock samples are analyzed within 24 hours of collection or refrigerated unshucked until analyzed.
K		1.5.5	Refrigerated storage of shellstock does not exceed 48 hours.
K		1.5.6	If shellstock is refrigerated, only live animals are used in the analysis.
K		1.5.7	If shellfish are shucked in a location other than the laboratory, they must be prepared according to steps 1-12 in "Preparation of Sample" section below.
PART II – ANALYSIS OF SHELLFISH FOR NSP TOXIN - MBA			
	2.1 Preparation of Sample		
C		**2.1.1**	**At least 12 animals are used per sample.**
O		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
K		2.1.3	Shellstock are opened by cutting the adductor muscles.
C		**2.1.4**	**Shell liquor is discarded.**
O		2.1.5	The inside of the shells is rinsed with fresh water to remove sand or other foreign material.
K		2.1.6	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
K		2.1.7	Damage to the body of the mollusk is minimized in the process of opening.
K		2.1.8	100 – 150 grams of meat are collected or all the available sample if there is less than 100 grams.
O		2.1.9	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K		2.1.10	Pieces of shell and drainings are discarded.
C		**2.1.11**	**Drained meats are blended at high speed until homogenous (60-120 seconds).**
C		**2.1.12**	**Shellfish homogenates are digested within 2 hours of blending.**
	2.2 Digestion of Sample		
K		2.2.1	All glassware used is clean and properly washed with a succession of at lea st three fresh water rinses, and a final distilled/deionized rinse to remove residual detergent.
K		2.2.2	100 grams (or entire sample amount if less than 100 grams is available) of homogenized sample is weighted into a beaker.

C		**2.2.3**	**1 ml of concentrated HCl and 5 g NaCl is added to the 100 gram homogenate and thoroughly mixed. (For samples <100 g, add reagents to obtain final concentrations of 0.12N HCl and 5% NaCl.)**
C		**2.2.4**	**The homogenate is brought to a boil and once 100 ± 1°C (sea level) is reached, gently boil for 5 minutes.**
O		2.2.5	The beaker is covered with a watch glass or equivalent during boiling to prevent excessive evaporation.
O		2.2.6	The homogenate is boiled under adequate ventilation (fume hood).
O		2.2.7	The boiled, acidified homogenate is cooled to room temperature or below in a refrigerator or in an ice bath.
	2.3 Extraction		
C		**2.3.1**	**All steps in the extraction procedure which involve any manipulation of diethyl ether are carried out under adequate.**
C		**2.3.2**	**100 ml of diethyl ether is added to the cooled, acidified homogenate in a stoppered centrifuge tube and shaken vigorously for 5 minutes.**
O		2.3.3	Centrifuge tubes are vented frequently while being shaken and before being centrifuged to avoid accidents.
C		**2.3.4**	**The content of the centrifuge tubes are centrifuged at 2000 rpm for 10 to 15 minutes.**
C		**2.3.5**	**The clear upper ether phase is transferred to a large separatory funnel.**
C		**2.3.6**	**The contents of the centrifuge tube are extracted three additional times for a total of four times, each time with 100 ml of diethyl ether. The upper phases are combined together in the separatory funnel (as in step 5).**
C		**2.3.7**	**The ether extract is transferred to a large, clean, dry pre-weighed beaker (discard any emulsion or tissue that may have settled in the funnel.)**
C		**2.3.8**	**Ether is evaporated to dryness.**
C		**2.3.9**	**The final lipid residue is weighted and the weight is recorded.**
	2.4 Bioassay		
C		**2.4.1**	**The volume of the lipid residue is adjusted by weight to 10 ml (9.17 g) per 100 g shellfish extracted using cottonseed oil. If a solvent with a density similar to cottonseed oil is used, the volume is adjusted to a weight 10 times the density of the solvent. Specify the weight to which the volume is adjusted to.**
K		2.4.2	A 25 gauge hypodermic needle is used for injection.
C		**2.4.3**	**Healthy male mice in the weight range of 17 to 23 grams from a stock colony are used for routine assays.** **Stock strain used: _____ Source of the mice: _____**
C		**2.4.4**	**Mice are allowed to acclimate for at least 24 hours prior to injection. In some cases up to 48 hours may be required.** **Typical length of the period of acclimation is: _____**
O		2.4.5	Mice are weighed to the nearest 0.1 gram.
C		**2.4.6**	**The extract is completely mixed before it is injected.**
C		**2.4.7**	**Mice are injected intraperitoneally with 1 ml of the lipid extract.**

C		**2.4.8 A total of 5 mice are injected with undiluted or diluted extract as appropriate per sample in routine assays.** **a. The extract is not diluted when all test/assaymice survive beyond 110 minutes of injection.** **b. The extract is diluted when 2 of 2 test mice or 3 of 5 assay mice survive for fewer than 110 minutes after injection** **c. When dilution is required, only dilutions which produce mean/median death times within 110 to 360 minutes of injection are used in the analysis.**
C		**2.4.9 The time of completed injection is recorded.**
C		**2.4.10 Mice are continuously observed for at least 6 hours (360 minutes).**

C		**2.4.11 If death occurs within the period of continuous observation, the time of death to the nearest minute is noted by the last gasping breath.**
K		2.4.12 If mice survive thetest, the time of death is recorded as ">" the period of continuous observation.
	2.5 Calculation of Toxicity	
C		**2.5.1 The death time of each mouse is converted to mouse units (MU) using Table 8 in *Recommended Procedures*, 4th Edition.**
O		2.5.2 Table 8 is interpolatedfor death times between 110 and 360 minutes that are not listed in the Table.
K		2.5.3 A weight correction in MU is made for each mouse injected using Table 8 in *Recommended Procedures*, 4th Edition.
O		2.5.4 Table 8 is interpolated to accommodate weights which are not listed.
C		**2.5.5 The death time for each mouse in MU is multiplied by a weight correction in MU to give the corrected mouse unit (CMU) for each mouse.**
C		**2.5.6 The mean corrected mouse unit of the array of corrected mouse units (CMU) is used when all the mice injected with diluted or undiluted extract die during the period of continuous observation.**
C		**2.5.7 The median corrected mouse unit of the array of corrected mouse units (CMU) is used when at least one mouse either survives the test or dies.**
C		**2.5.8 The concentration of toxin is determined by the formula: Mean or median CMU x Dilution Factor x 10.**
C		**2.5.9 When the time of death is known for certain for all mice injected, toxicity is determinate and the toxin concentration is reported as the number of mouse units per 100 grams of sample.**

LABORATORY:	DATE OF EVALUATION:
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**	

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
NEUROTOXIC SHELLFISH POISON COMPONENTS (NSP MBA): PART I and II	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the NSP Component

1. **Does Not Conform Status** The NSP MBA component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥ 3** or
   b. The total # of Key nonconformities is **≥ 6** or
   c. The total # of Critical, Key and Other is **≥ 10**

2. **Provisionally Conforms Status**: The NSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1** but **< 3**

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____Date:_____

LEO Signature:_____Date:_____

NSSP Form *3-NSP MBA Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Alkaline Phosphatase Probe

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating [PART III]
	Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation [PART III]
	Alkaline Phosphatase Probe Hybridization [PART III] - **Note: Temperature ranges for hybridization can be expanded with an appropriate study as described in the method.**

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (check those items which apply).
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**~~The QA plan is implemented.~~ The laboratory follows all internal policies and procedures while implementing the documented quality system.**
K	6		1.1.3	The Laboratory participates in a Vibrio proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well-lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of at least 0.1 pH units.
K	9		1.4.2	The pH electrodes being used consist of a pH half-cell and reference half-cell or equivalent combination electrode free from Ag/AgCl or contains an ion exchange barrier preventing passage of Ag ions into the solution which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment.
K	4		1.4.4	The pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	6		1.4.5 A minimum of two (2) standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
K	4, 17		1.4.6 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope *(Circle the method used).*
K	5, 15		1.4.7 The balances used provide a sensitivity of at least 0.01 g at the weights of use for direct plating and 0.1 g for MPN.
K	6		1.4.8 Balance calibrations are checked monthly according to manufacturer specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance calibrations is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12, 15**		**1.4.10 Refrigerator temperatures in which AP-probes are stored are maintained between 2 and 8 ºC.**
K	1		1.4.11 The temperature of general purpose refrigerators, those not containing AP-probes, are maintained between 0 and 4 ºC.
C	**2**		**1.4.12 Freezer temperatures are maintained at -15 ºC or below.**
K	6		1.4.13 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**12**		**1.4.14 The temperature of the incubator is maintained at 35 ± 2.0 ºC.**
C	**6**		**1.4.15 Working thermometers used in the air incubators are graduated in at least 0.5 ºC increments.**
K	5, 8		1.4.16 Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
C	**6**		**1.4.17 Temperature of the water bath is maintained appropriately under all loading conditions.**
C	**5**		**1.4.18 Working thermometers used in the water bath are graduated in at least 0.1 ºC increments.**
K	4, 6		1.4.19 Air incubator/water bath temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.20 All working thermometers are appropriately immersed.**
C	**5**		**1.4.21 Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**5, 6**		**1.4.22 A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0, 35, 42, 54 and/or 55 ºC (54 ºC for *Vibrio parahaemolyticus* and 55 ºC for *Vibrio vulnificus*). These calibration records (certificates of calibration) are maintained.**
K	3		1.4.23 Standards thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____

C	**5**		**1.4.24 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with at least an accuracy of ±0.05 ºC are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.25 The accuracy of working thermometers is checked annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Results are recorded and records maintained.
O	8		1.4.26 Appropriate pipet aids are available and used to inoculate samples.
K	7		1.4.27 Micropipettors are calibrated annually and checked for accuracy quarterly at volumes of use. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing	
K	5		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and sample.
O	5		1.5.3 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method of preparation is used to ensure the appropriate volumes of diluent.
C	**5**		**1.5.5 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1 mL aliquots; nor, are pipettes larger than 1.1 mL used to deliver 0.1 mL aliquots.**
K	5		1.5.6 In washing reusable pipets, glassware and labware, a succession of at least three (3) fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**8**		**1.5.7 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.8 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded, and records maintained.**
		1.6 Sterilization and Decontamination	
K	5		1.6.1 The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2 Routine autoclave maintenance is performed, and the records are maintained.
C	**19, 20, 21**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer, or an appropriate working temperature monitoring device.**

K	2, 5, 6		1.6.4	An autoclave standards thermometer (or data logger) has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. If in-house checks for accuracy of the standards thermometer will be conducted at the steam point, calibration of the autoclave standards thermometer at 100 °C is also recommended, but not required.
K	2, 10, 18		1.6.5	The autoclave standards thermometer (or data logger) is checked every five (5) years for accuracy at either 121 °C by a qualified calibration laboratory; or, is checked in-house at the steam point (100 °C) if it has been previously calibrated at both 100 °C and 121 °C. Any change in temperature at the steam point changes the calibrated temperature at 121 °C by the same magnitude. Date of most recent determination: _____
K	2, 8		1.6.6	Working autoclave thermometers (or data loggers) are checked against the autoclave standards thermometer at 121 ºC yearly. Date of last check:_____Method:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded, and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6, 8		1.6.9	Autoclave sterilization records including the length of sterilization cycle, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle the appropriate type or types*)
K	5, 8		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 ºC.
K	8		1.6.11	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	8		1.6.12	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded, and records maintained.
K	5		1.6.13	Reusable pipets are stored and sterilized in aluminum or stainless-steel containers.
K	5		1.6.14	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 ºC for two (2) hours.
C	**2**		**1.6.15**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded, and records maintained.**
C	**2**		**1.6.16**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded, and records maintained.**
C	**2**		**1.6.17**	**The sterility of pre-sterilized disposable pipettes, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded, and records maintained.**
K	8		1.6.18	Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.

		1.7 Media and Reagent Preparation	
C	**12, 15**	**1.7.1**	**Media and reagents are prepared from the individual components and pH adjusted appropriately, except in the case of TCBS, which is commercially dehydrated.**
K	1, 5, 8	1.7.2	Dehydrated media, and media and reagent components are properly stored in a cool, clean, dry place.
K	1	1.7.3	Media and components are labeled with the analyst's initials, date of receipt, date opened or date of preparation, if applicable (dye solutions).
C	**1, 2, 7**	**1.7.4**	**Caked or expired media or components are discarded.**
C	**6**	**1.7.5**	**Reagent water is distilled or deionized (*circle appropriate choice*), tested monthly and exceeds 0.5 megohms-cm resistivity (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 ºC. (*Circle the appropriate water quality descriptor determined*). Results are recorded and the records maintained.**
C	**6**	**1.7.6**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 mg/L). Results are recorded, and records maintained.** **Specify method of determination: _____**
K	6	1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded, and records maintained.
K	12	1.7.8	The volume and concentration of media (APW) in the tube is suitable for the amount of sample inoculated.
C	**2**	**1.7.9**	**The total time of exposure of the sugar containing agar VVA to autoclave temperatures does not exceed 45 minutes. Total exposure time of APW and T1N3 agar does not exceed 60 minutes. TCBS, CC and mCPC are not autoclaved.**
C	**1**	**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded, and records maintained.**
C	**1**	**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Positive *Vibrio parahaemolyticus* productivity control_____** **Negative *Vibrio parahaemolyticus* productivity control_____** **Positive *Vibrio vulnificus* productivity control_____** **Negative *Vibrio vulnificus* productivity control_____**
C	**6, 12**	**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded, and records are maintained.**
		1.8 Storage of Prepared Culture Media and Reagents	
K	5	1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	2	1.8.2	Stored media are labeled with the storage expiration date or sterilization date.

K	2		1.8.3	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	6		1.8.4	Storage under refrigeration of prepared agar plates in sealed plastic bags shall not exceed two (2) weeks.
K	6		1.8.5	Storage under refrigeration of prepared broth media with loose fitting closures shall not exceed one (1) month.
K	6		1.8.6	Storage under refrigeration of prepared broth media and diluent with screw-cap closures shall not exceed three (3) months.
K	12, 15		1.8.7	Refrigerated prepared plates are dried inverted before use to permit the sample to be completely absorbed into the medium to prevent colony spreading, for direct plating.
K	2, 6		1.8.8	All prepared broth media and diluent stored under refrigeration are warmed to room temperature prior to use, at temperatures that do not exceed the medium's incubation temperature.
K	15		1.8.9	Storage at room temperature of Lysis Solution, Ammonium Acetate Buffer, 20XSSC, 1XSSC/SDS, and 3XSSC/SDS for the hybridization procedure shall not exceed three (3) months.
K	15		1.8.10	Storage under refrigeration of Hybridization Buffer for the hybridization procedure shall not exceed one (1) week.
C	**15**		**1.8.11**	**NBT/BCIP solution and 1XSSC for the hybridization procedure should be made fresh the day of use.**
PART II – SHELLFISH SAMPLES				
		2.1 Sample Handling and Receipt		
C	**1, 5, 12, 15**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5, 15		2.1.2	Shellfish samples are received in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	1, 5		2.1.3	Samples are received labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5, 12, 15**		**2.1.4**	**Immediately after collection, samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 ºC with ice or cold packs for transport to the laboratory or rejected. Direct contact of the shellfish with ice in the transport container should be avoided. Once received, the samples are placed under refrigeration unless processed immediately.**
K	5, 15		2.1.5	If ice is used in sample transport, samples are rejected if melt water has come in contact with the samples.
C	**15**		**2.1.6**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 hours. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 hours once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 11		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2, 11		2.2.2	Blades of shucking knives are not corroded.
K	5, 11		2.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 11		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.

K	5, 11		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5, 11		2.2.6	Shellfish are allowed to drain in a clean container or on clean towels prior to opening.
K	2, 5, 11		2.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol, or gloves are donned. The gloves, if worn, are latex, nitrile and/or stainless-steel mesh to protect analyst's hands from injury.
C	**5, 11**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5, 11, 12, 15**		**2.2.9**	**The contents of the shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**12, 15**		**2.2.10**	**A representative sample of 10 to 14 shellfish is used for analysis.**
C	**2, 11**		**2.2.11**	**The quantity of meat and liquor is sufficient to cover the blender blades or additional shellfish are used in order to ensure sample homogeneity.**
K	5, 12, 13, 15		2.2.12	Either a 1:1 dilution is made, or the sample is homogenized without dilution. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	12, 14, 15		2.2.13	Sterile phosphate buffered saline (pH 7.4) or alkaline peptone water (APW) is used as the sample diluent. If APW is used, sample analysis is conducted immediately.
C	**12, 15**		**2.2.14**	**Samples are blended for 90-120 seconds until homogenous.**
PART III – ALKALINE PHOSPHATASE PROBE METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN SHELLFISH				
		3.1 Preparation of Samples for the Alkaline Phosphatase Probe Method: Direct Plating		
C	**2, 12, 15**		**3.1.1**	**For oyster samples, two tenths (0.20) of a gram of the initial 1:1 diluted homogenate (or 0.10 g of undiluted homogenate) and/or appropriate dilutions are used as inoculum. Dilutions are made in sterile PBS or APW. If APW is used, time from initial dilution until plating does not exceed 30 minutes.** **For samples other than oysters, 100 µl of the 1:10 dilution and/or subsequent dilutions should be used as inoculum.**
K	12, 15		3.1.2	For analysis of total *V. parahaemolyticus*, at least one (1) T1N3 plate is inoculated to be probed for the *tlh* gene. For pathogenic *V. parahaemolyticus*, at least two (2) T1N3 plates are inoculated to be probed for the *tdh* gene. For analysis of *V. vulnificus*, at least one (1) VVA plate is inoculated to be probed for the *vvhA* gene.
K	12, 15		3.1.3	Sterile cell spreaders are used to spread each inoculum evenly onto the dry T1N3 and/or VVA agar plates.

C	**2**		**3.1.4**	**For *V. parahaemolyticus* analysis, a *tdh*+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**		**3.1.5**	**The process control cultures accompany the samples throughout incubation and hybridization and color development phases of the method. Results are recorded, and records are maintained.**
C	**12, 15**		**3.1.6**	**Inoculated plates are incubated 16-24 hours at 35 ± 2 ºC. All plates are used for colony lifts and hybridization, except for those with confluent growth.**
		3.2 Preparation of Samples for the Alkaline Phosphatase Probe Method: APW Enrichment and Colony Isolation		
K	11, 12		3.2.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**12**		**3.2.2**	**The 1:10 dilution is prepared gravimetrically with sterile PBS. All successive dilutions are prepared volumetrically.**
C	**12, 16**		**3.2.3**	**Appropriate sample dilutions are inoculated into sterile APW.** **Specify dilution(s) used:_____** **Specify number of tubes per dilution:_____**
C	**2**		**3.2.4**	**For *V. parahaemolyticus* analysis, a tdh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non-*V. vulnificus* culture is used as a negative process control.**
C	**2**		**3.2.5**	**The process control cultures accompany the samples throughout incubation, isolation and confirmation. Results are recorded, and records are maintained.**
C	**12**		**3.2.6**	**Inoculated APW enrichment tubes are incubated at 35 ± 2.0 ºC.**
C	**12**		**3.2.7**	**Tubes are read after 18-24 hours of incubation. Clear tubes are negative. Turbid tubes are positive. Positive tubes are confirmed as *Vibrio parahaemolyticus* or *Vibrio vulnificus* as appropriate.**
K	12		3.2.8	A loopful from the top one (1) cm of APW tubes showing growth is streaked onto TCBS for *V. parahaemolyticus* and mCPC or CC agars for *V. vulnificus* isolation.
C	**12**		**3.2.9**	**TCBS plates are incubated at 35 ± 2 ºC and mCPC or CC plates are incubated at 35-40 ºC for 18-24 hours.**

C	**12**		**3.2.10**	**Presumptive colonies are selected meeting these phenotypic characteristics:** ***V. parahaemolyticus* appear on TCBS agar as round, opaque, green or bluish colonies, two (2) to three (3) mm in diameter. Interfering large, opaque and yellow colonies are avoided.** ***V. vulnificus* appear on mCPC or CC agar as round, flat, opaque, yellow colonies, one (1) to two (2) mm in diameter. Typical positives have "fried egg" appearance. Purple/blue colonies are avoided.**
C	**12**		**3.2.11**	**A sterile 96-well microtiter plate is filled with 100 µl/well of APW. Presumptive vibrios are picked from a selective agar plate using a sterile toothpick or wood transfer stick to individual wells. The plate is incubated 3-5 hours or overnight at 35 ± 2 °C. A 48-prong replicator is used to replicate/transfer isolates in the wells to an agar plate (T1N3 for *V. parahaemolyticus* and VVA for *V. vulnificus*).**
C	**12**		**3.2.12**	**Plates are incubated at 35 ± 2 ºC for 18-24 hours.**
		3.3 Alkaline Phosphatase Probe Hybridization: Filter Preparation		
C	**12, 15**		**3.3.1**	**VVA/T1N3 plates are overlaid with labeled (sample number, dilution) #541 Whatman filters for one (1) to 30 minutes.**
K	12, 15		3.3.2	Filters are transferred with colony side up to a plastic or glass Petri dish lid containing one (1) ml of lysis solution to wet the filter.
C	**12, 15**		**3.3.3**	**Filters are microwaved to dryness, but not brown. Microwave for 15-30 seconds/filter, depending on the wattage of the microwave. Additional heating cycles may be required.**
K	12, 15		3.3.4	Filters are neutralized for five (5) minutes in an appropriate vessel or container with ammonium acetate (4 ml/filter) on a shaker at room temperature.
C	**12, 15**		**3.3.5**	**#541 Whatman filters are rinsed two (2) times in 1X SSC buffer (10 ml/filter) for 1-2 minutes. Filters may be air dried and stored at this point.**
C	**12, 15**		**3.3.6**	**Up to 30 filters are incubated in proteinase K solution (10 ml/filter) for 30 minutes at 42 °C with shaking (~50 rpm).**
K	12, 15		3.3.7	Filters are rinsed three (3) times in 1X SSC (10 ml/filter) for 10 minutes at room temperature with shaking at 50-125 rpm. Filters may be air dried and stored at this point.
		3.4 Alkaline Phosphatase Probe Hybridization: Hybridization		
C	**12, 15**		**3.4.1**	**For total *V. parahaemolyticus* (*tlh*), the 5'AP-labeled probe 5'aa agc gga tta tgc aga agc act g 3' is used.** **For pathogenic *V. parahaemolyticus* (*tdh*), the 5'AP-labeled probe 5'gg ttc tat tcc aag taa aat gta ttt g 3' is used.** **For *V. vulnificus* (*vvhA*), the 5'AP-labelled probe 5'ga gct gtc acg gca gtt gga acc a 3' is used.**
C	**12, 15**		**3.4.2**	**Probes are stored in the refrigerator and are not frozen.**
K	12, 15		3.4.3	A maximum of five (5) filters to be hybridized with the same probe are added to a plastic bag.

C	**12, 15**		**3.4.4**	**Filters are presoaked in 10-15 ml of hybridization buffer for 30 minutes at 54-± 0.1 ºC for *V. parahaemolyticus* (*tlh* and *tdh*) or 55 ± 0.1 °C for *V. vulnificus* with shaking.**
C	**12, 15**		**3.4.5**	**Used buffer is discarded and 10 ml of fresh pre-warmed buffer per bag is added. Probe (final concentration of 0.5 pmol/ml) is quickly added to each bag and incubated for 1 hour at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**
K	15		3.4.6	Filters are removed from the bag(s) and transferred to an appropriate vessel or container. Up to 30 filters hybridized with the same probe can be combined.
C	**12, 15**		**3.4.7**	**Filters are rinsed two (2) times for 10 minutes each in 1X SSC – 1% SDS (for tlh and *Vibrio vulnificus*) or 3X SSC – 1% SDS (for tdh) (10 ml/filter) at 54 ± 0.1 ºC for *Vibrio parahaemolyticus* or 55 ± 0.1 ºC for *Vibrio vulnificus* with shaking.**
K	12, 15		3.4.8	Filters are rinsed five (5) times for five (5) minutes each in 1X SSC (10 ml/filter) at room temperature with shaking.
		3.5 Alkaline Phosphatase Probe Hybridization: Color development		
C	**12, 15**		**3.5.1**	**In a petri dish containing 20 ml of NBT/BCIP solution, filters (5 or fewer) are added and incubated with gentle shaking at room temperature, or at 35 ºC for faster results. The petri dish is kept covered to omit light.**
K	12, 15		3.5.2	Color development of the positive control is checked every 30 minutes. Reaction time varies.
K	12, 15		3.5.3	Filters are rinsed in tap or deionized/distilled water (10 ml/filter) three (3) times for 10 minutes each to stop color development.
C	**12, 15**		**3.5.4**	**Reactions of test sample colonies are compared to the positive and negative process control cultures. Positive reactions appear as purple or brown spots, yellow spots are considered negative reactions. Filters are stored in the dark.**
		3.6 Alkaline Phosphatase Probe Hybridization: Computation of Results		
C	**12, 15**		**3.6.1**	**For direct plating, probe-positive colonies are counted and multiplied by the plated dilution factor of the sample to determine the concentration. Note that filter colonies must correspond to colonies visible on the agar plate.**
K	15		3.6.2	For direct plating, results are reported as CFU/g of sample.
C	**12**		**3.6.3**	**For APW enrichment, upon identification of probe-positive colonies refer to the original positive APW dilutions and record MPN value as derived in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	12, 16		3.6.4	For APW enrichments, results are reported as MPN/g of sample or pass/fail in the case of PHP samples.

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LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
ALKALINE PHOSPHATASE PROBE MICROBIOLOGICAL COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-III	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Alkaline Phosphatase Probe Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The Alkaline Phosphatase Probe Microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform Provisionally Conforms Conforms

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form *9 – Alkaline Phosphatase Probe Checklist*, Rev June 2024

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist – Microbiology modifications for consistency
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to modify text in thirty-two (32) NSSP Microbiology Checklist items and to delete two (2) line items from the checklist. Modified checklist items are as follows: 1.2.4, 1.3.5, 1.4.1, 1.4.3, 1.4.4, 1.4.6, 1.4.7, 1.4.10, 1.4.23, 1.4.24, 1.4.25, 1.5.4, 1.5.10, 1.6.6, 1.6.8, 1.6.9, 1.6.13, 1.7.4, 1.7.6, 1.7.7, 1.7.8, 1.7.10, 1.7.11, 2.1.4, 3.1.2, 3.1.3, 3.2.1, 3.2.3, 3.2.9, 3.2.10, 3.2.11, and 3.7.2. The line items to be deleted are 3.11.6 and 4.3.6. After deletions, editorial renumbering of Sections 3.11 and 4.3 will be required. Specific edits are reflected in attached supporting documentation.
12. Public Health Significance	The proposed modifications are to improve consistency in the current NSSP Laboratory Evaluation checklist. Deletion of line items 3.11.6 and 4.3.6 remove the possibility of a laboratory receiving a double nonconformity during a technical evaluation.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
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TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**
C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Multiple Tube Fermentation Technique for Seawater (APHA) [PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	8, 11		1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s):
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and ~~shall have~~ at least three (3) months of experience in laboratory science.
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies per plate for a 15 minute exposure ~~and~~ determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment *(Indicate the adjustment used)*.

K	8		1.4.4 pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	11		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the electrode isopotential point (pH 7) and the second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (~~*Circle*~~ *Indicate the method used.)*
K	9		1.4.7	~~Balance~~ The balances used provide~~s~~ a sensitivity of at least 0.1 g at~~-~~ weights of use.
K	11,13		1.4.8	Balance calibrations are verified monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1, 39		1.4.10	Refrigerator temperature is maintained ~~at~~ between ~~0~~ 2 to 4~~8~~°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**9, 36**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are verified annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____

C	**9**		**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (~~*Circle*~~ *Indicate the thermometer type used.*)**
K	13, 32		1.4.24 The accuracy of working thermometers ~~are~~ is verified annually against the standards thermometer either at the temperatures at which they are used or by ice point determination. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. Results are recorded and records maintained.
O	11		1.4.25 Appropriate pipet aids are available and used to inoculate samples. ~~Mouth pipetting is not permitted.~~
		1.5 Labware and Glassware Washing	
O	9		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3 Sample containers are made of glass or some other inert material.
O	9		1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with ~~rubber stoppers.~~secure caps or screw caps with nontoxic liners.
K	9		1.5.5 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7 Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8 In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10 With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
K	9		1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2 Routine autoclave maintenance is performed and the records are maintained.

C	29, 32, 33		1.6.3	The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. ~~Sterilization~~Maximum temperature is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device. Results are recorded and records maintained.

K	11		1.6.4	An autoclave standards thermometer has been calibrated using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5	The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination _____
K	1		1.6.6	Working autoclave thermometers are verified against the autoclave standards thermometer at 121°C yearly. Date of last check _____ ~~Method~~ _____
K	32		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape and/or sterilization chemical indicators (e.g., strips, pouches) ~~is~~ are used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total ~~heat exposure time~~time in autoclave, and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (~~*Circle*~~ *Indicate the appropriate record type(s)* ~~*or types.*~~)
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures ≥160°C.
K	9		1.6.11	A thermometer capable of determining temperatures ≥160°C accurately is used to monitor the operation of the hot-air sterilizing oven.
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. ~~Records~~ Results are recorded and records are maintained.
K	11		1.6.14	Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15**	**The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16**	**The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17	Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18	Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
C	**2**		**1.6.19**	**The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20**	**The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**

K	18		1.6.21 Hardwood applicator transfer sticks or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization _____

C	**2**		**1.6.22**	**The sterility of the hardwood applicator transfer sticks/presterilized loops is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23	Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation		
K	3, 5		1.7.1	Media is commercially dehydrated except in the case of A-1 medium which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2	Media is prepared and sterilized according to manufacturer's instructions.
O	11		1.7.3	Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4	Dehydrated media and media components are labeled with date of receipt, ~~and~~ date opened~~.~~, and analyst initials.
C	**12**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**11**		**1.7.6**	**Reagent water is distilled or deionized (*~~circle appropriate choice~~indicate which is used*), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms- cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*~~Circle the appropriate~~ Indicate water quality descriptor determined.*) Results are recorded and the records maintained.**
C	**11**		**1.7.7**	**Reagent water for media and dilution preparation is analyzed for residual chlorine monthly and is at a non- detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** ~~**Specify method of determination** _____~~
K	11		1.7.8	Reagent water for media and diluent preparation contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	9		1.7.9	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	~~11~~**40**		**1.7.10**	~~**Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**~~**Sugar/Carbohydrate broths are removed from the autoclave immediately upon cycle completion with removal less than or equal to 45 minutes (depending on autoclave exhaust cycle).**
C	**1**		**1.7.11**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and the records maintained.**

C	**40**		**1.7.12 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____**

O	9		1.7.13	Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14	The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II – SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
~~O~~K	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium. (*Circle appropriate one.*)**
C	**9, 34**		**2.2.2**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**

C	**9**		**2.2.3**	**In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.6	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
K	9		2.2.8	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
K	9, 11		2.3.3	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. (*Circle the method of transfer.*)
C	**9**		**2.3.4**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium without salicin. Study records are maintained and are available upon request.**

C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**9, 34**		**2.5.4**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.5**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
C	**2**		**2.5.8**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation*. Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
C	**2,5**		**2.5.9**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.11**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition.
K	7		2.6.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5	Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**

C	**2**		**2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and the record is maintained.**
K	2, 11		2.7.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10 The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11 Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12 Forceps tips are clean.
O	11		2.7.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20 Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar	
K	11		2.8.1 Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2 The phosphate buffered saline is properly sterilized.**
K	23		2.8.3 A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4 Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar	
C	**24**		**2.9.1 mTEC agar is used.**
C	**23, 34**		**2.9.2 The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.3 The membrane is placed grid side up within the sterile filter apparatus.**

C	**23, 25**		**2.9.4 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.5 Sample volumes are filtered under vacuum.**
K	26		2.9.6 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.7 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.8 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.9 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**		**2.9.10 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.11 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ± 0.5°C for two (2) hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.12 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ± 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**

C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
K	9, 11, 21, 35		2.12.8 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.9 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 35**		**2.13.1 The sample is shaken vigorously (25 times in a 12 ”arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 35**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 35**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 35**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 35**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control**
C	**9, 11, 35**		**2.13.10 Inoculated plates are incubated inverted at 35 ± 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 35**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**

PART III – SHELLFISH SAMPLES				
		3.1 Collection and Transportation of Samples		
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	~~Shellstock~~ Shellfish samples are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for analysis..
K	9		3.1.3	~~Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.~~Shellfish samples, as received, are labeled with the collectors' company/processor, collectors' name, type of shellfish, the source (harvest area and sampling station), the time, date, and place (if applicable) of collection or are rejected for regulatory analysis.
C	**9**		**3.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination		
K	2,32		3.2.1	Shucking knives, scrub brushes and blender jars are ~~(~~autoclave~~)~~ sterilized for 15 minutes ~~prior to use.~~.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water, or clean gloves are donned, immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**, 32		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**, 32		**3.2.9**	**A representative sample of at least 12 shellfish are used for the analysis.~~Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.~~**
~~K~~C	2, 9		3.2.10	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container**~~At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.~~
~~K~~C	9		3.2.11	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity**~~A representative sample of at least 12 shellfish is used for the analysis.~~
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
C	**9**		**3.2.13**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**

K	9		3.2.14 APHA *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA	
C	**9**		**3.3.1 Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. (*Circle the medium used.*)**
K	9		3.3.2 Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.

C	**9**		**3.3.3**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.5	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.6**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____Negative Process control _____**
K	9		3.3.7	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
K	9, 11		3.4.2	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. (*Circle the method of transfer.*)
C	**9**		**3.4.3**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	15 to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**

C	**9**		**3.6.5 An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6 Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7 Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8 Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9 Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10 Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11 A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC	
K	9		3.7.1 Colony counts determined in accordance with Pa rt III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
C	**19**		**3.7.2 Colony counts are reported as ~~CFU~~SPC/gram~~s~~ of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP	
C	**2,3**		**3.8.1 Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2 Double strength modified MacConkey agar is used.
C	**3**		**3.8.3 Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4 Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5 Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6 The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7 The sample homogenate is cultured within two (2) minutes of blending.**
C	**2,3**		**3.8.8 Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9 Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10 The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**3, 13**		**3.8.12 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.13 Plates are stacked no more than three (3) high in the incubator.**

C	**2**		**3.8.14 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive Process Control: _____Negative Process Control:_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
Bacteriological Examination of Shellfish Meats for Male Specific Coliphage (MSC)			
		3.10 MSC Equipment and Supplies	
K	2		3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100mL.
K	2		3.10.2 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		3.10.3 The sterility of each batch/ lot of pre-sterilized or reusable syringes, filters and/or filter units is determined. Results are recorded and records maintained.
C	**2**		**3.10.4 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.5 The temperature of the incubator used is maintained at 36 ± 1°C.**
K	2		3.10.6 The temperature of the freezer is maintained at ≤- 15°C.
C	**1**		**3.10.7 The sterility of disposable 50 mL centrifuge tubes is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28, 31		3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28, 31		3.11.2 Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28, 31		3.11.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	2		3.11.4 Unsterilized soft agar is stored at ≤-15°C for up to three (3) months.
K	27, 28, 31		3.11.5 The soft agar is sterilized for 15 minutes at 121°C before use.
~~K~~	~~28, 31~~		~~3.11.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.~~
K	2, 27, 28, 31		3.11.~~7~~ 6 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		3.12 Preparation of Host Culture for MSC Analysis	
C	**28, 31**		**3.12.1 *E. coli* Famp ATCC 700891 is the bacterial host strain.**
K	27, 28, 31		3.12.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.

K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28, 31**		**3.12.4 After inoculation, the host cell growth broth culture is not shaken.**
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.13.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.14 MSC Sample Analysis	
C	**28, 31**		**3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.**
C	**27, 28, 31**		**3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.**
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	**27, 28, 31**		**3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.**
C	**27, 28, 31**		**3.14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28, 31**		**3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28, 31**		**3.14.11 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.**
K	27, 28, 31		3.14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive Control: _____
K	27, 28, 31		3.14.13 Room temperature growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.14.15 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28, 31		3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28, 31**		**3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.15 Computation of Results - MSC	
C	**27**		**3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28, 31		3.15.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	2		3.15.4 The MSC count is rounded off conventionally to give a whole number.
		PART IV – MSC WASTEWATER ANALYSIS	
		4.1 Collection and Transportation of Samples	
C	**11**		**4.1.1 Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**39**		**4.1.2 Effluent sample bottles must contain 0.2 mL of 10% sodium thiosulfate solution for every 200 mL of sample to inactivate any residual chlorine.**
K	1		4.1.3 Samples are identified with collector's name, sampling location, time and date of collection.
C	**9**		**4.1.4 Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**

O	1		4.1.5	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**4.1.6**	**Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies		
K	38		4.2.1	Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.
K	9		4.2.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		4.2.3	The sterility of each batch/lot of pre-sterilized or reusable syringes, filters, and/or filter units is determined. Results are recorded and records maintained.
C	**27, 28**		**4.2.4**	**The balance used provides a sensitivity of at least 0.01g.**
C	**27, 28**		**4.2.5**	**The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6	The temperature of the freezer is maintained at ≤ -15°C.
C	**28**		**4.2.7**	**The sterility of the transfer vessel utilized is determined with each lot. Results are recorded and records maintained.**
		4.3 MSC Media Preparation		
K	28		4.3.1	Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2.	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28		4.3.3	Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4	Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
K	27, 28		4.3.5	The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.
~~K~~	~~27, 28~~		~~4.3.6~~	~~Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.~~
K	27, 28		4.3.~~7~~6	Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis		
C	**28**		**4.4.1**	***E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2	Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3	Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**4.4.4**	**After inoculation, the host cell growth broth culture is not shaken.**

		4.5 MSC Sample Analysis		
C	**38**		**4.5.1**	**Wastewater sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10-2) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.**
K	27, 28		4.5.2	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.5.3	Two hundred microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample.
C	**27, 28**		**4.5.4**	**2.5 mL of sample is added to a tube of tempered soft agar.**
C	**27, 28**		**4.5.5**	**The tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.5.6**	**The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			**4.5.7**	**Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.**
K	27, 28		4.5.8	Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28		4.5.9	Room Temperature Growth broth is used as the negative control or blank.
K	27, 28		4.5.10	Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		4.5.11	A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28		4. 5.12	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**4.5.13**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.6 Computation of Results - MSC		
C	**38**		**4.6.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted. The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**38**		**4.6.2**	**When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
K	28		4.6.3	The formula used for determining the density of MSC in PFU/100 mL is: High Range: N * 1000 = PFU/100mL; Low Range: N * 5 = PFU/100mL; Where N is the total number of plaques counted on all plates.
O	9		4.6.4	The MSC count is rounded off conventionally to give a whole number.

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SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is ≥ **4 or**

   b. The total # of Key nonconformities is ≥ **13 or**

   c. The total # of Critical, Key and Other is ≥ **18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is ≥ **1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form *LAB-100 Microbiology,* Rev. June 2024

Proposal No. 2 7 - 1 4 5

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist – Microbiology Single Dilution MPN Reporting and Unnecessary Requirements
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The proposal is to replace the text as follows for Microbiology Checklist items 2.4.2, 2.6.2, and 3.5.2: Results from a single dilution series are calculated using the appropriate MPN table. Additionally, propose removing the following line items from the Microbiology Checklist: 1.4.25, 1.5.3, 1.5.4, 1.5.5, 3.2.14. Editorial renumbering will be addressed, as applicable.
12. Public Health Significance	The proposed modifications to the Checklist clarify reporting for single dilution MPN tests and remove outdated line items. Public health and laboratory integrity will not be affected by proposed modifications.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
~~CFSANDSSLEOS@FDA.HHS.GOV~~**HFPShellfishLEOS@FDA.HHS.GOV**

SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Multiple Tube Fermentation Technique for Seawater (APHA) [PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	8, 11		1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s):
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory science.
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4	pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	11		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the electrode isopotential point (pH 7) and the second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.)*
K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are verified monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**9, 36**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are verified annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____

C	**9**		**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 32		1.4.24 The accuracy of working thermometers are verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. Results are recorded and records maintained.
~~O~~	~~11~~		~~1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.~~
		1.5 Labware and Glassware Washing	
O	9		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
~~K~~	~~9~~		~~1.5.3 Sample containers are made of glass or some other inert material.~~
~~O~~	~~9~~		~~1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.~~
~~K~~	~~9~~		~~1.5.5 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.~~
C	**9**		**1.5.6 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7 Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8 In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10 With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
K	9		1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2 Routine autoclave maintenance is performed and the records are maintained.
C	**29, 32, 33**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**

K	11		1.6.4	An autoclave standards thermometer has been calibrated using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5	The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination _____
K	1		1.6.6	Working autoclave thermometers are verified against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	32		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.*)
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures ≥160°C.
K	9		1.6.11	A thermometer capable of determining temperatures ≥160°C accurately is used to monitor the operation of the hot-air sterilizing oven.
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11		1.6.13	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14	Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15**	**The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16**	**The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17	Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18	Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
C	**2**		**1.6.19**	**The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20**	**The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21	Hardwood applicator transfer sticks or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization _____

C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks/presterilized loops is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of A-1 medium which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared and sterilized according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.)* Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non- detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination _____**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	9		1.7.9 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10 Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.11 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**40**		**1.7.12 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____**

O	9		1.7.13	Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14	The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II – SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium. (*Circle appropriate one.*)**
C	**9, 34**		**2.2.2**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**

C	**9**		**2.2.3**	**In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.6	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.8	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
K	9, 11		2.3.3	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. (*Circle the method of transfer.*)
C	**9**		**2.3.4**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
K	7		2.4.2	~~Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".~~ Results from single dilution tests are calculated using the appropriate MPN table.
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**

C	**2, 30**		**2.5.2 A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium without salicin. Study records are maintained and are available upon request.**

C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**9, 34**		**2.5.4**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.5**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
C	**2**		**2.5.8**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation*. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**		**2.5.9**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.11**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition.
K	7		2.6.2	~~Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".~~ Results from single dilution tests are calculated using the appropriate MPN table."
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5	Colonies are counted with the aid of magnification.

C	**11, 23**		**2.7.6 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**

C	**2**		**2.7.7**	**Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8**	**When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and the record is maintained.**
K	2, 11		2.7.9	New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10**	**The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11	Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12	Forceps tips are clean.
O	11		2.7.13	Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14	Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15	If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16	Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17**	**Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18	A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19	The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20	Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar		
K	11		2.8.1	Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2**	**The phosphate buffered saline is properly sterilized.**
K	23		2.8.3	A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4	Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar		
C	**24**		**2.9.1**	**mTEC agar is used.**
C	**23, 34**		**2.9.2**	**The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.3**	**The membrane is placed grid side up within the sterile filter apparatus.**

C	**23, 25**		**2.9.4 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.5 Sample volumes are filtered under vacuum.**
K	26		2.9.6 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.7 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.8 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.9 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**		**2.9.10 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.11 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ± 0.5°C for two (2) hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.12 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ± 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**

C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
K	9, 11, 21, 35		2.12.8 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.9 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 35**		**2.13.1 The sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 35**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 35**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 35**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 35**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control**
C	**9, 11, 35**		**2.13.10 Inoculated plates are incubated inverted at 35 ± 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 35**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**

PART III – SHELLFISH SAMPLES				
3.1 Collection and Transportation of Samples				
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
3.2 Preparation of Shellfish for Examination				
K	2,32		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**, 32		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**, 32		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	2, 9		3.2.10	At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9		3.2.11	A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
C	**9**		**3.2.13**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
~~K~~	~~9~~		~~3.2.14~~	~~APHA *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.~~
3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA				
C	**9**		**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. (*Circle the medium used.*)**
K	9		3.3.2	Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.

C	**9**		**3.3.3**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.5	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.6**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control _____**
K	9		3.3.7	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
K	9, 11		3.4.2	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. (*Circle the method of transfer.*)
C	**9**		**3.4.3**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	~~Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".~~ Results from single dilution tests are calculated using the appropriate MPN table."
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	15 to 20 mL of tempered sterile plate count agar is used per plate.

C	**9**		**3.6.4 Agar tempering bath maintains the agar at 44-46°C.**

C	**9**		**3.6.5 An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6 Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7 Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8 Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9 Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10 Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11 A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC	
K	9		3.7.1 Colony counts determined in accordance with Pa rt III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
C	**19**		**3.7.2 Colony counts are reported as CFU/grams of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP	
C	**2,3**		**3.8.1 Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2 Double strength modified MacConkey agar is used.
C	**3**		**3.8.3 Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4 Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5 Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6 The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7 The sample homogenate is cultured within two (2) minutes of blending.**
C	**2,3**		**3.8.8 Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9 Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10 The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**3, 13**		**3.8.12 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.13 Plates are stacked no more than three (3) high in the incubator.**

C	**2**		**3.8.14 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive Process Control: _____ Negative Process Control:_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
Bacteriological Examination of Shellfish Meats for Male Specific Coliphage (MSC)			
		3.10 MSC Equipment and Supplies	
K	2		3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100mL.
K	2		3.10.2 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		3.10.3 The sterility of each batch/ lot of pre-sterilized or reusable syringes, filters and/or filter units is determined. Results are recorded and records maintained.
C	**2**		**3.10.4 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.5 The temperature of the incubator used is maintained at 36 ± 1°C.**
K	2		3.10.6 The temperature of the freezer is maintained at ≤-15°C.
C	**1**		**3.10.7 The sterility of disposable 50 mL centrifuge tubes is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28, 31		3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28, 31		3.11.2 Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28, 31		3.11.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	2		3.11.4 Unsterilized soft agar is stored at ≤-15°C for up to three (3) months.
K	27, 28, 31		3.11.5 The soft agar is sterilized for 15 minutes at 121°C before use.
K	28, 31		3.11.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.
K	2, 27, 28, 31		3.11.7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		3.12 Preparation of Host Culture for MSC Analysis	
C	**28, 31**		**3.12.1 *E. coli* Famp ATCC 700891 is the bacterial host strain.**
K	27, 28, 31		3.12.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.

K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28, 31**		**3.12.4 After inoculation, the host cell growth broth culture is not shaken.**
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.13.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.14 MSC Sample Analysis	
C	**28, 31**		**3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.**
C	**27, 28, 31**		**3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.**
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	**27, 28, 31**		**3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.**
C	**27, 28, 31**		**3.14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28, 31**		**3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28, 31**		**3.14.11 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.**
K	27, 28, 31		3.14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive Control: _____
K	27, 28, 31		3.14.13 Room temperature growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.14.15 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28, 31		3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28, 31**		**3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.15 Computation of Results - MSC	
C	**27**		**3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28, 31		3.15.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	2		3.15.4 The MSC count is rounded off conventionally to give a whole number.
		PART IV – MSC WASTEWATER ANALYSIS	
		4.1 Collection and Transportation of Samples	
C	**11**		**4.1.1 Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**39**		**4.1.2 Effluent sample bottles must contain 0.2 mL of 10% sodium thiosulfate solution for every 200 mL of sample to inactivate any residual chlorine.**
K	1		4.1.3 Samples are identified with collector's name, sampling location, time and date of collection.
C	**9**		**4.1.4 Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**

O	1		4.1.5	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**4.1.6**	**Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies		
K	38		4.2.1	Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.
K	9		4.2.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		4.2.3	The sterility of each batch/lot of pre-sterilized or reusable syringes, filters, and/or filter units is determined. Results are recorded and records maintained.
C	**27, 28**		**4.2.4**	**The balance used provides a sensitivity of at least 0.01g.**
C	**27, 28**		**4.2.5**	**The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6	The temperature of the freezer is maintained at ≤ -15°C.
C	**28**		**4.2.7**	**The sterility of the transfer vessel utilized is determined with each lot. Results are recorded and records maintained.**
		4.3 MSC Media Preparation		
K	28		4.3.1	Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2.	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28		4.3.3	Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4	Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
K	27, 28		4.3.5	The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.
K	27, 28		4.3.6	Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.
K	27, 28		4.3.7	Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis		
C	**28**		**4.4.1**	***E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2	Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3	Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**4.4.4**	**After inoculation, the host cell growth broth culture is not shaken.**

		4.5 MSC Sample Analysis		
C	**38**		**4.5.1**	**Wastewater sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10-2) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.**
K	27, 28		4.5.2	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.5.3	Two hundred microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample.
C	**27, 28**		**4.5.4**	**2.5 mL of sample is added to a tube of tempered soft agar.**
C	**27, 28**		**4.5.5**	**The tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.5.6**	**The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			**4.5.7**	**Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.**
K	27, 28		4.5.8	Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28		4.5.9	Room Temperature Growth broth is used as the negative control or blank.
K	27, 28		4.5.10	Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		4.5.11	A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28		4. 5.12	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**4.5.13**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.6 Computation of Results - MSC		
C	**38**		**4.6.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted. The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**38**		**4.6.2**	**When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
K	28		4.6.3	The formula used for determining the density of MSC in PFU/100 mL is: High Range: N * 1000 = PFU/100mL; Low Range: N * 5 = PFU/100mL; Where N is the total number of plaques counted on all plates.
O	9		4.6.4	The MSC count is rounded off conventionally to give a whole number.

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Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	PCR Microbiology Checklist –Updates for consistency
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to modify the PCR Microbiology checklist to improve consistency of NSSP requirements across multiple checklists. Specific edits are reflected in supporting documentation. Editorial renumbering of the checklist as required by edits.
12. Public Health Significance	The proposed modifications are to improve consistency in the current NSSP PCR Microbiology Laboratory Evaluation checklist. This action will help laboratories meet NSSP requirements when performing multiple methods.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

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U.S. FOOD AND DRUG ADMINISTRATION
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SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* detection in Oysters [PART IV] QuantStudio DX

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15-minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (Indicate the adjustment used).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two standard buffer solutions is used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Indicate the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1, 27		1.4.10	Refrigerator temperature is maintained between 2 to 8 °C.
C	**7**		**1.4.11**	**Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ 0.05 °C are used as the laboratory standards thermometer (*Indicate the thermometer type used*).**
K	3, 8		1.4.22	The accuracy of working thermometers is verified annually against the standards thermometer either at temperatures of which they are used or by ice point determination. Discard working temperature sensing devices that differ by >1oC from the reference/standard device.. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes used and accuracy is verified quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils and containers, are clean borosilicate glass, stainless steel or other noncorroding material.

K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	Autoclave(s) are of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**28, 29, 30**		**1.6.3**	**The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Maximum temperature ~~Sterilization~~ is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device. Results are recorded and records maintained.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are verified against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape and/or sterilization chemical indicators (e.g., strips, pouches) are used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total time in autoclave, and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Indicate the appropriate record type (s)*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.

K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records are maintained.
K	5		1.6.14	Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.
C	**2**		**1.6.16**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.18**	**The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19	Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation		
K	13, 14		1.7.1	Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2	Media components are properly stored in a cool dry place.
O	6		1.7.3	Dehydrated media and media components are labeled with date of receipt, date opened, expiration date, and analyst's initials.
C	**6**		**1.7.4**	**Caked or expired media or media components are discarded.**
C	**6**		**1.7.5**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (<0.1 ppm). Results are recorded and records are maintained**
C	**6**		**1.7.6**	**Reagent water is distilled or deionized (indicate which is used), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms- cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (Indicate water quality descriptor determined.) Results are recorded and the records are maintained.**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8	The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9**	**Non-sugar/carbohydrate media broths are removed from the autoclave immediately upon cycle completion with removal not to exceed 60 minutes.**
C	**1**		**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and records are maintained.**
C	**1**		**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.

K	8		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4	Storage under refrigeration of prepared culture media with loose fitting closures does not exceed 1 month.
K	6		1.8.5	Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6	All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II – SAMPLES AND PROCESSING				
		2.1 Sample Collection, Transportation and Receipt		
C	**2, 6**		**2.1.1**	**A representative sample of shellstock is collected.**
K	5		2.1.2	Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3	Shellfish samples, as received, are labeled with the collectors' company/processor, collectors' name, type of shellfish, the source (harvest area and sampling station), the time, date, and place (if applicable) of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 6		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2	Blades of shucking knives are not corroded.
K	5		2.2.3	The hands of the analyst are thoroughly washed with soap and water or clean gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6	Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**5**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9**	**A representative sample of at least 12 live shellfish are used for the analysis**
C	**5**		**2.2.10**	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container**
C	**2, 5**		**2.2.11**	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
		2.3 APW Enrichment		
K	5		2.3.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.

C	**5, 15**		**2.3.2**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**2.3.3**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution_____**
C	**2, 15, 27**		**2.3.4**	**For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **An uninoculated APW blank will serve as the uninoculated process control.** **The process control cultures accompany the samples throughout the method. Records are maintained.**
C	**13**		**2.3.5**	**Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**2.3.6**	**Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
PART III- PCR METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN OYSTERS SMARTCYCLER II AND AB 7500 FAST				
		3.1 PCR Reagents		
C	**14, 15**		**3.1.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.1.2	Fluorescent probes are stored in light occluding tubes or containers.

C	**14, 15, 18, 19**		**3.1.3 The PCR forward and reverse primers and probes are appropriate for the platform.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE/TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.1.4 Lyophilized forward and reverse primers, and probes, are reconstituted with TE buffer to produce a 100 µM stock solution.**
C	**14, 18**		**3.1.5 Primer and probe stock solutions are diluted with molecular grade, nuclease free water to produce a 10 µM working solution.**
C	**14, 18**		**3.1.6 Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**
C	**21, 22**		**3.1.7 Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.1.8 PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.2 DNA Extraction	
C	**14, 18**		**3.2.1 All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.2.2 Pipet tips have aerosol barriers.**
K	14, 18		3.2.3 Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.2.4 All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.2.5 Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.2.6 One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.2.7 Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.2.8 A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.2.9 After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**

K	14, 18		3.2.10	Frozen extracts are analyzed within 6 months of frozen storage.
		3.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**3.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.3.2**	**For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.3.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.3.4**	**Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.3.5**	**Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.4 PCR		
C	**14, 19**		**3.4.1**	**If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.4.2**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.4.3	Two (2) µL of APW blank extracted from the uninoculated process control is added to a reaction tube or plate well containing 23µL of Master Mix.
K	14, 19		3.4.4	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
K	14, 19		3.4.5	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
C	**14, 19**		**3.4.6**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix as a no template control. A no template control is included with each PCR run.**
C	**14, 19**		**3.4.7**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control. A positive PCR control is included with each PCR run.**
K	14, 19		3.4.8	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.4.9**	**After centrifugation, tubes or plates are inserted into the instrument.**
		3.5 PCR Amplification		
C	**14, 19**		**3.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		3.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.5.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.6 Computation of Results		
K	14, 19		3.6.1	All runs in which the positive PCR control generates a Ct value for the target(s) of interest and the no template control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**3.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**

C	**16**		**3.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control is considered invalid and should be re-tested.**
C	**13**		**3.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN results. Use the Dilution Selection Tool located in FDA BAM Appendix 2 to determine which dilutions to use in the final MPN.**
K	13		3.6.8	Results are reported as MPN/g of sample or pass/fail in the case of PHP samples.

PART IV- PCR METHOD FOR *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* DETECTION IN OYSTERS QUANTSTUDIO DX				
		4.1 PCR Reagents		
C	**14, 15**		**4.1.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		4.1.2	Fluorescent probes are stored in light occluding tubes or containers.
C	**26**		**4.1.3**	**The PCR forward and reverse primers, and probes target.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3' Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3' Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3' WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3' WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3' Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3' Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3' Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3' Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3' TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3' TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3' Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ **<u>For Vv Real-time PCR Method</u>** vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3' vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3' vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGBNFQ
C	**14, 18**		**4.1.4**	**Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**26**		**4.1.5**	**Storage of thawed working stocks of primers and probes are stored between 2-8°C, not to exceed 2 weeks.**

C	**26**		**4.1.6**	**Storage of aliquoted working stocks of primers and probes stored in -20°C manual defrost freezer does not exceed 1 year.**
C	**26**		**4.1.7**	**Taqman Environmental Mastermix 2.0 is stored in -20°C manual defrost freezer until first use. After first use, it is stored between 2-8°C.**
C	**26**		**4.1.8**	**Internal control (IC) is stored in -20°C manual defrost freezer until first use. After first use, they are stored between 2-8°C.**
		4.2 DNA Extraction		
C	**14, 18**		**4.2.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**4.2.2**	**Pipet tips have aerosol barriers.**
K	14, 18		4.2.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		4.2.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**4.2.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**26**		**4.2.6**	**Two-hundred (200) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**26**		**4.2.7**	**For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.**
K	26		4.2.8	Extracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen extracts are analyzed within 1 month of frozen storage.
C	**26**		**4.2.9**	**A tlh+ trh+ tdh+ *V. parahaemolyticus* (WA4647 or equivalent), a tlh+ tdh+ Orf8+ *V. parahaemolyticus* (BAA-240 or equivalent), and vvha+ *V. vulnificus* (ATCC 27562 or equivalent) cultures are extracted and combined to serve as the positive PCR (amplification) control.**
		4.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**4.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**26**		**4.3.2**	**For each reaction, add the specified amount of water, appropriate primer and probe concentrations, and Taqman Environmental Master Mix.**
K	14, 16, 18		4.3.3	The Master Mix is vortexed to mix constituents and then briefly spun immediately prior to dispensing aliquots to reaction tubes or plates.
C	**14, 16, 18, 26**		**4.3.4**	**Eighteen (18) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**4.3.5**	**Master Mix must be used on the day of preparation or stored at –20°C until time of use.**
		4.4 PCR		
C	**14, 19, 26**		**4.4.1**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 18µL of Master Mix for a total PCR reaction volume of 20µL.**
K	14, 19, 26		4.4.2	Two (2) µL of extracted blank APW from the uninoculated process control is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.3	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.4	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
C	**14, 19, 26**		**4.4.5**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as a no template control.**

C	**14, 19, 26**		**4.4.6**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as the positive PCR (amplification) control.**
K	14, 19, 26		4.4.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 30 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**16**		**4.4.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		4.5 PCR Amplification		
C	**14, 19**		**4.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		4.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**4.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		4.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**4.5.5**	**The analysis settings are adjusted as specified in the protocol.**

		4.6 Computation of Results		
K	14, 19		4.6.1	All runs in which the NTC has no amplification; positive control generates a Ct value for the target(s) of interest; and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	2		**4.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**4.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**4.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		4.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**4.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control (except NTC) is considered invalid and should be re-tested.**
C	**13**		**4.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN results. Use the Dilution Selection Tool located in FDA BAM Appendix 2 to determine which dilutions to use in the final MPN.**
K	13		4.6.8	Results are reported as MPN/g of sample or pass/fail in the case of PHP samples.

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LABORATORY:			DATE of EVALUATION:
SHELLFISH LABORATORY EVALUATION CHECKLIST **PCR Microbiology** **SUMMARY OF NONCONFORMITIES**			
Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PCR MICROBIOLOGY COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the PCR Microbiology Component:

1. **Does Not Conform Status**: The PCR Microbiology component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥4 or**
   b. The total # of Key nonconformities is **≥13 or**
   c. The total # of Critical, Key and Other is **≥18**
2. **Provisionally Conforms Status**: The PCR Microbiology component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *6 – PCR Microbiology Checklist*, Rev. December 2025

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	US Food & Drug Administration (FDA)
Affiliation	US Food & Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	CPK1, HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Checklist Modifications – Combined MBA for PSP and NSP Checklist
Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
Text of Proposal/ Requested Action	The requested action is to combine the MBA for PSP and MBA for NSP Checklists. Proposal 27-102 which rescinds the Scotia Rapid Test (SRT) received interim approval by the Executive Board, so the SRT is not addressed in this proposal beyond deleting the relevant line items. Part I: Quality Assurance is combined for both methods as there is significant overlap in the checklist line items. Line items that remain specific to the individual methods are documented appropriately. Part II: Collection and Transportation of Samples and Sample Processing is combined for both methods; the requirements are identical. Part III: Analysis of Shellfish for PSP Toxins; the MBA for PSP method requirements are now located in Sections 3.1 – 3.3. There are no changes to the specific requirements for MBA for PSP. Part IV: Analysis of Shellfish for NSP Toxin; the MBA for NSP method requirements are located in Sections 4.1 – 4.4. Several line items were updated or removed to improve the checklist. The changes improve clarity of the line items and/or combine/remove line items to avoid repetition. See specific edits to all line items in the supporting documentation. Editorial re-numbering will be addressed, as applicable
Public Health Significance	The proposed changes improve the MBA for NSP by adding missing references and aligning the checklist requirements with the MBA for PSP method. In addition, for laboratories that run both methods this will reduce the amount of work that is done to ensure they are meeting the requirements for both methods.
Cost Information	None

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
~~CFSANDSSLEOS@FDA.HHS.GOV~~**HFPSHELLFISHLEOS@FDA.HHS.GOV**

SHELLFISH LABORATORY EVALUATION CHECKLIST
Mouse Bioassay ~~and Scotia Rapid Test~~ for Paralytic Shellfish Poisoning (PSP) and Neurotoxic Shellfish Poisoning (NSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Mouse Bioassay for Paralytic Shellfish Poisoning (MBA PSP) [Part III]
	~~Scotia Rapid Test for Paralytic Shellfish Poisoning (SRT PSP)~~
	Mouse Bioassay for Neurotoxic Shellfish Poisoning (MBA NSP) [Part IV]

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	5, 6, 8		1.1.1	Written Plan adequately covers all of the following: (check √ those items which apply)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
				h. Animal care.
C	**6**		**1.1.2**	**The QA plan is implemented.**
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial/private laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
		1.3 Work Area		
O	5, 6		1.3.1	Adequate for the workload and storage.
O	5		1.3.2	Clean and well lighted.
O	5		1.3.3	Adequate temperature control.
O	5		1.3.4	All work surfaces are nonporous and easily cleaned.
C	**8**		**1.3.5**	**A separate, quiet area with adequate temperature control for mice acclimation and injection is maintained.**
		1.4 Laboratory Equipment		
O	2		1.4.1	The pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH paper in the appropriate range (i.e. 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	7		1.4.3	pH electrodes consist of pH half-cell and reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).

K	6		1.4.4	pH meter is calibrated daily when in use. Results are recorded and records are maintained.
K	5		1.4.5	Effect of temperature has been compensated for by an ATC probe; use of a triode or by manual adjustment.
K	5		1.4.6	A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.
K	6, 12		1.4.7	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of slope. (Circle method used).
K	2		1.4.8	The balances being used provide an appropriate sensitivity at the weights of use. a. [PSP Only] To prepare reference solution, the balance must have a sensitivity of at least 0.1 g at a load of 1 g. b. For sample extraction, the balance must have a sensitivity of at least 0.1 g at a load of 100 g. c. For gravimetric extract volume adjustment, the balance must have a sensitivity of at least (check if applicable): _____ [PSP Only] 0.1 g at a load of 200 g. _____ [NSP Only] 0.01 g at a load of 1g and 10g. ~~c.~~d. _____ To weigh mice for assay, the balance must have a sensitivity of at least 0.1 g at a load of 20 g.
K	4,5		1.4.9	The balance calibration is checked monthly according to the manufacturer's specifications using NIST Class S, ASTM Class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10	Refrigerator temperature is maintained between 0 and 4˚C.
K	5		1.4.11	Refrigerator temperature is monitored at least once daily on workdays. Results are recorded and records are maintained.
K	4		1.4.12	Freezer temperature is maintained within manufacturer's tolerance.
K	5		1.4.13	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records are maintained.
C	**10**		**1.4.14**	**All in-service thermometers are properly calibrated and immersed. Results are recorded and records are maintained.**
O	6		1.4.15	All glassware is clean.
C	**5**		**1.4.16**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records are maintained.**
C	**9**		**1.4.17**	**An alkaline or acid based detergent is used for washing glassware/labware.**
		1.5 Reagents and Reference Solution Preparation and Storage		
C	**5**		**1.5.1**	**Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm – cm resistance(2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 °C. (Circle the**

			appropriate water quality descriptor determined). Results are recorded and records are maintained.
K	5		1.5.2 Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (≤ 0.1 mg/L). Results are recorded and records are maintained. Specify method of determination _____.
C	**9**		**1.5.2~~1~~ [PSP Only] Any residual (unused) STX diHCl standard solution is never stored ~~—~~after the ampule has been opened.**
K	~~15~~14		1.5.3~~2~~ [PSP Only] PSP reference solution (1 µg/mL) is prepared gravimetrically and diluted ~~——~~with 0.001 M HCl solution.
K	9		1.5.4~~3~~ [PSP Only] Prepared PSP reference solution is stored under refrigeration in a sealed non-reactive container. Solution may be stored indefinitely as long as there is no detectable evaporation loss as determined by weight. If evaporation is detected, the solution is discarded appropriately. Records are maintained.
C	**~~14~~13**		**1.5.5~~4~~ [PSP Only] All working dilutions from the PSP reference solution are prepared gravimetrically using 0.001 M HCl.**
K	9		1.5.6~~5~~ [PSP Only] All working dilutions prepared from the PSP reference solution are discarded appropriately after use.
K	2~~5~~		1.5.7 [NSP Only] Concentrated (12N) HCl is used to acidify the homogenate.~~1.5.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm – cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 °C. (Circle the appropriate water quality descriptor determined). Results are recorded and records are maintained.~~
K	2~~5~~		1.5.8 [NSP Only] Reagent grade NaCl is used in the extraction procedure. ~~1.5.7 Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (≤ 0.1 mg/L). Results are recorded and records are maintained. Specify method of determination _____.~~
C	**2**		**1.5.9 [NSP Only] Diethyl ether purified for lipid extraction is used for extracting lipids from the shellfish homogenates.**
C	**2**		1.5.10 **[NSP Only] Cottonseed oil (0.917 g/ml) or a vegetable oil with a similar density (0.915 to 0.927 g/ml) is used as the toxin delivery system. Name and density of the oil if substituted for cottonseed oil.** **Name: _____ Density:**
		1.6 Bioassay Supplies	
K	2		1.6.1 Appropriate gauge hypodermic needle is used for injection. a. 26 gauge for PSP b. 25 gauge for NSP
C	**2**		**1.6.2 Healthy mice in the weight range of 17.0 -23.0 grams (19 - 21 grams is preferable) from a stock colony are used for routine assays. Previously injected mice are never re-used for a bioassay.** **Stock strain: _____ Source: _____**
C	**9**		**1.6.3 Mice are allowed to acclimate at least 24 hours prior to injection. In some cases, 48 hours may be required.**
K	9		1.6.4 Mice are weighed to the nearest 0.1 g.
PART II – SAMPLE COLLECTION AND PROCESSING			
		~~1.6~~2.1 Collection and Transportation of Samples and Sample Processing	

O	2		~~1.6~~2.1.1 Shellstock are collected in clean, waterproof, puncture resistant containers, loosely sealed.
K	2		~~1.6~~2.1.2 Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest a rea, sampling station, time, date and place (if applicable) of collection.
C	**2**		**~~1.6~~2.1.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	~~15~~14, 9		~~1.6~~2.1.4 Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**~~14~~13**		**~~1.6~~2.1.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
~~PART II – ANALYSIS OF SHELLFISH FOR PSP TOXINS – MOUSE BIOASSAY (MBA)~~			
		2.2~~1~~	**Preparation of Samples for Analysis – Homogenization**
C	**~~15~~14, 9**		**2.~~1~~2.1 At least 12 animals (or more to provide 100 g of shellfish meat) are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	2		2.~~1~~2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	2		2.~~1~~2.3 Shellstock are opened by cutting the adductor muscles.
O	2		2.~~1~~2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	2		2.~~1~~2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**2**		**2.~~1~~2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	2		2.~~1~~2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	2		2.~~1~~2.8 Pieces of shell and drainage are discarded.
C	**2**		**2.~~1~~2. 9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60 - 120 seconds).**
PART III – ANALYSIS OF SHELLFISH FOR PSP TOXINS – MOUSE BIOASSAY (MBA PSP)			
		3~~2~~.1~~2~~	**Preparation of Samples for Analysis – APHA/AOAC Digestion & Extraction**
K	~~15~~14, 9		3~~2~~.1~~2~~.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
K	2		3~~2~~.~~2~~1.2 100 grams of homogenized sample is weighed into a beaker.

K	2		32.21.3 The sample homogenate is extracted in a 1:1 weight/volume ratio by adding 0.1 M HCl or 0.18 M HCl (*circle the appropriate choice*).
K	2		32.21.4 Homogenate/acid mixture is stirred thoroughly before boiling to completely mix the contents.
C	**2**		**32.21.5 To prevent toxin transformation, the pH of the homogenate/acid mixture before boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
C	**2**		**32.21.6 The homogenate/acid mixture is promptly brought to its boiling point, then gently boiled at 100 ± 1 °C for 5 minutes.**
O	9		32.21.7 The homogenate/acid mixture is boiled under adequate ventilation (e.g. fume hood). A watch glass may be used to prevent excessive evaporation.
O	9		32.21.8 The homogenate/acid mixture is allowed to cool to room temperature.
C	**2**		**32.21.9 The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	2		32.21.10 The homogenate/acid mixture is adjusted gravimetrically to the pre-boiling weight using 0.001 M HC1.
K	2		32.21.11 The homogenate/acid mixture is allowed to separate by gravity or by centrifugation (e.g. centrifuged at 3,000 RPM for 5 minutes).
K	9		32.21.12 If the extracted sample cannot be assayed immediately, then the supernatant is decanted and stored in a sealed container under refrigeration for up to 24 hours or frozen for longer storage.
K	9		32.21.13 Refrigerated extracts are allowed to reach ambient temperature before being bioassayed ~~or tested by the SRT for PSP~~.
		2.33.2 Mouse Bioassay (MBA) for PSP	
~~K~~	~~2~~		~~2.3.1 A 26-gauge hypodermic needle is used for intraperitoneal injections.~~
~~C~~	~~2~~		~~**2.3.2 Healthy mice in the weight range of 17.0 - 23.0 grams (19 - 21 grams is preferable) from a stock colony are used for routine assays. Previously injected mice are never re-used for a bioassay.**~~ ~~**Stock strain:_____ Source:_____**~~
~~C~~	~~9~~		~~**2.3.3 Mice are allowed to acclimate at least 24 hours prior to injection. In some cases, 48 hours may be required.**~~
C	**2**		**3.2.1 Mice are injected intraperitoneally with 1 mL of extracted sample.**
C	**9**		**2.33.2.24 A conversion factor (CF) for the lab has been appropriately determined.** **Lab CF:_____ Date CF established:_____**
C	**2**		**2.3.53.2.3 The CF value is checked weekly if assays are done on one or several days during the week or once each day that assays are performed if they are performed less than once per week.** **Date of current CF check:_____ CF verified: yes/no (*circle choice*)**
C	**2**		**2.3.63.2.4 If the lab CF is not verified during a check, the lab follows the appropriate procedure for establishing a temporary CF to use for the day/week.**

C	**2, 9**		**~~2.3.7~~3.2.5 If the lab CF fails to be verified, the cause is investigated and the situation is corrected. If the cause cannot be determined with reasonable certainty and the lab CF fails to be verified > three times in a year, the lab CF is recalculated through a restandardization procedure.**
~~K~~	~~9~~		~~2.3.8 Mice are weighed to the nearest 0.1 g.~~
~~C~~	~~2~~		~~**2.3.9 Mice are injected intraperitoneally with 1 mL of extracted sample.**~~
K	2		~~2.3.10~~3.2.6 For CF checks, five mice are injected.
K	9		~~2.3.11~~3.2.7 For routine assays, three mice (two when both survive) are injected per sample.
C	**2**		**~~2.3.12~~3.2.8 Elapsed time post-injection is accurately determined and recorded.**
C	**2**		**~~2.3.13~~3.2.9 When death occurs, the time of death to the nearest second is noted at the last gasping breath and recorded.**
C	**9, 2**		**~~2.3.14~~3.2.10 Mice are continually observed for up to 20 minutes after injection, then periodically observed for a total time of up to 60 minutes after injection.**
C	**2**		**~~2.3.15~~3.2.11 If the median corrected mouse unit is greater than 1.92 (5 minutes), then the sample is diluted with 0.001 M HCl as appropriate to achieve a median corrected mouse unit, MCMU of 1.39-1.92 (a death time of 5-7 minutes).**
		~~2.4~~3.3	**Calculation of toxicity for ~~MBA~~PSP**
C	**2**		**~~2.4~~3.3.1 The death time for each mouse is converted to mouse units (MU) using Sommer's Table and recorded. Any mice surviving beyond 60 minutes are recorded as < 0.875 MU.**
C	**2**		**~~2.4~~3.3.2 The weight for each mouse is corrected to mouse units using the table of weights in Recommended Procedures (Table 7) and interpolated for weights not listed.**
C	**2**		**~~2.4~~3.3.3 The Corrected Mouse Unit (CMU) for each mouse injected is calculated as follows:** **Death time in MU x Weight correction in MU = CMU**
C	**2**		**~~2.4~~3.3.4 The Median Corrected Mouse Unit (MCMU) for each sample is calculated and used in the final toxicity calculation for that sample.**
C	**2**		**~~2.4~~3.3.5 The toxicity of each sample is calculated as follows:** **µg STX eq/100 g of sample = MCMU x CF x DF x 200 except when less than 100 grams of sample is used for analysis.** **In this case an adjustment for sample weight must be made such that the formula for calculating sample toxicity becomes:** **µg STX eq/100 grams of sample = MCMU x CF x DF x 200/Adjusted weight of the acidified sample x 200.** **Where:** **MCMU=Median Corrected Mouse Unit for the sample** **CF=Laboratory Conversion Factor** **DF=Dilution Factor (e.g. 1:1 dilution, DF=2)**
C	**11**		**~~2.4~~3.3.6 Any value equal to or greater than 80 µg STX eq/100 g of sample is actionable.**

PART ~~III~~ IV – ANALYSIS OF SHELLFISH FOR NSP TOXIN – MOUSE BIOASSAY (MBA NSP)~~ANALYSIS FOR PSP TOXINS – SCOTIA RAPID TEST (SRT)~~			
		~~3.1 Screening by Scotia Rapid Test (SRT)~~4.1 Digestion of Sample	
K	2	4.1.1	100 grams (or entire sample amount if less than 100 grams is available) of homogenized sample is weighted into a beaker.
C	2	**4.1.2**	**1 ml of concentrated HCl and 5 g NaCl is added to the 100 gram homogenate and thoroughly mixed. (For samples <100 g, add reagents to obtain final concentrations of 0.12N HCl and 5% NaCl.)**
C	2	**4.1.3**	**The homogenate is brought to a boil and once 100 + 1°C (sea level) is reached, gently boil for 5 minutes under adequate ventilation.** A watch glass may be used to prevent excessive evaporation.
O	9	4.1.4	The beaker is covered with a watch glass or equivalent during boiling to prevent excessive evaporation.
O	2	4.1.5	The boiled, acidified homogenate is cooled to room temperature or below in a refrigerator or in an ice bath.
		4.2 Extraction	
C	2, 9	**4.2.1**	**All steps in the extraction procedure that involve the use of diethyl ether are carried out under adequate ventilation with appropriate safety precautions.**
C	2	**4.2.2**	**100 ml of diethyl ether is added to the cooled, acidified homogenate in a stoppered centrifuge tube and shaken vigorously for 5 minutes.**
O	2	4.2.3	Centrifuge tubes are vented frequently while being shaken and before being centrifuged to avoid accidents.
C	2	**4.2.4**	**The acidified homogenate is extracted 4 separate times. Each time adding 100 mL of diethyl ether, shaking vigorously for 5 minutes and centrifuging at 2000 rpm for 10 to 15 minutes.**
C	2	**4.2.5**	**After each centrifugation step, the clear upper ether phase is transferred to a large separatory funnel.**
C	2	**4.2.6**	**The ether extract is transferred to a large, clean, dry pre-weighed beaker (discard any emulsion or tissue that may have settled in the funnel.)**
C	2	**4.2.7**	**Ether is evaporated to dryness and the final lipid residue is weighed. Weight is recorded.**
C	2	**4.2.8**	**The volume of the lipid residue is adjusted by weight to 10 ml (9.17 g) per 100 g shellfish extracted using cottonseed oil. If a solvent with a density similar to cottonseed oil is used, the volume is adjusted to a weight 10 times the density of the solvent. Specify the weight to which the volume is adjusted to.**
		4.3 Mouse Bioassay (MBA) for NSP	
C	2	**4.3.1**	**The extract is completely mixed before it is injected.**

C	2		**4.3.2 Mice are injected intraperitoneally with 1 ml of the lipid extract.**
C	2		**4.3.3 A total of 5 mice are injected with undiluted or diluted extract as appropriate per sample in routine assays.** **a. The extract is not diluted when all test/assay mice survive beyond 110 minutes of injection.** **b. The extract is diluted when 2 of 2 test mice or 3 of 5 assay mice survive for fewer than 110 minutes after injection.** **c. When dilution is required, only dilutions which produce mean/median death times within 110 to 360 minutes of injection are used in the analysis.**
C	2		**4.3.4 The time of completed injection is recorded.**
C	2		**4.3.5 Mice are continuously observed for at least 6 hours (360 minutes).**
C	2		**4.3.6 If death occurs within the period of continuous observation, the time of death to the nearest minute is noted by the last gasping breath.**
K	2		4.3.7 If mice survive the test, the time of death is recorded as ">" the period of continuous observation.
		4.4 Calculation of toxicity for NSP	
C	2		**4.4.1 The death time of each mouse is converted to mouse units (MU) using Table 8 in *Recommended Procedures*, 4th Edition.**
O	2		4.4.2 Table 8 is interpolated for death times between 110 and 360 minutes that are not listed in the Table.
K	2		4.4.3 A weight correction in MU is determined for each mouse injected using Table 8 in *Recommended Procedures*, 4th Edition. Correction factors for weights not listed are interpolated from Table 8.
C	2		**4.4.4 The death time for each mouse in MU is multiplied by a weight correction in MU to give the corrected mouse unit (CMU) for each mouse.**
C	2		**4.4.5 The mean CMU of the array is used when all the mice injected with diluted or undiluted extract die during the period of continuous observation (death times are determinate) .**
C	2		**4.4.6 The median CMU of the array is used when 100% mortality is observed and death times are indeterminate or if less than 100% mortality is observed.**
C	2		**4.4.7 The concentration of toxin is determined by the formula:** **Mean or median CMU x Dilution Factor x 10** **Mean or median as determined in 4.4.5 and 4.4.6** **Dilution Factor (DF) = if applicable (DF is 1 for undiluted sample)** **10 = lipid residue volume after adjusting to 10 mL with cottonseed oil**

C	2		**4.4.8 Results are reported following APHA Recommended Procedures.** **When the time of death is known for certain for all mice injected, toxicity is determinate and the toxin concentration is reported as:** **Number of mouse units/100 grams of shellfish meats**
~~K~~	~~9~~		~~3.1.1 Before beginning any screening, the following items are recorded for the SRT kit in use.~~ ~~a. Date received.~~ ~~b. Batch/lot numbers for all kit components (test strip and PSP AOAC buffer).~~ ~~c. Expiration dates for all kit components.~~ ~~d. Date opened and/or used.~~
~~K~~	~~13~~		~~3.1.2 When placed into service, all kit components are within the accepted expiration dates.~~
~~C~~	~~13~~		~~**3.1.3 The desiccant pouch inside the test strip wrapping is blue in color, indicating suitability for use. Any test strip wrapping containing a pink desiccant pouch is discarded.**~~
~~K~~	~~13~~		~~3.1.4 All kit components are stored according to the manufacturer's recommendations.~~
~~C~~	~~9~~		~~**3.1.5 A positive control of 80 µg STX eq/100 g of sample is used to test new kit lots and buffers. Results are recorded and records maintained.**~~
~~C~~	~~9~~		~~**3.1.6 Micropipettes with appropriate ranges for the volumes being measured are used.**~~
~~K~~	~~9~~		~~3.1.7 All micropipettes are maintained and calibrated according to manufacturer's instructions. Results are recorded and records maintained.~~
~~C~~	~~13~~		~~**3.1.8 400 µL of buffer solution is accurately transferred to a small tube.**~~
~~C~~	~~13~~		~~**3.1.9 100 µL of sample extract is accurately added to the buffer.**~~
~~K~~	~~13~~		~~3.1.10 The buffer/sample mixture is carefully mixed by inserting the tip of the micropipette into the mixture and pipetting up and down at least three times.~~
~~C~~	~~13~~		~~**3.1.11 100 µL of the thoroughly mixed solution is added to the test strip sample well.**~~
~~K~~	~~9~~		~~3.1.12 Micropipette tips are not reused.~~
~~K~~	~~13~~		~~3.1.13 Inoculated test strips are allowed to react with the sample mixture for the period of time recommended by the manufacturer.~~
~~C~~	~~13~~		~~**3.1.14 The test strip result is interpreted according to the instruction card provided by the manufacturer, which is specific to each batch/lot of test strips. Results are recorded and records are maintained.**~~
~~K~~	~~13~~		~~3.1.15 If a test result is interpreted as invalid; the pH of the sample extract is checked and adjusted as needed to fall between pH 2.0 – 4.0. Fresh PSP AOAC buffer is used to re-test the sample on a new test strip.~~
~~C~~	~~13~~		~~**3.1.16 If the same sample is interpreted as invalid on two different test strips, then the sample is assumed to contain interfering substances, and an alternative test method is used.**~~
~~C~~	~~11~~		~~**3.1.17 Any positive result on a SRT is actionable.**~~

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LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist Modifications – Microbiology and PCR Sterility
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	PCR Checklist item 1.6.16 and Microbiology Checklist item 1.6.15: (Critical) The sterility of reusable items is determined with each load sterilized. Results are recorded and records maintained. Sterility is determined by an appropriate microbiological method, biological indicators and/or chemical integrators. Indicators and/or integrators appropriate for cycle and conditions are used and appropriately placed throughout the autoclave and/or product to verify the sterility of autoclave sterilized consumables. Autoclave sterilized materials are considered sterile as per manufacturer IFU of autoclave packages and/or indicators/integrators used. Upon expiration, the items may be re-packaged, autoclaved, and verified. Results are recorded and records maintained. PCR Checklist item 1.6.17 and Microbiology Checklist item 1.6.16: (Critical) The sterility of pre-sterilized consumables is determined with each lot received. Sterility is verified by an appropriate microbiological method, or a traceable certification of sterilization is obtained. Results are recorded and records maintained. Certificates are maintained in accordance with the records management plan. PCR Checklist item 1.6.18 and Microbiology Checklist items 1.6.19, 1.6.20, 1.6.22, 2.7.4, 2.11.2 will be deleted to avoid checklist redundancy. Editorial updates to items 2.7.10, 2.11.7, 3.10.3, 3.10.7, 4.2.3, and 4.2.7 are also proposed to reflect the sterility requirement. Editorial re-numbering will be addressed, as applicable.
12. Public Health Significance	Historically, pre-sterilized items required sterility verification testing by NSSP laboratories. This proposal is a paradigm shift; instead of testing all pre-sterilized items before use, NSSP laboratories will be allowed to retain the traceable certification of sterilization as proof of sterility. The proposed changes clarify the acceptable methods for sterility verification and remove redundancy in the checklists, as all pre-sterilized and autoclaved sterilized items share the same requirements.

13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
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SHELLFISH AND AQUACULTURE POLICY BRANCH
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SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K – Key O – Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast

PART I – QUALITY ASSURANCE				
CODE	**REF**	**ITEM**		
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15 minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11**	**Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 ± 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5°C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22	All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.

K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3**	**The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14	Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.
C	**2**		**1.6.16**	~~**The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**~~ The sterility of reusable items is determined with each load sterilized. Results are recorded and records maintained. Sterility is determined by an appropriate microbiological method, biological indicators and/or chemical

			integrators. Indicators and/or integrators appropriate for cycle and conditions are used and appropriately placed throughout the autoclave and/or product to verify the sterility of autoclave sterilized consumables. Autoclave sterilized materials are considered sterile as per manufacturer IFU of autoclave packages and/or indicators/integrators used. Upon expiration, the items may be re-packaged, autoclaved, and verified. Results are recorded and records maintained.

C	**2**		**1.6.17**	~~**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.**~~ ~~**If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**~~ The sterility of pre-sterilized consumables is determined with each lot received. Sterility is verified by an appropriate microbiological method, or a traceable certification of sterilization is obtained. Results are recorded and records maintained. Certificates are maintained in accordance with the records management plan.
C	**2**		**1.6.18**	~~**The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**~~
K	8		1.6.19	Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation		
K	13, 14		1.7.1	Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2	Media components are properly stored in a cool dry place.
O	6		1.7.3	Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**6**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**6**		**1.7.6**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (≤0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8	The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9**	**Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.

K	5		1.8.3	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4	Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5	Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6	All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II – SAMPLES				
		2.1 Sample Collection, Transportation and Receipt		
C	**2, 6**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5		2.1.2	Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3	Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 6		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2	Blades of shucking knives are not corroded.
K	5		2.2.3	The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6	Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7	Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9**	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10**	**A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11**	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14**	**Samples are blended for 60 to 120 seconds until homogenous.**
PART III- PCR METHOD FOR VIBRIO VULNIFICUS AND VIBRIO PARAHAEMOLYTICUS DETECTION IN OYSTERS				
		3.1 APW Enrichment		

K	5		3.1.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**3.1.2**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**3.1.3**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution _____**

C	**2, 15**		**3.1.4**	**For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **The process control cultures accompany the samples throughout incubation, isolation, and confirmation. Records are maintained.**
C	**13**		**3.1.5**	**Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**3.1.6**	**Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
		3.2 PCR Reagents		
C	**14, 15**		**3.2.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.2.2	Fluorescent probes are stored in light occluding tubes or containers.
C	**14, 15, 18, 19**		**3.2.3**	**The PCR forward and reverse primers and probes are appropriate for the platform.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE/TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.2.4**	**Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**

C	**14, 18**		**3.2.5**	**Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.2.6**	**Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**
C	**21, 22**		**3.2.7**	**Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.2.8**	**PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.3 DNA Extraction		
C	**14, 18**		**3.3.1**	**All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.3.2**	**Pipet tips have aerosol barriers.**
K	14, 18		3.3.3	Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.3.4	All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.3.5**	**Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.3.6**	**One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.3.7**	**Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.3.8	A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.3.9**	**After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**
K	14, 18		3.3.10	Frozen extracts are analyzed within 6 months of frozen storage.
		3.4 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**3.4.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.4.2**	**For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.4.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.4.4**	**Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.4.5**	**Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.5 PCR		
C	**14, 19**		**3.5.1**	**If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.5.2**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.5.3	Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix for each batch of Master Mix prepared as a no template control.
C	**14, 19**		**3.5.4**	**Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.**
C	**14, 19**		**3.5.5**	**Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.**

O	14, 19		3.5.6	Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control.
K	14, 19		3.5.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**16**		**3.5.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		3.6 PCR Amplification		
C	**14, 19**		**3.6.1**	**The appropriate instrument platform is used for the protocol.**
K	16		3.6.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.6.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.6.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.6.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.7 Computation of Results		
K	14, 19		3.7.1	All runs in which the positive control generates a Ct value for the target(s) of interest and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.7.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**3.7.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**3.7.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.7.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.7.6**	**Any reaction in which no Ct value is generated for the target(s) of interest or the internal control is considered invalid and should be re-tested.**
C	**13**		**3.7.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.7.8	For APW enrichment, results are reported as MPN/g of sample.

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PCR MICROBIOLOGY COMPONENT: (Part I-III)	
A. Results Total # of Critical (C) Nonconformities in Parts I-III Total # of Key (K) Nonconformities in Parts I-III Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-III	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the PCR Microbiology Component:

1. **Does Not Conform Status**: The PCR Microbiological component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥4 or**
   b. The total # of Key nonconformities is **≥13 or**
   c. The total # of Critical, Key and Other is **≥18**
2. **Provisionally Conforms Status**: The PCR microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *6 – PCR Microbiology Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**
C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Multiple Tube Fermentation Technique for Seawater (APHA) [PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	8, 11		1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s):
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory science.
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4	pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	11		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the electrode isopotential point (pH 7) and the second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.)*
K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are verified monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**9, 36**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are verified annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____

C	**9**		**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 32		1.4.24 The accuracy of working thermometers are verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. Results are recorded and records maintained.
O	11		1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing	
O	9		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3 Sample containers are made of glass or some other inert material.
O	9		1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.
K	9		1.5.5 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7 Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8 In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10 With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
K	9		1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2 Routine autoclave maintenance is performed and the records are maintained.
C	**29, 32, 33**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**

K	11		1.6.4 An autoclave standards thermometer has been calibrated using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5 The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination _____
K	1		1.6.6 Working autoclave thermometers are verified against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	32		1.6.7 Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8 Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9 Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.*)
K	11		1.6.10 For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures ≥160°C.
K	9		1.6.11 A thermometer capable of determining temperatures ≥160°C accurately is used to monitor the operation of the hot-air sterilizing oven.
K	13		1.6.12 Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11		1.6.13 Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14 Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15 ~~The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.~~ The sterility of reusable items is determined with each load sterilized. Results are recorded and records maintained. Sterility is determined by an appropriate microbiological method, biological indicators and/or chemical integrators. Indicators and/or integrators appropriate for cycle and conditions are used and appropriately placed throughout the autoclave and/or product to verify the sterility of autoclave sterilized consumables. Autoclave sterilized materials are considered sterile as per manufacturer IFU of autoclave packages and/or indicators/integrators used. Upon expiration, the items may be re-packaged, autoclaved, and verified. Results are recorded and records maintained.**
C	**1**		**1.6.16 ~~The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.~~The sterility of pre-sterilized consumables is determined with each lot received. Sterility is verified by an appropriate microbiological method, or a traceable certification of sterilization is**

			obtained. Results are recorded and records maintained. Certificates are maintained in accordance with the records management plan.
K	9		1.6.17 Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18 Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
~~C~~	~~2~~		~~1.6.19 The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.~~
~~C~~	~~2~~		~~1.6.20 The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.~~
K	18		1.6.21 Hardwood applicator transfer sticks or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization _____

C	**2**		~~**1.6.22 The sterility of the hardwood applicator transfer sticks/presterilized loops is checked routinely. Results are recorded and the records maintained.**~~
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of A-1 medium which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared and sterilized according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.)* Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non- detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination _____**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	9		1.7.9 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10 Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.11 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**40**		**1.7.12 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____** **Media: _____Positive Control: _____Negative Control: _____**

O	9		1.7.13	Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14	The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II – SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium. (*Circle appropriate one.*)**
C	**9, 34**		**2.2.2**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**

C	**9**		**2.2.3**	**In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.6	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
K	9		2.2.8	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
K	9, 11		2.3.3	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. (*Circle the method of transfer.*)
C	**9**		**2.3.4**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium without salicin. Study records are maintained and are available upon request.**

C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**9, 34**		**2.5.4**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.5**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
C	**2**		**2.5.8**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation*. Results are recorded and the records maintained.** **Positive process control _____Negative process control _____**
C	**2,5**		**2.5.9**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.11**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition.
K	7		2.6.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		~~**2.7.4**~~	~~**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**~~
K	11		2.7.5	Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**

C	**2**		**2.7.7 Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8 When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and the record is maintained.**
K	2, 11		2.7.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10 ~~The sterility of each lot or autoclave batch of~~ Sterile membrane filters are used. ~~checked before use.~~**
K	2		2.7.11 Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12 Forceps tips are clean.
O	11		2.7.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20 Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar	
K	11		2.8.1 Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2 The phosphate buffered saline is properly sterilized.**
K	23		2.8.3 A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4 Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar	
C	**24**		**2.9.1 mTEC agar is used.**
C	**23, 34**		**2.9.2 The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.3 The membrane is placed grid side up within the sterile filter apparatus.**

C	**23, 25**		**2.9.4 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.5 Sample volumes are filtered under vacuum.**
K	26		2.9.6 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.7 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.8 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.9 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**		**2.9.10 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.11 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ± 0.5°C for two (2) hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.12 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ± 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**~~2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.~~**

C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 ~~The sterility of each lot and autoclave batch of~~ Sterile membrane filters are used. ~~is verified before use.~~**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
K	9, 11, 21, 35		2.12.8 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.9 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 35**		**2.13.1 The sample is shaken vigorously (25 times in a 12 ”arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 35**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 35**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 35**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 35**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control**
C	**9, 11, 35**		**2.13.10 Inoculated plates are incubated inverted at 35 ± 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 35**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**

PART III – SHELLFISH SAMPLES				
3.1 Collection and Transportation of Samples				
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
3.2 Preparation of Shellfish for Examination				
K	2,32		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**, 32		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**, 32		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	2, 9		3.2.10	At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9		3.2.11	A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
C	**9**		**3.2.13**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.14	APHA *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA				
C	**9**		**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. (*Circle the medium used.*)**
K	9		3.3.2	Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.

C	**9**		**3.3.3**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.5	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.6**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____Negative Process control _____**
K	9		3.3.7	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
K	9, 11		3.4.2	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. (*Circle the method of transfer.*)
C	**9**		**3.4.3**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	15 to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**

C	**9**		**3.6.5 An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6 Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7 Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8 Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9 Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10 Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11 A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC	
K	9		3.7.1 Colony counts determined in accordance with Pa rt III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
C	**19**		**3.7.2 Colony counts are reported as CFU/grams of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP	
C	**2,3**		**3.8.1 Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2 Double strength modified MacConkey agar is used.
C	**3**		**3.8.3 Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4 Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5 Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6 The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7 The sample homogenate is cultured within two (2) minutes of blending.**
C	**2,3**		**3.8.8 Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9 Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10 The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**3, 13**		**3.8.12 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.13 Plates are stacked no more than three (3) high in the incubator.**

C	**2**		**3.8.14 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive Process Control: _____Negative Process Control:_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
Bacteriological Examination of Shellfish Meats for Male Specific Coliphage (MSC)			
		3.10 MSC Equipment and Supplies	
K	2		3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100mL.
K	2		3.10.2 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		3.10.3 ~~The sterility of each batch/ lot of pre-sterilized or reusable~~ Sterile syringes, filters and/or filter units are used. ~~is determined. Results are recorded and records maintained.~~
C	**2**		**3.10.4 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.5 The temperature of the incubator used is maintained at 36 ± 1°C.**
K	2		3.10.6 The temperature of the freezer is maintained at ≤- 15°C.
C	**1**		**3.10.7 ~~The sterility of disposable~~ Sterile 50 mL centrifuge tubes are used. ~~is determined with each lot. Results are recorded and records maintained~~.**
		3.11 MSC Media Preparation	
K	28, 31		3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28, 31		3.11.2 Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28, 31		3.11.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	2		3.11.4 Unsterilized soft agar is stored at ≤-15°C for up to three (3) months.
K	27, 28, 31		3.11.5 The soft agar is sterilized for 15 minutes at 121°C before use.
K	28, 31		3.11.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.
K	2, 27, 28, 31		3.11.7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		3.12 Preparation of Host Culture for MSC Analysis	
C	**28, 31**		**3.12.1 *E. coli* Famp ATCC 700891 is the bacterial host strain.**
K	27, 28, 31		3.12.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.

K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28, 31**		**3.12.4 After inoculation, the host cell growth broth culture is not shaken.**
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.13.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.14 MSC Sample Analysis	
C	**28, 31**		**3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.**
C	**27, 28, 31**		**3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.**
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	**27, 28, 31**		**3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.**
C	**27, 28, 31**		**3.14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28, 31**		**3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28, 31**		**3.14.11 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.**
K	27, 28, 31		3.14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive Control: _____
K	27, 28, 31		3.14.13 Room temperature growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.14.15 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28, 31		3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28, 31**		**3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.15 Computation of Results - MSC	
C	**27**		**3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28, 31		3.15.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	2		3.15.4 The MSC count is rounded off conventionally to give a whole number.
		PART IV – MSC WASTEWATER ANALYSIS	
		4.1 Collection and Transportation of Samples	
C	**11**		**4.1.1 Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**39**		**4.1.2 Effluent sample bottles must contain 0.2 mL of 10% sodium thiosulfate solution for every 200 mL of sample to inactivate any residual chlorine.**
K	1		4.1.3 Samples are identified with collector's name, sampling location, time and date of collection.
C	**9**		**4.1.4 Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**

O	1		4.1.5	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**4.1.6**	**Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies		
K	38		4.2.1	Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.
K	9		4.2.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		4.2.3	~~The sterility of each batch/lot of pre-sterilized or~~ Sterile syringes, filters, and/or filter units are used. ~~is determined. Results are recorded and records maintained.~~
C	**27, 28**		**4.2.4**	**The balance used provides a sensitivity of at least 0.01g.**
C	**27, 28**		**4.2.5**	**The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6	The temperature of the freezer is maintained at ≤ -15°C.
C	**28**		**4.2.7**	**~~The sterility of the~~ Sterile transfer vessels are used. ~~utilized is determined with each lot. Results are recorded and records maintained.~~**
		4.3 MSC Media Preparation		
K	28		4.3.1	Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2.	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28		4.3.3	Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4	Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
K	27, 28		4.3.5	The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.
K	27, 28		4.3.6	Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.
K	27, 28		4.3.7	Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis		
C	**28**		**4.4.1**	***E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2	Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3	Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**4.4.4**	**After inoculation, the host cell growth broth culture is not shaken.**

		4.5 MSC Sample Analysis		
C	**38**		**4.5.1**	**Wastewater sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10-2) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.**
K	27, 28		4.5.2	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.5.3	Two hundred microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample.
C	**27, 28**		**4.5.4**	**2.5 mL of sample is added to a tube of tempered soft agar.**
C	**27, 28**		**4.5.5**	**The tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.5.6**	**The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			**4.5.7**	**Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.**
K	27, 28		4.5.8	Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28		4.5.9	Room Temperature Growth broth is used as the negative control or blank.
K	27, 28		4.5.10	Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		4.5.11	A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28		4. 5.12	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**4.5.13**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.6 Computation of Results - MSC		
C	**38**		**4.6.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted. The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**38**		**4.6.2**	**When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
K	28		4.6.3	The formula used for determining the density of MSC in PFU/100 mL is: High Range: N * 1000 = PFU/100mL; Low Range: N * 5 = PFU/100mL; Where N is the total number of plaques counted on all plates.
O	9		4.6.4	The MSC count is rounded off conventionally to give a whole number.

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SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is ≥ **4 or**

   b. The total # of Key nonconformities is ≥ **13 or**

   c. The total # of Critical, Key and Other is ≥ **18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is ≥ **1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date:_____

LEO Signature: _____ Date:_____

NSSP Form *LAB-100 Microbiology,* Rev. June 2024

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
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6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist Modifications - Collection and Transportation of Samples
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to modify text of temperature requirements for sample transport and receipt on the Microbiology and PCR Microbiology checklists. The proposal edits four (4) line items, updates criticality codes, and adds a line item on the NSSP Microbiology Checklist. On the PCR Microbiology checklist, the proposal edits one (1) line item, adds one (1) line item and one (1) reference, and updates criticality codes. Specific edits are reflected in attached supporting documentation. Editorial re-numbering will be addressed, as applicable.
12. Public Health Significance	The proposed modifications are to improve understanding and promote consistency in the current NSSP Microbiology and NSSP PCR Microbiology Laboratory Evaluation checklists. These proposed updates will simplify and clarify the sample receipt and temperature requirements for the collection and transportation of seawater and shellfish samples.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
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SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Multiple Tube Fermentation Technique for Seawater (APHA) [PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	8, 11		1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s):
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory science.
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4	pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	11		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the electrode isopotential point (pH 7) and the second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.)*
K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are verified monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**9, 36**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are verified annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____

C	**9**		**1.4.23**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 32		1.4.24	The accuracy of working thermometers are verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. Results are recorded and records maintained.
O	11		1.4.25	Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing		
O	9		1.5.1	Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2	Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3	Sample containers are made of glass or some other inert material.
O	9		1.5.4	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.
K	9		1.5.5	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6**	**Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7	Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8	In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10**	**With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	9		1.6.1	Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2	Routine autoclave maintenance is performed and the records are maintained.
C	**29, 32, 33**		**1.6.3**	**The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**

K	11		1.6.4	An autoclave standards thermometer has been calibrated using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5	The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination _____
K	1		1.6.6	Working autoclave thermometers are verified against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	32		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.*)
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures ≥160°C.
K	9		1.6.11	A thermometer capable of determining temperatures ≥160°C accurately is used to monitor the operation of the hot-air sterilizing oven.
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11		1.6.13	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14	Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15**	**The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16**	**The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17	Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18	Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
C	**2**		**1.6.19**	**The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20**	**The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21	Hardwood applicator transfer sticks or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization _____

C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks/presterilized loops is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of A-1 medium which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared and sterilized according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.)* Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non- detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination _____**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	9		1.7.9 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10 Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.11 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**40**		**1.7.12 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____**

O	9		1.7.13	Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14	The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II – SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
~~C~~K	9		2.1.3	Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) ~~capable of maintaining a temperature of 0 to 10°C~~ with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.
~~O~~C	1		**2.1.4**	**A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.**
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium. (*Circle appropriate one.*)**
C	**9, 34**		**2.2.2**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**

C	**9**		**2.2.3**	**In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.6	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.8	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
K	9, 11		2.3.3	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. (*Circle the method of transfer.*)
C	**9**		**2.3.4**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium without salicin. Study records are maintained and are available upon request.**

C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**9, 34**		**2.5.4**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.5**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
C	**2**		**2.5.8**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation*. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**		**2.5.9**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.11**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition.
K	7		2.6.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5	Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**

C	**2**		**2.7.7**	**Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8**	**When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and the record is maintained.**
K	2, 11		2.7.9	New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10**	**The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11	Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12	Forceps tips are clean.
O	11		2.7.13	Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14	Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15	If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16	Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17**	**Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18	A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19	The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20	Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar		
K	11		2.8.1	Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2**	**The phosphate buffered saline is properly sterilized.**
K	23		2.8.3	A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4	Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar		
C	**24**		**2.9.1**	**mTEC agar is used.**
C	**23, 34**		**2.9.2**	**The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.3**	**The membrane is placed grid side up within the sterile filter apparatus.**

C	**23, 25**		**2.9.4**	**Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.5**	**Sample volumes are filtered under vacuum.**
K	26		2.9.6	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.7**	**The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.8**	**The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.9**	**Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**		**2.9.10**	**Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.11**	**Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ± 0.5°C for two (2) hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.12**	**After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ± 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar		
C	**23**		**2.10.1**	**All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2**	**Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3**	**When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4**	**The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5**	**Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment		
C	**9, 11, 21**		**2.11.1**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**

C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
K	9, 11, 21, 35		2.12.8 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.9 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 35**		**2.13.1 The sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 35**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 35**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 35**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 35**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control _____**
C	**9, 11, 35**		**2.13.10 Inoculated plates are incubated inverted at 35 ± 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 35**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**

PART III – SHELLFISH SAMPLES				
		3.1 Collection and Transportation of Samples		
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
~~C~~K	9		3.1.4	Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) ~~which is maintained between 0 and 10°C~~ with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.
C	9		**3.1.5**	**~~The temperature of the shellfish samples or a temperature control (TC) is taken upon receipt in the laboratory. Temperature must be between 0-10°C or less than that of the shellfish or TC at the time of collection. Results are recorded and maintained.~~ The temperature of the shellstock samples or a seawater temperature control (TC) is taken upon receipt in the laboratory. Results are recorded and maintained.** **a. When a seawater TC is used to document temperature at receipt it must be:** **i. between 0-10°C or** **ii. less than the temperature of the growing area at the time of collection.** **b. When the temperature of shellstock is used to document temperature at receipt it must be:** **i. between 0-10°C or** **ii. less than the temperature of the shellstock or growing area at the time of collection.**
C	**1**		**3.1.6~~5~~**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination		
K	2,32		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**, 32		**3.2.8**	**Shellstock are not shucked directly through the hinge.**

C	**9**, 32		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	2, 9		3.2.10	At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9		3.2.11	A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
C	**9**		**3.2.13**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.14	APHA *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA		
C	**9**		**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. (*Circle the medium used.*)**
K	9		3.3.2	Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.3**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.5	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.6**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control _____**
K	9		3.3.7	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
K	9, 11		3.4.2	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. (*Circle the method of transfer.*)
C	**9**		**3.4.3**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**

		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	15 to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**
C	**9**		**3.6.5**	**An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6	Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7**	**Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8	Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9	Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10	Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11	A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC		
K	9		3.7.1	Colony counts determined in accordance with Pa rt III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
C	**19**		**3.7.2**	**Colony counts are reported as CFU/grams of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP		
C	**2,3**		**3.8.1**	**Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2	Double strength modified MacConkey agar is used.
C	**3**		**3.8.3**	**Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4	Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5	Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6**	**The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**

C	**9**		**3.8.7**	**The sample homogenate is cultured within two (2) minutes of blending.**
C	**2,3**		**3.8.8**	**Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9	Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10	The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**3, 13**		**3.8.12**	**When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.13**	**Plates are stacked no more than three (3) high in the incubator.**
C	**2**		**3.8.14**	**Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive Process Control: _____ Negative Process Control:_____**
		3.9 Computation of Results - ETCP		
K	11		3.9.1	Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2	A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3**	**All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4**	**Results are reported as CFU/100 grams of sample.**
Bacteriological Examination of Shellfish Meats for Male Specific Coliphage (MSC)				
		3.10 MSC Equipment and Supplies		
K	2		3.10.1	Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100mL.
K	2		3.10.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		3.10.3	The sterility of each batch/ lot of pre-sterilized or reusable syringes, filters and/or filter units is determined. Results are recorded and records maintained.
C	**2**		**3.10.4**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28, 31**		**3.10.5**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
K	2		3.10.6	The temperature of the freezer is maintained at ≤-15°C.
C	**1**		**3.10.7**	**The sterility of disposable 50 mL centrifuge tubes is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28, 31		3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28, 31		3.11.2	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.

O	27, 28, 31		3.11.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	2		3.11.4 Unsterilized soft agar is stored at ≤-15°C for up to three (3) months.
K	27, 28, 31		3.11.5 The soft agar is sterilized for 15 minutes at 121°C before use.
K	28, 31		3.11.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.
K	2, 27, 28, 31		3.11.7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		3.12 Preparation of Host Culture for MSC Analysis	
C	**28, 31**		**3.12.1 *E. coli* Famp ATCC 700891 is the bacterial host strain.**
K	27, 28, 31		3.12.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28, 31**		**3.12.4 After inoculation, the host cell growth broth culture is not shaken.**
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.13.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.**
C	**28, 31**		**3.13.13 Samples are blended at high speed for 180 seconds.**
		3.14 MSC Sample Analysis	
C	**28, 31**		**3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.**

C	**27, 28, 31**		**3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.**
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	**27, 28, 31**		**3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.**
C	**27, 28, 31**		**3.14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28, 31**		**3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28, 31**		**3.14.11 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.**
K	27, 28, 31		3.14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained. Positive Control: _____
K	27, 28, 31		3.14.13 Room temperature growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.14.15 A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28, 31		3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28, 31**		**3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.15 Computation of Results - MSC	
C	**27**		**3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
K	28, 31		3.15.3 The formula used for determining the density of MSC in PFU/100 grams is: (0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.
O	2		3.15.4 The MSC count is rounded off conventionally to give a whole number.
PART IV – MSC WASTEWATER ANALYSIS			
		4.1 Collection and Transportation of Samples	

C	**11**		**4.1.1**	**Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**39**		**4.1.2**	**Effluent sample bottles must contain 0.2 mL of 10% sodium thiosulfate solution for every 200 mL of sample to inactivate any residual chlorine.**
K	1		4.1.3	Samples are identified with collector's name, sampling location, time and date of collection.
~~C~~K	9		4.1.4	Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) ~~capable of maintaining a temperature of 0 to 10°C~~ with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.
~~O~~C	**1**		**4.1.5**	**A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. ~~Temperature should be equivalent or less than that of the growing area waters.~~ Temperature must be between 0-10°C or less than temperature at collection if collected at the time of sampling. Results are recorded and maintained. ~~Results are recorded and maintained~~.**
C	**9**		**4.1.6**	**Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies		
K	38		4.2.1	Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.
K	9		4.2.2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
K	1		4.2.3	The sterility of each batch/lot of pre-sterilized or reusable syringes, filters, and/or filter units is determined. Results are recorded and records maintained.
C	**27, 28**		**4.2.4**	**The balance used provides a sensitivity of at least 0.01g.**
C	**27, 28**		**4.2.5**	**The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6	The temperature of the freezer is maintained at ≤ -15°C.
C	**28**		**4.2.7**	**The sterility of the transfer vessel utilized is determined with each lot. Results are recorded and records maintained.**
		4.3 MSC Media Preparation		
K	28		4.3.1	Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2.	Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
O	27, 28		4.3.3	Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4	Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
K	27, 28		4.3.5	The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.
K	27, 28		4.3.6	Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.

K	27, 28		4.3.7	Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis		
C	**28**		**4.4.1**	***E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2	Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3	Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	**27, 28**		**4.4.4**	**After inoculation, the host cell growth broth culture is not shaken.**

		4.5 MSC Sample Analysis		
C	**38**		**4.5.1**	**Wastewater sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10-2) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.**
K	27, 28		4.5.2	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.5.3	Two hundred microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample.
C	**27, 28**		**4.5.4**	**2.5 mL of sample is added to a tube of tempered soft agar.**
C	**27, 28**		**4.5.5**	**The tube contents is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.5.6**	**The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C			**4.5.7**	**Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.**
K	27, 28		4.5.8	Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28		4.5.9	Room Temperature Growth broth is used as the negative control or blank.
K	27, 28		4.5.10	Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		4.5.11	A negative control plate is plated at the end of each set of samples analyzed.
K	27, 28		4. 5.12	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**4.5.13**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.6 Computation of Results - MSC		
C	**38**		**4.6.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted. The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**38**		**4.6.2**	**When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
K	28		4.6.3	The formula used for determining the density of MSC in PFU/100 mL is: High Range: N * 1000 = PFU/100mL; Low Range: N * 5 = PFU/100mL; Where N is the total number of plaques counted on all plates.
O	9		4.6.4	The MSC count is rounded off conventionally to give a whole number.

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SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
MICROBIOLOGICAL COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the Microbiological Component:

1. **Does Not Conform Status**: The Microbiological component of this laboratory is not in conformity with NSSP requirements if:

   a. The total # of Critical nonconformities is **≥ 4 or**

   b. The total # of Key nonconformities is **≥ 13 or**

   c. The total # of Critical, Key and Other is **≥ 18**

2. **Provisionally Conforms Status**: The microbiological component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

NSSP Form *LAB-100 Microbiology,* Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* detection in Oysters [PART IV] QuantStudio DX

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15-minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperatures are maintained between 0 and 4 °C, except for reagent refrigerators which are maintained between 2 and 8 °C.
C	**7**		**1.4.11**	**Freezer temperature is maintained at -15 °C or below.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22	All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes used and checked for accuracy quarterly. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.
K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.

K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3**	**The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.
K	5		1.6.14	Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15	Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.

C	**2**		**1.6.16**	**The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.17**	**The sterility of autoclave sterilized disposable pipet tips and microcentrifuge tubes is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized pipet tips and microcentrifuge tubes are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.18**	**The sterility of presterilized disposable pipets, pipet tips and microcentrifuge tubes is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.19	Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation		
K	13, 14		1.7.1	Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	6		1.7.2	Media components are properly stored in a cool dry place.
O	6		1.7.3	Media components are labeled with the analyst's initials, date of receipt and date opened.
O	6		1.7.4	Dehydrated media are labeled with date of receipt and date opened.
C	**6**		**1.7.5**	**Caked or expired media or media components are discarded.**
C	**6**		**1.7.6**	**Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (<0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7	Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8	The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9**	**Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10**	**Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11**	**Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12**	**The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared Culture Media		
K	5		1.8.1	Prepared culture media are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2	Stored media are labeled with the storage expiration date or sterilization date.
K	5		1.8.3	Storage of prepared culture media at room temperature does not exceed 7 days.
K	2		1.8.4	Storage under refrigeration of prepared broth media with loose fitting closures does not exceed 1 month.
K	6		1.8.5	Storage under refrigeration of prepared culture media with screw- cap closures does not exceed 3 months.
K	11		1.8.6	All prepared broth media stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
		PART II – SAMPLES AND PROCESSING		
		2.1 Sample Collection, Transportation and Receipt		
C	**2, 6**		**2.1.1**	**A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**

K	5		2.1.2	Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3	Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
~~C~~K	5		2.1.4	Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) ~~which is maintained between 2 and 10 °C~~ with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately. For IQF samples, if samples are received frozen, they are stored in the freezer unless defrosting is initiated immediately.
~~K~~C	5, 27		**2.1.6**	**The temperature of the shellfish samples or a seawater temperature control (TC) is taken upon receipt in the laboratory.** **a. When a seawater TC is used to document temperature at receipt it must be:** **i. between 2-10°C or** **ii. if received within 5h of collection, less than that of the TC at the time of collection.** **b. When the temperature of shellstock is used to document temperature at receipt it must be:** **i. between 2-10°C or** **ii. if received within 5h of collection, less than that of the shellfish at the time of collection.** **c. For IQF samples, samples may be transported under frozen conditions.** **Results are recorded and maintained.**
C	**1**		**2.1.5**	**Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis		
K	2, 6		2.2.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2	Blades of shucking knives are not corroded.
K	5		2.2.3	The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5	Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6	Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7	Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8**	**Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9**	**The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10**	**A representative sample of at least 12 shellfish is used for analysis**
C	**2, 5**		**2.2.11**	**A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12	The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13		2.2.13	Sterile phosphate buffered saline (pH 7.4) is used as the sample diluent.
C	**5**		**2.2.14**	**Samples are blended for 60 to 120 seconds until homogenous.**

		2.3 APW Enrichment		
K	5		2.3.1	Sterile phosphate buffered saline (PBS) is used as the sample diluent.
C	**5, 15**		**2.3.2**	**The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**2.3.3**	**Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution_____**
C	**2, 15, 23**		**2.3.4**	**For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture diluted to <10^3 per ml is used as a positive process control. A non *V. vulnificus* culture is used as a negative process control.** **An uninoculated APW blank will serve as the uninoculated process control.** **The process control cultures accompany the samples throughout the method. Records are maintained.**
C	**13**		**2.3.5**	**Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**2.3.6**	**Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
PART III- PCR METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN OYSTERS SMARTCYCLER II AND AB 7500 FAST				
		3.1 PCR Reagents		
C	**14, 15**		**3.1.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		3.1.2	Fluorescent probes are stored in light occluding tubes or containers.

C	**14, 15, 18, 19**		**3.1.3 The PCR forward and reverse primers and probes are appropriate for the platform.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE/TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **<u>For Vv Real-time PCR Method</u>** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.1.4 Lyophilized forward and reverse primers, and probes, are reconstituted with TE buffer to produce a 0.1 mM stock solution.**
C	**14, 18**		**3.1.5 Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.**
C	**14, 18**		**3.1.6 Reconstituted primers and probes are stored in a -20 °C manual defrost freezer for up to 5 freeze thaw cycles, not to exceed two years.**
C	**21, 22**		**3.1.7 Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.**
C	**21, 22**		**3.1.8 PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20°C manual defrost freezer until first use. After first use, they can be stored between 2-8 °C.**
		3.2 DNA Extraction	
C	**14, 18**		**3.2.1 All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**3.2.2 Pipet tips have aerosol barriers.**
K	14, 18		3.2.3 Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.2.4 All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.2.5 Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**14, 18**		**3.2.6 One thousand (1000) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**14, 18**		**3.2.7 Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.2.8 A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.2.9 After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boil preps may be refrigerated not to exceed 72 hours.**

K	14, 18		3.2.10	Frozen extracts are analyzed within 6 months of frozen storage.
		3.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**3.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.3.2**	**For each reaction, add the specified amount of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, and internal control DNA is added.**
K	14, 21, 18		3.3.3	The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.3.4**	**Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.3.5**	**Master Mix must be used on the day of preparation or stored at –20 °C until time of use.**
		3.4 PCR		
C	**14, 19**		**3.4.1**	**If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.4.2**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.4.3	Two (2) µL of APW blank extracted from the uninoculated process control is added to a reaction tube or plate well containing 23µL of Master Mix.
K	14, 19		3.4.4	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
K	14, 19		3.4.5	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
C	**14, 19**		**3.4.6**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix as a no template control. A no template control is included with each PCR run.**
C	**14, 19**		**3.4.7**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control. A positive PCR control is included with each PCR run.**
K	14, 19		3.4.8	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	16		**3.4.9**	**After centrifugation, tubes or plates are inserted into the instrument.**
		3.5 PCR Amplification		
C	**14, 19**		**3.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		3.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**3.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		3.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**3.5.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.6 Computation of Results		
K	14, 19		3.6.1	All runs in which the positive PCR control generates a Ct value for the target(s) of interest and the no template control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.
C	**2**		**3.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**3.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**

C	**16**		**3.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		3.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**3.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control is considered invalid and should be re-tested.**
C	**13**		**3.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from either the calculator or MPN table in conjunction with dilution selection tool in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		3.6.8	For APW enrichment, results are reported as MPN/g of sample.

PART IV- PCR METHOD FOR *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* DETECTION IN OYSTERS QUANTSTUDIO DX				
		4.1 PCR Reagents		
C	**14, 15**		**4.1.1**	**Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	14, 15		4.1.2	Fluorescent probes are stored in light occluding tubes or containers.

C	**26**		**4.1.3 The PCR forward and reverse primers, and probes target.** **<u>For Total and Pathogenic Vp Real-time PCR Method</u>** Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3' Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3' Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3' WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3' WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3' Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3' Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3' Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3' Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3' TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3' TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3' Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ **<u>For Vv Real-time PCR Method</u>** vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3' vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3' vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGBNFQ
C	**14, 18**		**4.1.4 Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a 0.1 mM stock solution.**
C	**26**		**4.1.5 Storage of thawed working stocks of primers and probes are stored between 2-8°C, not to exceed 2 weeks.**
C	**26**		**4.1.6 Storage of aliquoted working stocks of primers and probes stored in -20°C manual defrost freezer does not exceed 1 year.**
C	**26**		**4.1.7 Taqman Environmental Mastermix 2.0 is stored in -20°C manual defrost freezer until first use. After first use, it is stored between 2-8°C.**
C	**26**		**4.1.8 Internal control (IC) is stored in -20°C manual defrost freezer until first use. After first use, they are stored between 2-8°C.**
		4.2 DNA Extraction	
C	**14, 18**		**4.2.1 All microcentrifuge tubes and pipet tips are sterile.**
C	**14, 18**		**4.2.2 Pipet tips have aerosol barriers.**
K	14, 18		4.2.3 Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		4.2.4 All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**4.2.5 Aseptic technique is observed throughout the extraction and PCR analysis.**
C	**26**		**4.2.6 Two-hundred (200) µL aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	**26**		**4.2.7 For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.**
K	26		4.2.8 Extracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen extracts are analyzed within 1 month of frozen storage.

C	**26**		**4.2.9**	**A tlh+ trh+ tdh+ *V. parahaemolyticus* (WA4647 or equivalent), a tlh+ tdh+ Orf8+ *V. parahaemolyticus* (BAA-240 or equivalent), and vvha+ *V. vulnificus* (ATCC 27562 or equivalent) cultures are extracted and combined to serve as the positive PCR (amplification) control.**
		4.3 Preparation of the Master Mix for PCR		
C	**14, 16, 18**		**4.3.1**	**Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**26**		**4.3.2**	**For each reaction, add the specified amount of water, appropriate primer and probe concentrations, and Taqman Environmental Master Mix.**
K	14, 16, 18		4.3.3	The Master Mix is vortexed to mix constituents and then briefly spun immediately prior to dispensing aliquots to reaction tubes or plates.
C	**14, 16, 18, 26**		**4.3.4**	**Eighteen (18) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**4.3.5**	**Master Mix must be used on the day of preparation or stored at –20°C until time of use.**
		4.4 PCR		
C	**14, 19, 26**		**4.4.1**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 18µL of Master Mix for a total PCR reaction volume of 20µL.**
K	14, 19, 26		4.4.2	Two (2) µL of extracted blank APW from the uninoculated process control is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.3	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
K	14, 19, 26		4.4.4	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
C	**14, 19, 26**		**4.4.5**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as a no template control.**
C	**14, 19, 26**		**4.4.6**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 18µL of Master Mix for each PCR run as the positive PCR (amplification) control.**
K	14, 19, 26		4.4.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 30 seconds to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**16**		**4.4.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		4.5 PCR Amplification		
C	**14, 19**		**4.5.1**	**The appropriate instrument platform is used for the protocol.**
K	16		4.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**		**4.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	14, 19		4.5.4	Optical calibrations for the dyes being used are current, per the instrument manufacturer's recommendations.
C	**14, 19**		**4.5.5**	**The analysis settings are adjusted as specified in the protocol.**

		4.6 Computation of Results		
K	14, 19		4.6.1	All runs in which the NTC has no amplification; positive control generates a Ct value for the target(s) of interest; and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.

C	2		**4.6.2**	**Data is quality checked by the analyst.**
C	**14, 19**		**4.6.3**	**All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.**
C	**16**		**4.6.4**	**Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.**
K	16		4.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.
C	**16**		**4.6.6**	**Any reaction in which no Ct value is generated for the target(s) of interest and the internal control (except NTC) is considered invalid and should be re-tested.**
C	**13**		**4.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	13		4.6.8	For APW enrichment, results are reported as MPN/g of sample.

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PCR MICROBIOLOGY COMPONENT: (Part I-IV)	
A. Results Total # of Critical (C) Nonconformities in Parts I-IV Total # of Key (K) Nonconformities in Parts I-IV Total # of Critical (C), Key (K), and Other (O) Nonconformities in Parts I-IV	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the PCR Microbiology Component:

1. **Does Not Conform Status**: The PCR Microbiology component of this laboratory is not in conformity with NSSP requirements if:
   a. The total # of Critical nonconformities is **≥4 or**
   b. The total # of Key nonconformities is **≥13 or**
   c. The total # of Critical, Key and Other is **≥18**
2. **Provisionally Conforms Status**: The PCR Microbiology component of this laboratory is determined to be provisionally conforming to NSSP requirements if the number of critical nonconformities is **≥ 1 but ≤ 3**.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *6 – PCR Microbiology Checklist*, Rev. December 2025

Proposal No. 2 7 - 1 5 0 _____

_____ **Date**

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist – PCR Microbiology Technical Modifications
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The proposal is to modify the PCR Microbiology checklist to improve the accuracy and clarity of technical checklist requirements. In addition, some modifications correct incomplete and/or inaccurate checklist requirements. Specific edits are reflected in supporting documentation. Editorial renumbering of the checklist as required by edits.
12. Public Health Significance	The proposed modifications will clarify checklist line items which will help laboratories understand and meet NSSP requirements, thereby increasing likelihood to conform to requirements.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast
	MPN Real-time PCR method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* detection in Oysters [PART IV] QuantStudio DX

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	4, 6		1.1.1	Written Plan (Check √ those items which apply).
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures (SOPs).
				d. Internal quality control measures for equipment, their calibration maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**4**		**1.1.2**	**The QA plan is implemented.**
K	6		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify the program(s): _____
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or equivalent discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	4, 6		1.3.1	Adequate for workload and storage.
K	6		1.3.2	Clean, well lighted.
K	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	6		1.3.5	Microbiological quality of the air contains fewer than 15 colonies/plate for a 15-minute exposure determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
K	5		1.4.1	To determine the pH of prepared media and reagents, the pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	6		1.4.3	The effect of temperature on the pH is compensated for by an internal/external ATC probe or by manual adjustment (*Circle the appropriate type of adjustment*).
K	4		1.4.4	The pH meter is calibrated daily or with each use as per product literature. Results are recorded and records maintained.

K	6		1.4.5	A minimum of two standard buffer solutions are used to calibrate the pH meter. The first is near the electrode isopotential point (pH 7). The second is near the expected sample pH (i.e. pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	4		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope (*Circle the method used*).
K	5		1.4.7	The balances used provide a sensitivity of at least 0.1 g at the weights of use.
K	6		1.4.8	Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	6		1.4.9	Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperatures are maintained between ~~0 and 4~~2 to 8 °C.~~, except for sample storage and reagent refrigerators which are maintained between 2 and 8 °C.~~
C	**7**		**1.4.11**	**Freezer temperature is maintained at -20~~15~~ °C or below, and is manual defrost~~.~~.**
O	7		1.4.12	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**5**		**1.4.13**	**The temperature of the incubator is maintained at 35 +/- 2.0 °C.**
K	6		1.4.14	Thermometers used in the air incubators are graduated at no greater than 0.5 °C increments.
K	5		1.4.15	Working thermometers are located on top and bottom shelves of use in the air incubator or appropriately placed based on the results of spatial temperature checks.
K	4, 6		1.4.16	Air incubator temperatures are taken twice daily on workdays. Results are recorded and records maintained.
C	**3**		**1.4.17**	**All working thermometers are appropriately immersed.**
C	**2, 20**		**1.4.18**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**6, 20**		**1.4.19**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at the points 0 and 35. These calibration records are maintained.**
K	3, 5		1.4.20	Standard thermometers are checked annually for accuracy by ice point determination. Results are recorded and maintained. Date of most recent determination: _____
C	**2, 20**		**1.4.21**	**Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy of ≤ 0.05 °C are used as the laboratory standards thermometer (*Circle the thermometer type used*).**
K	3, 8		1.4.22	All working thermometers are checked annually against the standards thermometer at temperature(s) of use. Results are recorded and records maintained.
O	6		1.4.23	Appropriate pipet aids are available and used to inoculate samples.
K	2		1.4.24	Micropipettors are calibrated annually at appropriate volumes ~~used~~ and checked for accuracy quarterly spanning volumes of use. Results are recorded and records maintained.
		1.5 Labware and Glassware Washing		
K	5		1.5.1	Utensils, containers, glassware and plasticware are clean borosilicate glass, stainless steel or other noncorroding material.

K	5		1.5.2	Culture tubes are new and of a suitable size to accommodate the volume for nutritive ingredients and sample.
K	5		1.5.3	Dilution bottles and tubes are made of borosilicate glass or plastic and closed with secure caps or screw caps with nontoxic liners.
K	5		1.5.4	Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
K	5		1.5.5	In washing reusable pipets, glassware and labware, a succession of at least three fresh water rinses plus a final rinse of deionized water is used to thoroughly rinse off all detergent.
C	**2**		**1.5.6**	**An alkaline or acidic detergent is used for washing glassware/labware.**
C	**6**		**1.5.7**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination		
K	5		1.6.1	The autoclave is of sufficient size to accommodate the workload.
K	4		1.6.2	Routine autoclave maintenance is performed and the records maintained.
C	**6, 20**		**1.6.3**	**The autoclave provides a sterilizing temperature of 121 ± 2 °C as determined for each load using a calibrated maximum registering thermometer. As an alternative, an appropriate temperature monitoring device is used in place of the maximum registering thermometer when these are unavailable due to the ban on mercury.**
K	6		1.6.4	An autoclave standards thermometer has been calibrated by a qualified calibration laboratory using a primary standard traceable to NIST or an equivalent authority at 121 °C. Calibration at 100 °C, the steam point is also recommended but not required.
K	10		1.6.5	The autoclave standards thermometer is checked every five years for accuracy at either 121 °C or at 100 °C, the steam point if the thermometer has been previously calibrated at this temperature. Date of most recent determination: _____
K	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121 °C yearly. Date of last check:_____
K	6		1.6.7	Spore strips/suspensions appropriate for use in an autoclave media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	6		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	6		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings (*Circle the appropriate type or types*).
K	6		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures in the range of 160 to 180 °C.
K	5		1.6.11	A thermometer capable of determining temperatures accurately in the range of 160 to 180 °C is used to monitor the operation of the hot air sterilizing oven.
K	8		1.6.12	Records of temperature and exposure times are maintained for the operation of the hot-air sterilizing oven.
K	6		1.6.13	Spore strips/suspensions appropriate for use in dry heat are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Results are recorded and records maintained.

K	5		1.6.14 Reusable pipets are stored and sterilized in aluminum or stainless steel containers.
K	5		1.6.15 Reusable pipets (in canisters) are sterilized in a hot-air oven at 170 °C for 2 hours.
~~C~~	~~2~~		~~1.6.16 The sterility of reusable pipets is determined with each load sterilized. Results are recorded and records maintained.~~
C	**2**		**1.6.167 The sterility of autoclave sterilized ~~disposable pipet tips and microcentrifuge tubes~~consumables is determined with each load sterilized. Results are recorded and records maintained.** **If presterilized ~~pipet tips and microcentrifuge tubes~~consumables are purchased certificate should be maintained and sterility confirmed as in 1.6.18.**
C	**2**		**1.6.178 The sterility of presterilized consumables~~disposable pipets, pipet tips and microcentrifuge tubes~~ is determined with each lot received. Results are recorded and records maintained.**
K	8		1.6.198 Spent broth cultures and agar plates are properly decontaminated before disposal.
		1.7 Media Preparation	
K	13, 14		1.7.1 Alkaline peptone water (APW) is prepared from the individual components and pH adjusted appropriately.
K	13, 26		1.7.2 The ~~P~~phosphate buffered saline (PBS) is appropriate for the method and prepared from individual components and pH adjusted accordingly.
K	6		1.7.32 Dehydrated m~~M~~edia and media components are properly stored in a cool, clean dry place.
O	6		1.7.3 Dehydrated media and m~~M~~edia components are labeled with date of receipt, date opened, expiration date, and analyst's initials.~~the analyst's initials, date of receipt and date opened.~~
~~O~~	~~6~~		~~1.7.4 Dehydrated media are labeled with date of receipt and date opened.~~
C	**6**		**1.7.5 Caked or expired media or media components are discarded.**
C	**6**		**1.7.6 Reagent water for media and diluent preparation is analyzed for residual chlorine monthly and is at a non-detectable level (<0.1 ppm). Results are recorded and records maintained**
K	6		1.7.7 Reagent water for media and diluent preparation contains <100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records maintained.
K	5		1.7.8 The volume and concentration of media in the tube is suitable for the amount of sample inoculated.
C	**6**		**1.7.9 Media broths are not in the autoclave for more than 60 minutes.**
C	**1**		**1.7.10 Media and diluent sterility is determined for each load sterilized. Results are recorded and records maintained.**
C	**1**		**1.7.11 Media productivity is determined using media-appropriate positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.**
C	**6**		**1.7.12 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer requirements and/or method tolerance. Results are recorded and records are maintained.**
		1.8 Storage of Prepared ~~Culture~~ Media	
K	5		1.8.1 Prepared culture media and sample diluent are stored in a cool, clean, dry place where excessive evaporation and the danger of contamination is minimized.
K	8		1.8.2 Stored media and sample diluent are labeled with the storage expiration date or sterilization date.
K	5		1.8.3 Storage of prepared culture media and sample diluent at room temperature does not exceed 7 days.

K	2		1.8.4 Storage under refrigeration of prepared ~~broth~~ culture media and sample diluent with loose fitting closures does not exceed 1 month.
K	6		1.8.5 Storage under refrigeration of prepared culture media and sample diluent with screw- cap closures does not exceed 3 months.
K	11		1.8.6 All prepared broth media and sample diluent stored under refrigeration is warmed to room temperature prior to use, without exceeding incubation temperature.
PART II – SAMPLES AND PROCESSING			
		2.1 Sample Collection, Transportation and Receipt	
C	**2, 6**		**2.1.1 A representative sample is collected and a chain of custody documenting the history of the sample(s) from collection to final disposal has been established.**
K	5		2.1.2 Shellfish samples as received are collected in clean, waterproof, puncture resistant containers loosely sealed or are rejected for regulatory analysis.
K	5		2.1.3 Shellfish samples as received are labeled with the collector's (or if PHP, company/processor and collector's) name, the source, the time and date of collection or are rejected for regulatory analysis.
C	**5**		**2.1.4 Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 2 and 10 °C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**2.1.5 Analysis of the samples is initiated as soon as possible after collection, but not to exceed 36 h. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36 h once removed from the freezer.**
		2.2 Preparation of Samples for Analysis	
K	2, 6		2.2.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes.
O	2		2.2.2 Blades of shucking knives are not corroded.
K	5		2.2.3 The hands of the analyst are thoroughly washed with soap and water or new gloves are donned, immediately prior to cleaning the shells of debris.
O	2		2.2.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	5		2.2.5 Shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
K	5		2.2.6 Samples are allowed to drain in a clean container or on clean towels prior to opening
K	5, 15		2.2.7 Immediately prior to shucking, the hands or gloved hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol. The gloves if worn are latex, nitrile and/or stainless steel mesh to protect analyst's hands from injury.
C	**5**		**2.2.8 Shellfish are not shucked through the hinge.**
C	**5**		**2.2.9 The contents of the sample (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
C	**5**		**2.2.10 A representative sample of at least 12 live shellfish is used for analysis**
C	**2, 5**		**2.2.11 A quantity of meat and liquor is sufficient to cover the blender blades or additional oysters are used in order to ensure sample homogeneity.**
K	2, 13		2.2.12 The sample can be processed directly or a 1:1 dilution of shellfish:diluent made. If a dilution is made, the sample is weighed to the nearest 0.1 g and an equal amount, by weight, of diluent is added.
K	13, 26		2.2.13 Sterile phosphate buffered saline ~~(pH 7.4)~~ is used as the sample diluent and is appropriate for the method.
C	**5**		**2.2.14 Samples are blended for 60 to 120 seconds until homogenous.**
		2.3 APW Enrichment	
K	13~~5~~, 26		2.3.1 Sterile phosphate buffered saline (PBS) is used as the sample diluent and is

			appropriate for the method.
C	**5, 15**		**2.3.2 The 1:10 dilution is prepared gravimetrically with PBS. All successive dilutions are prepared volumetrically.** **For example, if an initial 1:1 dilution of the sample was used for blending, the 1:10 dilution is prepared by adding 20 g of sample homogenate to 80 ml of PBS. If the homogenate was not diluted, the 1:10 dilution is prepared by adding 10 g of sample homogenate to 90 ml of PBS.**
C	**17**		**2.3.3 Appropriate sample dilutions are inoculated into APW.** **Specify dilution(s) used _____** **Specify number of tubes per dilution_____**

C	**2, 15, 23**		**2.3.4 For *V. parahaemolyticus* analysis, a tdh+, trh+ *V. parahaemolyticus* culture is diluted so that the inoculum used for the positive process control is < 100 CFU. ~~to <10^3 per ml is used as a positive process control.~~ A non-*V. parahaemolyticus* culture is used as a negative process control.** **For *V. vulnificus* analysis, a *V. vulnificus* culture is diluted so that the inoculum used for the positive process control is < 100 CFU. ~~to <10^3 per ml is used as a positive process control.~~ A non-*V. vulnificus* culture is used as a negative process control.** **An uninoculated APW blank will serve as the uninoculated process control.** **The process control cultures accompany the samples throughout the method. Records are maintained.**
C	**13**		**2.3.5 Inoculated APW enrichment tubes are incubated at 35 +/- 2 °C.**
C	**13**		**2.3.6 Tubes are read after 18 – 24 hours of incubation. Clear tubes are negative. Turbid tubes are positive and shall be further processed.**
PART III- PCR METHOD FOR *VIBRIO VULNIFICUS* AND *VIBRIO PARAHAEMOLYTICUS* DETECTION IN OYSTERS SMARTCYCLER II AND AB 7500 FAST			
		3.1 PCR Reagents	
C	**~~14, 15~~16, 27**		**3.1.1 Lyophilized primers and probes are stored according to manufacturer's instructions.**
K	~~14, 15~~16, 27		3.1.2 Fluorescent probes are stored in light occluding tubes or containers.

C	**14, 15, 18, 19**		**3.1.3 The PCR forward and reverse primers and probes are appropriate for the platform.** **For Total and Pathogenic Vp Real-time PCR Method** tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ tlh_1043: JOE/TEXAS RED-5'- CGCTCGCGTTCACGAAACCGT-3'-BHQ2 IAC_109: CY5-5'- TCTCATGCGTCTCCCTGGTGAATGTG-3'- BHQ2 trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3' trh_292R: 5'-TGTTTACCGTCATATAGGCGCTT-3' tdh_89F: 5'-TCCCTTTTCCTGCCCCC-3' tdh_321R: 5'-CGCTGCCATTGTATAGTCTTTATC-3' tlh_884F: 5'-ACTCAACACAAGAAGAGATCGACAA-3' tlh_1091R: 5'-GATGAGCGGTTGATGTCCAAA-3' IAC_46F: 5'-GACATCGATATGGGTGCCG-3' IAC_186R: 5'-CGAGACGATGCAGCCATTC-3' **For Vv Real-time PCR Method** vvhF: 5'-TGTTTATGGTGAGAACGGTGACA-3' vvhR: 5'-TTCTTTATCTAGGCCCCAAACTTG-3' vvh Probe: Cy5-5'-CCGTTAACCGAACCACCCGCAA-3'-IAbRQ IAC 46F: 5'-GACATCGATATGGGTGCCG-3' IAC 186R: 5'-CGAGACGATGCAGCCATTC-3' IC Probe: JOE-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-IABkFQ
C	**14, 18**		**3.1.4 Lyophilized forward and reverse primers, and probes, are reconstituted with TE buffer to produce a ~~0.~~100 µ~~m~~M stock solution.**
C	**14, 18**		**3.1.5 Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a ~~0.0~~10 µ~~m~~M working solution.**
C	**~~14, 18~~16, 27**		**3.1.6 Reconstituted primers and probes are stored ~~in a -20 °C manual defrost freezer~~frozen for up to 5 freeze thaw cycles, not to ~~exceed~~ exceed manufacturer's recommended storage time~~two years.~~**

~~C~~	**~~21, 22~~**		**~~3.1.7 Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use. After first use, can be stored between 2-8 °C.~~**
C	**21, 22**		**3.1.~~8~~7 PCR reagents (Platinum *Taq* DNA, dNTPs, buffer, MgCl2, fluorescent dyes) are stored ~~in -20°C manual defrost freezer~~frozen until first use. After first use, they can be stored ~~between 2-8 °C~~refrigerated.**
C	**22, 28**		**3.1.9 Internal amplification control (IAC) DNA is stored frozen until first use and then stored ~~between 2-8 °C~~refrigerated.**
		3.2 DNA Extraction	
C	**14, 18**		**3.2.1 ~~All~~Sterile consumables, including pipet tips with aerosol barriers and serological pipets with cotton plugs, are used for DNA extraction. ~~microcentrifuge tubes and pipet tips are sterile.~~**
~~C~~	**~~14, 18~~**		**~~3.2.2 Pipet tips have aerosol barriers.~~**
K	14, 18		3.2.2~~3~~ Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		3.2.3~~4~~ All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected ~~immediately~~ prior to DNA extraction, Master Mix preparation and PCR analysis.
C	**14, 18**		**3.2.4~~5~~ Aseptic technique is observed throughout the extraction and PCR analysis.**

C	**14, 18**		**3.2.56 One thousand (1000) µL aliquots from each positive APW enrichment tube, including allthe process controls, are extracted.**
C	**14, 18**		**3.2.67 Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.**
K	14, 18		3.2.78 A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.
C	**14, 18**		**3.2.89 After boiling, tubes are chilled in ice or immediately frozen in a manual defrost freezer for future analysis. Boiled DNA extracts preps may be refrigerated not to exceedfor up to 72 hours.**
K	14, 18		3.2.9 10 Frozen DNA extracts are analyzed within 6 months of frozen storage.
		3.3 Preparation of the Master Mix for PCR	
C	**14, 16, 18**		**3.3.1 Nuclease-free consumables, including pipet tips with aerosol barriers, are used in Master Mix preparation.Nuclease-free microcentrifuge tubes and pipette tips, with filters, are used in Master Mix preparation.**
C	**14, 16, 18**		**3.3.2 For each reaction, add the specified amountappropriate concentration of water, buffer, MgCl2, dNTPs, specific primers, nuclease probes, *Taq*, ROX, and internal controlIAC DNA is added.**
K	14, 21, 18		3.3.3 The Master Mix is gently vortexed to mix constituents and then briefly spun.
C	**14, 16, 18**		**3.3.4 Twenty-three (23) µL of Master Mix is used for each PCR reaction.**
C	**14, 16, 18**		**3.3.5 Master Mix must be used on the day of preparation or stored at -20 °Cfrozen until time of use.**
		3.4 PCR	
C	**14, 19**		**3.4.1 If previously frozen, the DNA extracts are completely thawed at temperatures no warmer than room temperature. Immediately prior to use, DNA extracts are centrifuged at >5,000 x g for 2 minutes to remove particulate matter and cell debris.**
C	**14, 19**		**3.4.2 Two (2) µL of DNA template extract is are added to each reaction tube or plate well containing 23 µL of Master Mix for a total PCR reaction volume of 25 µL.**
K	14, 19		3.4.3 Two (2) µL of APW blankDNA template extracted from the uninoculated process control (APW blank) is added to a reaction tube or plate well containing 23µL of Master Mix.
K	14, 19		3.4.4 Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
K	14, 19		3.4.5 Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 23 µL of Master Mix.
C	**14, 19**		**3.4.6 Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 23 µL of Master Mix as a no template control (NTC). A no template controlNTC is included with each PCR run.**
C	**14, 19**		**3.4.7 Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 23 µL of Master Mix as the positive PCR control. A positive PCR control is included with each PCR run.**
K	14, 19		3.4.8 Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for 3-5 seconds briefly to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	1614, 19		**3.4.9 After centrifugation, tubes or plates are inserted into the instrument.**

		3.5 PCR Amplification	
C	**14, 19**	**3.5.1**	**The appropriate instrument platform is used for the protocol.**
K	~~16~~29	3.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**14, 19**	**3.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	~~14, 19~~29	3.5.4	Optical calibrations for the dyes being used are maintained~~current~~, per the instrument manufacturer's recommendations. Records are maintained.
C	**14, 19**	**3.5.5**	**The analysis settings are adjusted as specified in the protocol.**
		3.6 Computation of Results	
~~K~~	~~14, 19~~	~~3.6.1~~	~~All runs in which the positive PCR control generates a Ct value for the target(s) of interest and the no template control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.~~
C	**14, 19**	**3.6.1**	**A PCR run is considered valid if the following criteria are met:** **The positive PCR control reaction generates a Ct value for the target(s) of interest; and** **T~~t~~he ~~no template control (~~NTC~~)~~ reaction generates no Ct value for the target(s) of interest but generates a Ct value for the ~~internal control~~IAC.** **If the PCR run is invalid, ~~the DNA extracts are~~all samples and process controls may be retested.**
C	**14, 19**	**3.6.2**	**A set of samples is considered valid if the following criteria are met:** **The positive process control reaction generates a Ct value for the target(s) of interest; and** **~~t~~The negative process control reaction generates no Ct value for the target(s) of interest but generates a Ct value for the ~~internal control~~IAC; and** **~~t~~The uninoculated process control (APW blank) reaction generates no Ct value for the target(s) of interest but generates a Ct value for the ~~internal control~~IAC.**
C	**2**	**3.6.3~~2~~**	**Data is quality checked by the analyst.**
C	**~~14,~~ 19, 21**	**3.6.3**	**All ~~valid~~ reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are ~~considered to be~~reported as positive.~~All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.~~**
C	**~~16~~19, 21**	**3.6.4**	**Any reaction in a valid run which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescence~~t~~ data.~~Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.~~**
K	~~16~~19, 21	3.6.5	All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the ~~internal control~~ IAC are ~~c~~Considered to be reported as negative.
C	**~~16~~19, 21**	**3.6.6**	**Any reaction in a valid run which no Ct value is generated for the target(s) of interest and no Ct value is generated for the ~~internal control~~IAC is considered invalid and ~~should~~ may be re-tested.**
C	**~~13~~32**	**3.6.7**	**Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from either the calculator or MPN table in conjunction with dilution selection tool in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**
K	~~13~~32	3.6.8	For APW enrichment, results are reported as MPN/g of sample.

PART IV- PCR METHOD FOR *VIBRIO PARAHAEMOLYTICUS* AND *VIBRIO VULNIFICUS* DETECTION IN OYSTERS QUANTSTUDIO DX			
		4.1 PCR Reagents	
C	~~14, 15~~16, 27		**4.1.1 Lyophilized and stock solutions of primers and probes are stored according to manufacturer's instructions.**
K	~~14, 15~~16, 27		4.1.2 Fluorescent probes are stored in light occluding tubes or containers.
C	**26**		**4.1.3 The PCR forward and reverse primers and probes:. ~~, and probes target.~~** **~~For Total and Pathogenic Vp Real-time PCR Method~~Multiplex 1** ~~Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3'~~ ~~Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3' Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3'~~ Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3' Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3' Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3' WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3' WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3' vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3' vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGBNFQ ~~Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3'~~ ~~Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3' Tlh F: 5' CCG CTG ACA ATC GCT TCT C 3'~~ ~~Tlh R: 5' TTT GAT CTG GCT GCA TTG CT 3' TDH F: 5' TAT CCA TGT TGG CTG CAT TC 3'~~ ~~TDH R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3'~~ ~~Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ~~ ~~WA IC Probe :VIC 5' CGT AAG ACA ATC TGA TAG TAG T 3' MGBNFQ~~ ~~Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ~~ ~~Tlh Probe: 6FAM 5'ACC ACA CGA TCT GGA GCA ACG ACG MGBNFQ TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ~~**Multiplex 2** TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3' TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3' TDH Probe 3' VIC TGT CCC TTT TCC TGC CCC CGG 5' MGBNFQ Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3' Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3' Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3' Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3' Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3' Orf8 Probe: NED 5' TCC TGC TGT ACT TTT AG 3' MGBNFQ **~~For Vv Real-time PCR Method~~** ~~vvha-F: 5' GAT CGT TGT TTG ACC GTA AAC G 3'~~ ~~vvha-R 5' TGC TAA GTT CGC ACC ACA CTG T 3'~~ ~~vvha Probe: NED 3' CAA AAC GCT CAC AGT CG 5' MGBNFQ~~
C	**14, 18, 26**		**4.1.4 Lyophilized forward and reverse primers, and probes, are hydrated with TE buffer to produce a ~~0.~~100 ~~mM~~ µM stock solution.**

C	26		4.1.5 TStorage of thawed primer/probe mixesworking stocks of primers and probes are stored refrigerated between 2-8°C, not to exceed 2 weeks.
C	26		4.1.6 AStorage of aliquoted primer/probe mixes working stocks of primers and probes are stored in -20°C manual defrost freezerfrozen, does not to exceed 1 year.
C	26		4.1.7 Taqman Environmental Mastermix 2.0 is stored frozen in -20°C manual defrost freezer until first use. After first use, it is can be stored between 2-8°Crefrigerated.
C	26		4.1.8 Internal control (IC) is stored frozen in -20°C manual defrost freezer until first use. After first use, it they aret is can be stored between 2-8°Crefrigerated.
		4.2 DNA Extraction	
C	14, 18		4.2.1 All microcentrifuge tubes and pipet tips are sterile.
C	14, 1826		4.2.1 terile pipet tips with aerosol barriers or serological pipets with cotton plugs, are used for DNA extraction.
C	26, 3114, 18		4.2.2 All MagNAPure 96 consumables meet manufacturer specificationsPipet tips have aerosol barriers.
K	14, 18		4.2.3 Latex or nitrile gloves are worn throughout the extraction and PCR preparation process.
K	14, 18		4.2.4 All work surfaces, centrifuge racks and equipment used in PCR analysis are disinfected immediately prior to DNA extraction, Master Mix preparation and PCR analysis.
C	14, 18		4.2.5 Aseptic technique is observed throughout the extraction and PCR analysis.

C	26		4.2.6 Two-hundred (200) µL aliquots from each positive APW enrichment tube, including allthe process controls, are extracted.
C	26, 31		4.2.7 DNA is extracted using the MagNaPure 96 DNA and Viral NA Small Volume kit on the Roche MagNAPure 96 instrument.
C	26		4.2.87 For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.
K	26		4.2.98 Eextracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen DNA extracts are analyzed within 1 month of frozen storage.
C	26		4.2.109 A tlh+ trh+ tdh+ *V. parahaemolyticus* (WA4647 or equivalent), a tlh+ tdh+ Orf8+ *V. parahaemolyticus* (BAA-240 or equivalent), and vvha+ *V. vulnificus* (ATCC 27562 or equivalent) cultures are extracted and combined to serve as the positive PCR (amplification) control.
		4.3 Preparation of the Master Mix for PCR	
C	14, 16, 18		4.3.1 Nuclease-free microcentrifuge tubesconsumables, and including pipette tips, with aerosol barriersfilters, are used in Master Mix preparation.
C	26		4.3.2 For each reaction, add the appropriate concentration specified amount of water, appropriate primers and probes concentrations, and Taqman Environmental Master Mix.
K	14, 16, 1826		4.3.3 The Master Mix is gently vortexed to mix constituents and then briefly spun. immediately prior to dispensing aliquots to reaction tubes or plates.
C	14, 16, 18, 26		4.3.4 Eighteen (18) µL of Master Mix is used for each PCR reaction.
C	14, 16, 1819, 26		4.3.5 Master Mix must be used on the day of preparation or stored frozenat -20°C until time of use.
		4.4 PCR	

C	**~~14, 19,~~ 26**		**4.4.1**	**Two (2) µL of DNA template is added to each reaction tube or plate well containing 18µL of Master Mix for a total PCR reaction volume of 20µL.**
K	~~14, 19,~~ 26		4.4.2	Two (2) µL of DNA template extracted ~~extracted blank APW blank extracted~~ from the uninoculated process control (APW blank) is added to a reaction tube or plate well containing 18µL of Master Mix.
K	~~14, 19,~~ 26		4.4.3	Two (2) µL of DNA template extracted from the negative process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
K	~~14, 19,~~ 26		4.4.4	Two (2) µL of DNA template extracted from the positive process control culture is added to a reaction tube or plate well containing 18µL of Master Mix.
C	**~~14, 19,~~ 26**		**4.4.5**	**Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing 18µL of Master Mix ~~for each PCR run~~ as a no template control (NTC). A NTC is included with each PCR run.**
C	**~~14, 19,~~ 26**		**4.4.6**	**Two (2) µL of DNA template extracted from the positive control culture (prepared separately from the positive process control) is added to a reaction tube or plate well containing 18µL of Master Mix ~~for each PCR run~~ as the positive PCR ~~(amplification)~~ control. A positive PCR control is included with each PCR run.**
K	~~14, 19,~~ 26		4.4.7	Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged ~~for 30 seconds~~briefly to ensure that all reagents and the DNA template are in the bottom of the tube to optimize the PCR amplification process.
C	**~~16~~26**		**4.4.8**	**After centrifugation, tubes or plates are inserted into the instrument.**
		4.5 PCR Amplification		
C	**~~14, 19~~26**		**4.5.1**	**The appropriate instrument platform is used for the protocol.**
K	~~16~~30		4.5.2	Manufacturer's instructions are followed in operating the instrument.
C	**~~14, 19~~26**		**4.5.3**	**The PCR cycle parameters used are appropriate for the protocol.**
K	~~14, 19~~30		4.5.4	Optical calibrations for the dyes being used are maintained~~current~~, per the instrument manufacturer's recommendations. Records are maintained.
C	**~~14, 19~~26**		**4.5.5**	**The analysis settings are adjusted as specified in the protocol.**

		4.6 Computation of Results		
C~~K~~	**26~~14, 19~~**		**4.6.1**	**A PCR run is considered valid if the following criteria are met:** **The positive PCR control reaction generates a Ct value for the target(s) of interest; and** **T~~t~~he no template control (NTC) reaction generates no Ct value for the target(s) of interest~~but generates a Ct value for tIC~~.** ~~4.6.1 All runs in which the NTC has no amplification; positive control generates a Ct value for the target(s) of interest; and the negative control reaction generates no Ct value for the target(s), but a Ct value for the internal control are considered valid.~~ If the PCR run is invalid, all samples and process controls may be retested.
C	**26**		**4.6.2**	**A set of samples is considered valid if the following criteria are met:** **The positive process control reaction generates a Ct value for the target(s) of interest; and** **~~t~~The negative process control reaction generates no Ct value for the target(s) of interest but generates a Ct value for the ~~internal control~~IC; and** **~~t~~The uninoculated process control (APW blank) reaction generates no Ct value for the target(s) of interest but generates a Ct value for the ~~the~~**

			~~internal control~~**IC.** **IC is only run with Multiplex 1, so validity of sample set is based on those results.**
~~C~~	~~26~~		~~4.6.3 A reaction is considered valid if the following criteria are met:~~ ~~A Ct value is generated for the target(s) of interest or~~ ~~no Ct value is generated for the target(s) of interest, but a Ct value is generated for the internal control.~~
C~~C~~	2~~2~~		**4.6.3~~4~~ Data is quality checked by the analyst.**~~4.6.2Data is quality checked by the analyst.~~
C~~C~~	~~14, 19~~14~~21,~~ ~~19~~		**4.6.4~~5~~ All ~~valid~~ reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are ~~considered to be~~reported as positive.**~~4.6.3 All reactions in a valid run which generate a Ct value for the target(s) of interest with a sigmoidal amplification curve are considered to be positive.~~
C	~~16~~21		**4.6.5~~6~~ Any reaction in a valid run which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescence~~t~~ data.**
~~C~~	~~16~~		~~Any sample which does not demonstrate a sigmoidal amplification curve may have a reported positive/negative determination that is discrepant from the instrument if appropriately justified using the raw fluorescent data.~~
K~~K~~	~~16~~16~~21~~		~~3.6.5~~4.6.6 All ~~valid~~ reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are ~~Considered to be~~reported as negative.~~4.6.5 All reactions in a valid run which do not generate a Ct value for the target(s) of interest, but do generate a Ct value for the internal control are considered negative.~~
C~~C~~	~~16~~1~~2~~1~~6~~		**4.6.7 Any sample reaction in a valid run in which no Ct value is generated for the target(s) of interest and the ~~internal control~~IC with Multiplex 1 is considered invalid and ~~should~~may be re-extracted.**~~4.6.6 Any reaction in which no Ct value is generated for the target(s) of interest and the internal control (except NTC) is considered invalid and should be re-tested.~~
C~~C~~	~~13~~1~~3~~2~~32~~		**4.6.8 Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from either the calculator or MPN table in conjunction with dilution selection tool in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).**~~4.6.7 Upon determination of positive reactions, refer to the original positive dilutions of APW and record MPN values as derived from the calculator in Appendix 2 of the FDA Bacteriological Analytical Manual (BAM).~~
K~~K~~	~~13~~1~~3~~2~~3~~		4.6.9 For APW enrichment, results are reported as MPN/g of sample.~~4.6.8 For APW enrichment, results are reported as MPN/g of sample.~~

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LABORATORY:	DATE of EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST
PCR Microbiology

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	NSSP Biotoxin-related Laboratory Evaluation Checklists: Collection and Transportation of Samples Contingency Plan Edits
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory @.01 Quality Assurance .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to modify the Collection and Transportation of Samples contingency plan checklist line items in multiple checklists. The proposed edits are applicable to the following NSSP Laboratory Evaluation Checklists: 1. Mouse Bioassay and Scotia Rapid Test for Paralytic Shellfish Poisoning (PSP), line item 1.6.4 2. Paralytic Shellfish Poisoning (PSP HPLC-PCOX), line item 1.6.4 3. Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV, line item 1.6.4 4. MARBIONC Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA, line item 1.6.4 5. Diarrhetic Shellfish Poisoning Toxins (DSP) LC-MS/MS, line item 1.6.4 6. Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP), line item 2.1.5 Modified Text for Sample Collection and Transportation section: ~~Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur~~ When ~~are anticipated~~ the time from collection to initiation of extraction is anticipated to exceed 24 hours, the sample submitter(s) and laboratory implement ~~has~~ an appropriate written contingency plan ~~in place~~ to handle these samples and protect sample integrity. ~~For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt~~. The written contingency plan must ~~also~~ address~~es~~ field ~~and/or laboratory~~ practices and sample processing that ensures the sample and extract integrity ~~of the sample or extract~~ until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed

	The specific checklists and text are in the accompanying documents.
12. Public Health Significance	The proposed modifications to the requirement line item will clarify the laboratory's sample contingency plan standard requirement and improve consistency in NSSP laboratories. The current requirement has been misinterpreted, and the proposed modifications provide direct language to define the requirement.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

PUBLIC HEALTH SERVICE
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SHELLFISH LABORATORY EVALUATION CHECKLIST
Mouse Bioassay and Scotia Rapid Test for Paralytic Shellfish Poisoning (PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Mouse Bioassay for Paralytic Shellfish Poisoning (MBA PSP)
	Scotia Rapid Test for Paralytic Shellfish Poisoning (SRT PSP)

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	5, 6, 8		1.1.1 Written Plan adequately covers all of the following: (check √ those items which apply)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
			h. Animal care.
C	**6**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experiencerequirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meet the state/county educational and experiencerequirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst(s) meets the state/county educational and experiencerequirements for processing samples in a public health laboratory.
		1.3 Work Area	
O	5, 6		1.3.1 Adequate for the workload and storage.
O	5		1.3.2 Clean and well lighted.
O	5		1.3.3 Adequate temperature control.
O	5		1.3.4 All work surfaces are nonporous and easily cleaned.
C	**8**		**1.3.5 A separate, quiet area with adequate temperature control for mice acclimation and injection is maintained.**
		1.4 Laboratory Equipment	
O	2		1.4.1 The pH meter has a standard accuracy of 0.1 pH units.
K	9		1.4.2 pH paper in the appropriate range (i.e. 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	7		1.4.3 pH electrodes consist of pH half-cell and reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).
K	6		1.4.4 pH meter is calibrated daily when in use. Results are recorded and records are maintained.

K	5		1.4.5	Effect of temperature has been compensated for by an ATC probe; use of a triode or by manual adjustment.
K	5		1.4.6	A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.
K	6, 12		1.4.7	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of slope. (Circle method used).
K	2		1.4.8	The balances being used provide an appropriate sensitivity at the weights of use. a. To prepare reference solution, the balance must have a sensitivity of at least 0.1 g at a load of 1 g. b. For sample extraction, the balance must have a sensitivity of at least 0.1 g at a load of 100 g. c. For gravimetric extract volume adjustment, the balance must have a sensitivity of at least 0.1 g at a load of 200 g. d. To weigh mice for assay, the balance must have a sensitivity of at least 0.1 g at a load of 20 g.
K	4,5		1.4.9	The balance calibration is checked monthly according to the manufacturer's specifications using NIST Class S, ASTM Class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10	Refrigerator temperature is maintained between 0 and 4˚C.
K	5		1.4.11	Refrigerator temperature is monitored at least once daily on workdays. Results are recorded and records are maintained.
K	4		1.4.12	Freezer temperature is maintained within manufacturer's tolerance.
K	5		1.4.13	Freezer temperature is monitored at least once daily on workdays. Results are recorded and records are maintained.
C	**10**		**1.4.14**	**All in-service thermometers are properly calibrated and immersed. Results are recorded and records are maintained.**
O	6		1.4.15	All glassware is clean.
C	**5**		**1.4.16**	**With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records are maintained.**
C	**9**		**1.4.17**	**An alkaline or acid based detergent is used for washing glassware/labware.**
		1.5 Reagents and Reference Solution Preparation and Storage		
C	**9**		**1.5.1**	**Any residual (unused) STX diHCl standard solution is never stored after the ampule has been opened.**
K	15		1.5.2	PSP reference solution (1 µg/mL) is prepared gravimetrically and diluted with 0.001 M HCl solution.
K	9		1.5.3	Prepared PSP reference solution is stored under refrigeration in a sealed non-reactive container. Solution may be stored indefinitely as long as there is no detectable evaporation loss as determined by weight. If evaporation is detected, the solution is discarded appropriately. Records are maintained.
C	**14**		**1.5.4**	**All working dilutions from the PSP reference solution are prepared gravimetrically using 0.001 M HCl.**

K	9		1.5.5	All working dilutions prepared from the PSP reference solution are discarded appropriately after use.
C	**5**		**1.5.6**	**Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm – cm resistance(2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25 °C. (Circle the appropriate water quality descriptor determined). Results are recorded and records are maintained.**
K	5		1.5.7	Reagent water is analyzed for residual chlorine monthly and is at a non-detectable level (≤ 0.1 mg/L). Results are recorded and records are maintained. Specify method of determination_____.
K	5		1.5.8	Reagent water contains < 100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained.
		1.6 Collection and Transportation of Samples		
O	2		1.6.1	Shellstock are collected in clean, waterproof, puncture resistant containers, loosely sealed.
K	2		1.6.2	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**2**		**1.6.3**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	15, 9		1.6.4	~~Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur,~~ When the time from collection to initiation of extraction is anticipated to exceed 24 hours, the sample submitter(s) and ~~the~~ laboratory implement ~~has~~ an appropriate written contingency plan ~~in place~~ to handle these samples and protect sample integrity. ~~For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt.~~ The written contingency plan must ~~also~~ address~~es~~ field ~~and/or~~ practices and ~~laboratory~~ sample processing that ensure~~s~~ the sample and extract integrity ~~of the sample or extract~~ until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**14**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – ANALYSIS OF SHELLFISH FOR PSP TOXINS – MOUSE BIOASSAY (MBA)				
		2.1 Preparation of Samples for Analysis – Homogenization		
C	**15, 9**		**2.1.1**	**At least 12 animals (or more to provide 100 g of shellfish meat) are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	2		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	2		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	2		2.1.4	The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.

O	2		2.1.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.

C	**2**		**2.1.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	2		2.1.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	2		2.1.8	Pieces of shell and drainage are discarded.
C	**2**		**2.1. 9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60 - 120 seconds).**
		2.2 Preparation of Samples for Analysis – APHA/AOAC Digestion & Extraction		
K	15, 9		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
K	2		2.2.2	100 grams of homogenized sample is weighed into a beaker.
K	2		2.2.3	The sample homogenate is extracted in a 1:1 weight/volume ratio by adding 0.1 M HCl or 0.18 M HCl (*circlethe appropriate choice*).
K	2		2.2.4	Homogenate/acid mixture is stirred thoroughly before boiling to completely mix the contents.
C	**2**		**2.2.5**	**To prevent toxin transformation, the pH of the homogenate/acid mixture before boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
C	**2**		**2.2.6**	**The homogenate/acid mixture is promptly brought to its boiling point, then gently boiled at 100 ± 1 ºC for 5 minutes.**
O	9		2.2.7	The homogenate/acid mixture is boiled under adequate ventilation (e.g. fume hood).
O	9		2.2.8	The homogenate/acid mixture is allowed to cool to room temperature.
C	**2**		**2.2.9**	**The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	2		2.2.10	The homogenate/acid mixture is adjusted gravimetrically to the pre-boiling weight using 0.001 M HC1.
K	2		2.2.11	The homogenate/acid mixture is allowed to separate by gravity or by centrifugation (e.g. centrifuged at 3,000 RPM for 5 minutes).
K	9		2.2.12	If the extracted sample cannot be assayed immediately, then the supernatant is decanted and stored in a sealed container under refrigeration for up to 24 hours or frozen for longer storage.
K	9		2.2.13	Refrigerated extracts areallowed to reach ambient temperature before being bioassayed or tested by the SRT for PSP.
		2.3 Mouse Bioassay (MBA) for PSP		
K	2		2.3.1	A 26-gauge hypodermic needle is used for intraperitoneal injections.
C	**2**		**2.3.2**	**Healthy mice in the weight range of 17.0 -23.0 grams (19 - 21 grams is preferable) from a stock colony are used forroutine assays. Previouslyinjected mice are never re-used for a bioassay.** **Stock strain:_____ Source:_____**

C	**9**		**2.3.3**	**Mice are allowed to acclimate at least 24 hours prior to injection. In some cases, 48 hours may be required.**

C	**9**		**2.3.4**	**A conversion factor (CF) for the lab has been appropriately determined.** **Lab CF:_____Date CF established:_____**
C	**2**		**2.3.5**	**The CF value is checkedweekly if assays are done on one or several days during the week or once each day that assays are performed if they are performed less than once per week.** **Date of current CF check:_____ CF verified: yes/no (*circle choice*)**
C	**2**		**2.3.6**	**If the lab CF is not verified during a check, the lab follows the appropriate procedure for establishing a temporary CF to use for the day/week.**
C	**2, 9**		**2.3.7**	**If the lab CF fails to be verified, the cause is investigated and the situation is corrected. If the cause cannot be determined with reasonable certainty and the lab CF fails to be verified > three times in a year, the lab CF is recalculated through a restandardization procedure.**
K	9		2.3.8	Mice are weighed to the nearest 0.1 g.
C	**2**		**2.3.9**	**Mice are injected intraperitoneally with 1 mL of extracted sample.**
K	2		2.3.10	For CF checks, five mice are injected.
K	9		2.3.11	For routine assays, three mice (two when both survive) are injected per sample.
C	**2**		**2.3.12**	**Elapsed time post-injection is accurately determined and recorded.**
C	**2**		**2.3.13**	**When death occurs, the time of death to the nearest second is noted at the last gasping breath and recorded.**
C	**9, 2**		**2.3.14**	**Mice are continually observed for up to 20 minutes after injection, then periodically observed for a total time of up to 60 minutes after injection.**
C	**2**		**2.3.15**	**If the median corrected mouse unit is greater than 1.92 (5 minutes), then the sample is diluted with0.001 M HCl as appropriate to achieve a median corrected mouse unit, MCMU of 1.39-1.92 (a death time of 5-7 minutes).**
		2.4 Calculation of toxicity for MBA		
C	**2**		**2.4.1**	**The death time for each mouse is converted to mouse units (MU) using Sommer's Table and recorded. Any mice surviving beyond 60 minutes are recorded as < 0.875 MU.**
C	**2**		**2.4.2**	**The weight for each mouse is corrected to mouse units using the table of weights in Recommended Procedures (Table 7) and interpolated for weights not listed.**
C	**2**		**2.4.3**	**The Corrected Mouse Unit (CMU) for each mouse injected is calculated as follows:** **Death time in MU x Weight correction in MU = CMU**
C	**2**		**2.4.4**	**The Median Corrected Mouse Unit (MCMU) for each sample is calculated and used in the final toxicity calculation for that sample.**

C	**2**		**2.4.5 The toxicity of each sample is calculated as follows:** **µg STX eq/100 g of sample = MCMU x CF x DF x 200 except when less than 100 grams of sample is used for analysis.** **In this case an adjustment for sample weight must be made such that the formula for calculating sample toxicity becomes:** **µg STX eq/100 grams of sample = MCMU x CF x DF x 200/Adjusted weight of the acidified sample x 200.** **Where:** **MCMU=Median Corrected Mouse Unit for the sample** **CF=Laboratory Conversion Factor** **DF=Dilution Factor (e.g. 1:1 dilution, DF=2)**
C	**11**		**2.4.6 Any value equal to or greater than 80 µg STX eq/l00 g of sample is actionable.**
PART III – ANALYSIS FOR PSP TOXINS – SCOTIA RAPID TEST (SRT)			
		3.1 Screening by Scotia Rapid Test (SRT)	
K	9		3.1.1 Before beginning any screening, the following items are recorded for the SRT kit in use. a. Date received. b. Batch/lot numbers for all kit components (test strip and PSP AOAC buffer). c. Expiration dates for all kit components. d. Date opened and/or used.
K	13		3.1.2 When placed into service, all kit components are within the accepted expiration dates.
C	**13**		**3.1.3 The desiccant pouch inside the test strip wrapping is blue in color, indicating suitability for use. Any test strip wrapping containing a pink desiccant pouch is discarded.**
K	13		3.1.4 All kit components are stored according to the manufacturer's recommendations.
C	**9**		**3.1.5 A positive control of 80 µg STX eq/100 g of sample is used to test new kit lots and buffers. Results are recorded and records maintained.**
C	**9**		**3.1.6 Micropipettes with appropriate ranges for the volumes being measured are used.**
K	9		3.1.7 All micropipettes are maintained and calibrated according to manufacturer's instructions. Results are recorded and records maintained.
C	**13**		**3.1.8 400 µL of buffer solution is accurately transferred to a small tube.**
C	**13**		**3.1.9 100 µL of sample extract is accurately added to the buffer.**
K	13		3.1.10 The buffer/sample mixture is carefully mixed by inserting the tip of the micropipette into the mixture and pipetting up and down at least three times.
C	**13**		**3.1.11 100 µL of the thoroughly mixed solution is added to the test strip sample well.**
K	9		3.1.12 Micropipette tips are not reused.
K	13		3.1.13 Inoculated test strips are allowed to react with the sample mixture for the period of time recommended by the manufacturer.

C	**13**		**3.1.14**	**The test strip result is interpreted according to the instruction card provided by the manufacturer, which is specific to each batch/lot of test strips. Results are recorded and records are maintained.**
K	13		3.1.15	If a test result is interpreted as invalid; the pH of thesample extract is checked and adjusted as needed to fall between pH 2.0 – 4.0. Fresh PSP AOAC buffer is used to re-test the sample on a newtest strip.
C	**13**		**3.1.16**	**If the same sample is interpreted as invalid on two different test strips, then the sample is assumed to contain interfering substances, and an alternative test method is used.**
C	**11**		**3.1.17**	**Any positive result on a SRT is actionable.**

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LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PARALYTIC SHELLFISH POISON COMPONENT: PARTS I, II, III	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the PSP, MBA and/or SRT Component

1. **Conforms Status:** The PSP, MBA and/or SRT component of this Laboratory is in conformity with NSSP requirements if all of the following apply.

   a. No Critical nonconformities.
   b. and <6 Key nonconformities.
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The PSP, MBA and/or SRT component of this Laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.

   a. the number of Critical nonconformities is ≥ 1 but < 4,
   b. and <6 Key nonconformities.
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status:** The PSP, MBA and/or SRT component of this Laboratory is not in conformity with NSSP requirements when any of the following apply.

   a. The total # of Critical nonconformities is ≥4.
   b. or total # of Key nonconformities is ≥ 6.
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

NSSP Form *2 – Mouse Bioassay and Scotia Rapid Test for PSP Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Paralytic Shellfish Poisoning (PSP HPLC-PCOX)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check √ those that apply)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
			g. External performance assessment.
C	**5**		**1.1.2 QA Plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	**State's Human Resources Department**		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	**USDA Microbiology & EELAP**		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	**5**		**1.2.5 LC-Operator must be competent in the operation and maintenance of a basic liquid chromatography system.**
		1.3 Work Area	
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment.	
O	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit.
K	6		1.4.2 pH paper in the appropriate range (i.e. 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	10		1.4.3 pH electrodes consist of pH half-cell and reference half-cell or equivalent combination electrode/triode (free from Ag/AgCl or contains an ion exchange barrier to prevent passage of Ag ions into the medium that may result in inaccurate pH readings).
K	5		1.4.4 pH meter is calibrated daily when in use. Results are recorded and records are maintained.
K	8		1.4.5 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	8		1.4.6 A minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e. pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded.

K	5, 11		1.4.7 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	6		1.4.8 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8, 9		1.4.9 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.10 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.11 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	1		1.4.12 Freezer temperature is maintained at -20 ºC or below.
K	8		1.4.13 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**13**		**1.4.14 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.15 All glassware is clean.
K	3		1.4.16 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. binary mobile phase system delivering a pulse-free flow of 0.5-2.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 5-30 µL injections, d. temperature controlled column compartment capable of controlling temperature between 10 – 50 ºC, and e. fluorescence detector able to achieve the required sensitivity at an excitation wavelength (λ) of 330 nm and emission of 390 nm.
K	3, 4		1.4.17 The post-column reaction system used is equipped with the following: a. reactor module capable of maintaining 85 ºC, b. dual reagent pumps capable of delivering accurate flows of 0.4 mL/min, and c. if applicable, a reaction coil (knitted or equivalent) having a total volume of 1 mL and a length of 5 m x 0.5 mm.
K	6		1.4.18 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.19 A boiling water bath with sufficient volume to cover the sample/acid mixture is used for extraction.
O	3		1.4.20 Centrifuge capable of holding 50 mL polypropylene tubes.
K	3		1.4.21 Microcentrifuge capable of holding 1.5 mL microcentrifuge tubes and generating a minimum of 16000 g or equivalent is used.
		1.5 Reagents and Reference Solution Preparation and Storage	
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
C	**8**		**1.5.2 Water is glass distilled or deionized and exceeds 0.5 megaohm resistance or is less than 2 µSiemens/cm conductivity at 25 ºC to be tested and recorded monthly for resistance or conductivity and the results are recorded.**
K	8		1.5.3 Water is analyzed for residual chlorine monthly and is at a nondetectable level (≤0.1 ppm) Results are recorded and records are maintained.

K	8		1.5.4 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained.
K	8		1.5.5 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.7 The binary mobile phase system used to analyze the GTX and STX toxins consists of:** **1.5.7.1 Mobile Phase A, which contains 11 mM heptane sulfonate and 5.5 mM phosphoric acid (H_3PO_4), pH 7.1.** **1.5.7.2 Mobile Phase B, which contains 11 mM heptane sulfonate, 16.5 mM H_3PO_4 and11.5% acetonitrile (MeCN), pH 7.1.**
C	**3**		**1.5.8 The binary mobile phase system used to analyze the C toxins consists of:** **1.5.8.1 Mobile Phase A, which contains 2 mM tetrabutyl ammonium phosphate, pH 5.8.** **1.5.8.2 Mobile Phase B, which contains 2 mM tetrabutyl ammonium phosphate in 4% acetonitrile, pH 5.8.**
C	**3**		**1.5.8 The post-column oxidant consists of 100 mM H_3PO_4 and 5 mM periodic acid (H_5IO_6), pH 7.8.**
C	**3**		**1.5.9 The post-column acid used is 0.75 M nitric acid (HNO_3).**
C	**3**		**1.5.10 The heptane sulphonate used in mobile phase A and mobile phase B to analyze for GTX and STX toxins is prepared the day of use or refrigerated for up to one week.**
C	**3**		**1.5.11 The pH of mobile phases and the post-column oxidant are adjusted as follows:** **a. Mobile phase A and mobile phase B for the GTX and STX toxins are adjusted to 7.1 with ammonium hydroxide (NH_4OH),** **b. Mobile phase A and mobile phase B for the C toxins are adjusted to 5.8 in one direction only with 10% acetic acid (HOAc) if too basic or 1% NH_4OH if too acidic, and** **c. The post-column oxidant is adjusted to 7.8 with 5 M sodium hydroxide (NaOH).** **d. Mobile phases and post-column reagents are filtered before use if the HPLC does not have a degreaser.**
C	**3,7**		**1.5.12 Only certified reference materials are used for standard solutions. Source of the reference standard:**
C	**7**		**1.5.13 NRC Zero-Mus or a negative control matched matrix is used as a matrix blank as appropriate. Source of the negative matrix:_____**
C	**7**		**1.5.14 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.15 All standards used are within expiration date.**
C	**3**		**1.5.16 All standards are prepared gravimetrically.**
K	3		1.5.17 Intermediate mixes of primary standards are made up in 0.003 M HCl for the GTX/STX toxins or pH 5 glass distilled/deionized water for the C toxins labeled with the date of preparation and the expiration date and stored appropriately. The pH of the glass distilled/deionized water is adjusted when necessary by the dropwise addition of 10% acetic acid (HOAc).

C	**3**		**1.5.18**	**Working standards are made up from primary standard or intermediate mixes by dilution with toxin-free, deproteinated, matrix matched extracts.**
C	**7**		**1.5.19**	**Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.20**	**Quality Control shellfish tissues are stored frozen.**
C	**7**		**1.5.21**	**Working standards are labeled with the date of preparation, stored appropriately and used within 3 months of preparation.**
		1.6	**Collection and Transportation of Samples**	
O	6		1.6.1	Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6		1.6.2	Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**6**		**1.6.3**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	14		1.6.4	~~Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur,~~ When the time from collection to initiation of extraction is anticipated to exceed 24 hours, the sample submitter(s) and laboratory ~~has~~ implement an appropriate written contingency plan ~~in place~~ to handle these samples and protect sample integrity. ~~For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt.~~ The written contingency plan must ~~also~~ addresses ~~es~~ field ~~and/or laboratory~~ practices and sample processing that ensure~~s~~ the sample and extract integrity ~~of the sample or extract~~ until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**6**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR PSP TOXINS				
		2.1	**Preparation of Sample**	
C	**6**		**2.1.1**	**At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	6		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4	The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**6**		**2.1.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8	Pieces of shell and drainage are discarded.

C	**6**		**2.1.9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**

		2.2 Digestion of Sample		
K	6		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**3**		**2.2.2**	**Five (5) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
K	3		2.2.3	The sample homogenate is extracted in a 1:1 w/v ratio with 0.1 M HCl.
K	3		2.2.4	Homogenate/acid mixture is vortexed thoroughly before boiling to completely mix the contents.
C	**3**		**2.2.5**	**To prevent toxin transformation, the pH of the homogenate/acid mixture before boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of either 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH.**
C	**3**		**2.2.6**	**Samples in capped 50 mL polypropylene centrifuge tubes are extracted in a boiling water bath for 5 minutes.**
K	3		2.2.7	The pH of the cooled mixture after boiling is 3.0 ± 1.0, adjusted if necessary with the dropwise addition of 5 M HCl. Any sample with a pH of less than 2.0 is discarded and extracted again.
K	3		2.2.8	The homogenate/acid mixture is allowed to separate by gravity or by centrifugation.
		2.3 Deproteination		
C	**3**		**2.3.1**	**500 µL of sample extract is deproteinated with 25 µL of 30% trichloroacetic acid, vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
C	**3**		**2.3.2**	**The pH of the deproteinated extract is adjusted with 35 µL of 1.0 M NaOH vortexed thoroughly and centrifuged at ~ 16,000 *g* for 5 minutes.**
K	3		2.3.3	An aliquot of the deproteinated supernatant is filtered through a 0.2 µm filter.
		2.4 Analysis		
C	**2**		**2.4.1**	**A standard calibration curve (of at least six concentrations) is performed upon initial instrument set up, following any major hardware maintenance activity, or when the continuing calibration verification (CCV) indicates significant drift (> 30% for individual toxin) from the calibration. Results are recorded and records are maintained.**
K	3		2.4.2	10 µL is injected for GTX/STX toxins and 5 µL is injected for C-toxins.
K	3		2.4.3	Samples are stored in the sample compartment of the autosampler at 4 °C during analysis. Otherwise samples must be analyzed within 20 hours if the autosampler is held at room temperature.
K	3		2.4.4	A column heater that is capable of maintaining 30-40 °C for the GTX/STX toxins and 10-20 °C for the C toxins is used in the analysis.
C	**3**		**2.4.5**	**The appropriate analytical column is used.** **a. GTX/STX Toxins: Agilent Zorbax Bonus-RP column, 4.6 mm x 150 mm, 3.5 µm or equivalent.** **b. C Toxins: Thermo BetaBasic 8, 4.6 mm x 250 mm, 5 µm or equivalent.**

		2.5 System Suitability	
K	2	2.5.1	The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for each individual toxin.

C	**3**	**2.5.2**	**The resolution and retention time criteria that must be met are:** **a. For GTX and STX toxins, the matrix peak must be at least 70% baseline resolved between GTX3 and GTX2.** **b. For GTX and STX toxins, GTX5 must be at least 40% baseline resolved between dcGTX3 and dcGTX2.** **c. For GTX and STX toxins, dcSTX and STX must be at least 70% baseline resolved.** **d. For GTX and STX toxins, the retention time of GTX4 must be between 5 and 7 minutes.** **e. For the C toxins, C2 must be at least 70% baseline resolved between C1 and C2.** **f. For the C toxins, the retention time of C1 must be between 4 and 7 minutes.**
C	**2**	**2.5.3**	**Daily injection schedules must include the adequate frequency of injection standards based on an assessment of individual standard toxin variability. Variability in peak response must be less than 10% for calculation of toxicity in samples.**
		2.6 Calculation of Toxicity	
C	**4**	**2.6.1**	**The toxicity of the individual toxins is calculated as follows:** $\mu gSTXdiHCleq/100g = \mu MM \times \frac{333333.33}{111111111111} \times \frac{FFFFFFFF}{EEEEEE.FFFFFF} \times \frac{WWEE+VVFFFF}{WWEE} \times RRRRRREE \times 111111$ **Where:** **µM = Concentration of toxin in the extract, in µM;** **Fvol = Final volume of the deproteinized extract (e.g. 560 µL);** **Ext.vol = Volume of crude extract used (e.g. 500 µL);** **Wt = Weight of sample used;** **Vol = Volume of acid extractant used (e.g. 5 mL); and** **ReTx = Relative toxicity of toxin vs. Saxitoxin.**
C	**3**	**2.6.2**	**The individual toxicities for each toxin are summed to obtain the overall sample toxicity in µg STX equivalents/100 g (µg/100 g).**
C	**12**	**2.6.3**	**Any value at or above 80 µg STX equivalents /100 g of meat is actionable.**

Toxin	ReTx	Toxin	ReTx
GTX1	0.9940	NEO	0.9243
GTX2	0.3592	STX	1.0000
GTX3	0.6379	dcSTX	0.5131
GTX4	0.7261	C1	0.0060
GTX5	0.0644	C2	0.0963
dcGTX2	0.1538	C3	0.0133
dcGTX3	0.3766	C4	0.0576

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1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. AOAC Official Methods of Analysis (2011). AOAC Official Method 2011.02 Paralytic Shellfish Toxins in Mussels, Clams, Oysters, and Scallops Post-Column Oxidation (PCOX) Method.
4. Oshima, Y. 1995. J. AOAC Int. 78: 528-532.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16: 54-60.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2011. *NSSP Guide to the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PARALYTIC SHELLFISH POISON (PSP), PCOX COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the PSP, PCOX Component

1. **Conforms Status:** The PSP, PCOX component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

2. **Provisionally Conforms Status:** The PSP, PCOX component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. the number of critical nonconformities is **≥ 1** but **< 4**.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The PSP, PCOX component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is **≥ 4**.
   b. or the total # of Key nonconformities is **≥ 6.**
   c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____Date:_____

LEO Signature:_____Date:_____

NSSP Form *4 – PSP HPLC PCOX Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Domoic Acid (Amnesic Shellfish Poisoning; ASP) HPLC-UV

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	5, 8		1.1.1 Written Plan adequately covers all the following: (check √ those that apply)
			a. Organization of the laboratory.
			b. Staff training requirements.
			c. Standard operating procedures.
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety.
			f. Internal performance assessment.
C	**5**		**1.1.2 QA Plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
C	**5**		**1.2.5 LC-Operator must be trained in the operation and maintenance of a basic liquid chromatography system.**
		1.3 Work Area	
O	5, 8		1.3.1 Adequate for workload and storage.
O	8		1.3.2 Clean and well lighted.
O	8		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment	
K	6		1.4.1 The pH meter has a standard accuracy of 0.1 unit. [Only applicable if using the sample cleanup procedure]
K	5		1.4.2 The pH meter is calibrated daily when in use. Results are recorded and records are maintained. [Only applicable if using the sample cleanup procedure]
K	8		1.4.3 Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment. [Only applicable if using the sample cleanup procedure]
K	8		1.4.4 The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions is used to calibrate the pH meter. The first must be near the electrode isopotential point (pH 7). The second must be near the expected sample pH (i.e., pH 2, 4 or 11) as appropriate. Standard buffer solutions are used once and discarded. [Only applicable if using the sample cleanup procedure]

K	5, 11		1.4.5 Electrode acceptability is determined daily or with each use following either slope or millivolt procedure. [Only applicable if using the sample cleanup procedure]
K	6, 2		1.4.6 The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	8		1.4.7 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.8 Refrigerator temperature is maintained between 0 and 4 ºC.
K	8		1.4.9 Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K	4,15		1.4.10 Freezer temperature is maintained at -10 ºC or below.
K	8		1.4.11 Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	**13**		**1.4.12 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.13 All glassware is clean.
K	4		1.4.14 A high performance liquid chromatography system (HPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 1.0 mL/min, b. solvent degasser, c. autosampler (refrigerated preferred) with loop suitable for 20 µL injections, d. temperature controlled column compartment capable of controlling temperature at 40 ºC, e. ultraviolet detector/diode array detector able to achieve the required sensitivity at a wavelength (A) of 242 nm, and f. a data collection system (e.g., computer, integrator).
K	2		1.4.15 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	4		1.4.16 A solid phase extraction (SPE) vacuum manifold capable of holding 3 mL cartridges is used. [Only applicable if using the sample cleanup procedure]
O	4		1.4.17 A centrifuge capable of holding 50 mL polypropylene tubes is used.
		1.5 Reagents and Reference Solution Preparation and Storage	
C	**4, 15**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	8		1.5.2 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	8		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**4, 15**		**1.5.4 The mobile phase system used to analyze domoic acid consists of: 10% aqueous acetonitrile (v/v) and 0.1% trifluoroacetic acid (TFA).**
O	4		1.5.5 Mobile phase is filtered before use if the HPLC does not have a degasser.

C	**7**		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
K	4, 15		1.5.7 A cartridge wash solution is made up of 1 volume acetonitrile to 9 volumes of water (i.e., 10% aqueous acetonitrile). [Only applicable if using the sample cleanup procedure]
K	4		1.5.8 Citrate buffer (0.5 M, pH 3.2) is made up by dissolving 40.4 g citric acid monohydrate and 14 g triammonium citrate in 400 mL water, then adding 50 mL acetonitrile and diluting the total to 500 mL with water [or equivalent buffer]. [Only applicable if using the sample cleanup procedure]
C	**7**		**1.5.9 NRC CRM Zero-Mus or a negative control is used as a blank to ensure that there is no carry over between samples/standards. Source of the negative control: _____**
C	**7**		**1.5.10 All primary standards are stored appropriately as per supplier recommendations.**
C	**7**		**1.5.11 All standards used are within expiration date.**
C	**2**		**1.5.12 All standards are prepared either gravimetrically or using positive displacement pipettes.**
C	**4, 15**		**1.5.13 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 50% aqueous methanol). Dilution with toxin-free, cartridge wash solution (aqueous acetonitrile) is allowed if using the diluted crude sample or the sample cleanup procedure.**
C	**7**		**1.5.14 Zero-Mus is stored according to manufacturer's instructions.**
C	**2**		**1.5.15 Quality Control shellfish tissues are stored frozen.**
		1.6 Collection and Transportation of Samples	
O	6, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	6, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**6, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**
K	14, 2		1.6.4 ~~Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur,~~ When the time from collection to initiation of extraction is anticipated to exceed 24 hours, the sample submitter(s) and ~~-~~laboratory implement ~~has~~ an appropriate written contingency plan ~~in place~~ to handle these samples and protect sample integrity. ~~For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt.~~ The written contingency plan must~~also~~ address~~es~~ field practices and~~/or~~ sample processing that ensure the sample and extract integrity ~~laboratory processing that ensures the integrity of the sample or extract~~ until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted;

			b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.

C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – EXAMINATION OF SHELLFISH FOR ASP TOXINS			
		2.1 Preparation of Sample	
C	**6, 1**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., 3 geoduck gut balls).**
O	6		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	6		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	6		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	6		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**6**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	6		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	6		2.1.8 Pieces of shell and drainage are discarded.
C	**2, 6**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meat s and their drip loss liquid or thawed homogenates with their freeze- thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction	
K	4,6		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**4**		**2.2.2 Four (4) grams of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**4**		**2.2.3 The sample homogenate is extracted with 16 mL extraction solvent (1:1 methanol:water [also referred to as 50% aqueous methanol]).**
K	4, 15		2.2.4 Homogenate/extract mixture is centrifuged and filtered before analysis.
K	4, 15		2.2.5 The filtered extract is injected into the HPLC or loaded into the autosampler immediately.

K	4		2.2.6 When crude samples are diluted, dilutions are made by diluting 1 mL of filtered sample supernatant into a 5 mL volumetric flask and diluted with water to 5 mL.
K	4, 15		2.2.7 Crude extracts are sealed tightly and stored at -10 ºC or below.
		2.3 Sample Cleanup (Optional)	
O	4, 15		2.3.1 Three (3) mL SAX cartridges (500 mg silica derivatized with quaternary ammonium silane) are used for cleanup.
K	4		2.3.2 The SAX cartridge is conditioned with 6 mL methanol, followed by 3 mL water, followed by 3 mL extraction solvent (1:1 methanol:water).
C	**4, 15**		**2.3.3 The cartridge is not allowed to run dry during conditioning through sample loading.**
K	4, 15		2.3.4 Five (5) mL of filtered extract is loaded onto the cartridge and flowed slowly (~1 drop/s) until sample meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.5 Five (5) mL of wash solution (1:9 acetonitrile:water) is loaded to the cartridge and washed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4		2.3.6 0.5 mL of citrate buffer (0.5 M, pH 3.2) is loaded to the cartridge and flowed slowly (~1 drop/s) until meniscus reaches the top of cartridge packing, discarding effluent.
K	4, 15		2.3.7 A 2 mL volumetric tube is placed under the cartridge and any domoic acid is eluted into the tube by loading and flowing as much citrate buffer as needed slowly (~ 1 drop/s) until the 2 mL mark is reached on the tube.
C	**4, 15**		**2.3.8 The solution is thoroughly mixed before withdrawing an aliquot for analysis.**
K	4, 15		2.3.9 The cleaned up extract is injected into the HPLC or loaded into the autosampler immediately.
		2.4 Analysis	
C	**2**		**2.4.1 A standard calibration curve (of at least six concentrations) is performed daily. Results are recorded and records are maintained.**
K	4, 15		2.4.2 Twenty (20) µL of extract is injected for analysis.
K	2		2.4.3 Samples are stored in the sample compartment of the autosampler at 4°C during analysis. Otherwise samples must be analyzed within 9 hours if the autosampler is held at room temperature.
K	4, 15		2.4.4 A column heater is used and the temperature is maintained at 40 °C during the analysis.
C	**4**		**2.4.5 The appropriate analytical column is used: 25 cm x 4.6 mm id packed with 5 µm Vydac 201TP octadecylsilica or equivalent.**
K	2		2.4.6 The column is stored following the manufacturer's instructions when not in use.
O	2		2.4.7 If a precolumn in-line filter and/or a compatible guard column (e.g., 201GCC54T) are/is used, rejection criteria are established to determine when to change the filter/guard column.
C	**2**		**2.4.8 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.**
		2.5 System Suitability	

C	**2**		**2.5.1 The correlation coefficient for the linear regression of the calibration standards must be ≥ 0.990 for domoic acid.**
C	**3**		**2.5.2 The resolution and retention time criteria must ensure complete baseline resolution of L-tryptophan and domoic acid.**
K	2		2.5.3 Peak asymmetry is routinely monitored to evaluate the performance of the column. Results are recorded and records maintained.
C	**2**		**2.5.4 The column is replaced when a measure of peak asymmetry becomes <0.9 or >1.3.**
C	**2,4**		**2.5.5 Daily injection schedules must include the adequate frequency of injection standards and extraction blanks based on an assessment of individual standard toxin variability and lack of carry over.**
C	**2**		**2.5.6 Repeated injections of calibrated standards/samples agree within ±5% (as determined through the use of the coefficient of variation).**

			2.6 Calculation of Sample Toxicity
C	**4, 15**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** **µg/g domoic acid (DA) = DA injected × $\frac{VV}{WW}$ × (F)** **where:** **DA injected = the concentration in µg/ml of the extract injected;** **V = total volume of homogenate and extraction solvent (mL);** **W = weight (g) of tissue homogenate extracted (e.g., 4 g); and** **F = dilution factor (e.g., if SAX cleanup or crude sample dilution are performed).** **The concentration of DA injected may be determined using the nearest standard or the equation of the day's standard curve.**
C	**4, 15**		**2.6.2 Calculated domoic acid concentrations include the sum of domoic acid and epimer peaks, when the epimer represents 5% or more of the peak area.**
C	**12**		**2.6.3 Any value at or above 20 ppm (mg/kg or µg/g) domoic acid is actionable.**

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice. 21 CFR 58.
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4. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1995. J. AOAC Int. 78(2): 543-554.
5. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
6. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
7. Consult reference standard product literature.
8. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
9. American Public Health Association. 192. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
10. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16: 54-60.
11. Consult pH electrode product literature.
12. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2015. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
13. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
14. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.
15. Quilliam, M.A., M. Xie, and W.R. Hardstaff. 1991. A Rapid Extraction and Clean-up Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Bioscience, Technical Report #64, National Research Council Canada #33001.

LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
AMNESIC SHELLFISH POISON (ASP or domoic acid) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the ASP (domoic acid) Component

1. **Conforms Status: The ASP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The ASP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The ASP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥ 4**.
   - b. or the total # of Key nonconformities is **≥ 6.**
   - c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____Date:_____

LEO Signature:_____Date:_____

NSSP Form *5 – Domoic Acid HPLC-UV Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
MARBIONC Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C – Critical K - Key O - Other N/A - Not Applicable

PART I – QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	3, 6		1.1.1	Written Plan adequately covers all the following: (check √ those that apply)
				a. Organization of the Laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**3**		**1.1.2**	**QA Plan is implemented.**
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three months of experience in laboratory sciences.
		1.3 Work Area		
O	3, 6		1.3.1	Adequate for workload and storage.
O	6		1.3.2	Clean and well lighted.
O	6		1.3.3	Adequate temperature control.
O	6		1.3.4	All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment		
O	4		1.4.1	The pH meter has a standard accuracy of 0.1 unit.
K	4		1.4.2	pH paper in the appropriate range (i.e., 1-4), if used, is used with minimum accuracy of 0.5 pH units.
K	3		1.4.3	The pH meter is ca libra ted daily when in use. Results are recorded, and records are maintained.
K	6		1.4.4	Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	6		1.4.5	The pH meter manufacturer instructions are followed for calibration or a minimum of two standard buffer solutions (pH 7 and 10) is used to calibrate the pH meter. Standard buffer solutions are used once and discarded.
K	3, 7		1.4.6	Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.
K	2, 4		1.4.7	The balances being used provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	6		1.4.8	The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded, and records are maintained.

K	1		1.4.9 Refrigerator temperature is maintained between 0 and 4 ºC.
K	6		1.4.10 Refrigerator temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
K	11		1.4.11 Freezer temperature is maintained at -10 ºC or below.
K	6		1.4.12 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
C	**9**		**1.4.13 All in-service thermometers are properly calibrated and immersed.**
K	5		1.4.14 All glassware is clean.
C	**11**		**1.4.15 Absorbance Microplate reader equipped with filter for measurement at 450 nm is used.**
O	2		1.4.16 Absorbance Microplate reader performance is evaluated at least annually using manufacturer instructions or a check standard microplate at the appropriate wavelength (450) to assess alignment, accuracy, reproducibility, and linearity. Method used:_____
K	2		1.4.17 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded, and records are maintained.
O	11		1.4.18 A centrifuge capable of holding 15 mL or 50 mL polypropylene tubes is used.
		1.5 Reagents and Reference Solution Preparation and Storage	
C	**11**		**1.5.1 All solvents and reagents used are ACS grade materials or better.**
O	6		1.5.2 Water contains < 100 CFU/ml as determined monthly using the heterotrophic plate count method. Results are recorded, and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	6		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**11**		**1.5.4 Brevetoxin-3 (BTX-3 or PbTx-3) provided with the MARBIONC ELISA kit is used as the reference standard.**
C	**11**		**1.5.5 Stock standard solution is made by diluting brevetoxin-3 reference standard to 1 µg/ml in 100% methanol in a volumetric flask.**
C	**11**		**1.5.6 Working standard solution (100 ng/ml) is made by diluting 1 ml of stock solution to 10 ml in a volumetric flask using 100% methanol.**
K	11		1.5.7 Extraction solvent (80% methanol) is made by adding 800 ml of methanol to a 1 L graduated cylinder and bringing the total volume to 1 L with water.
K	11		1.5.8 Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are used within 1 week of preparation. pH of prepared media is determined to ensure it is consistent with manufacturers requirements. Results are recorded, and records are maintained.
K	11		1.5.9 Phosphate Buffered Saline, pH 7.4 and Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 are stored in refrigerator for no longer than 1 week and brought to room temperature before use.
K	11		1.5.10 Gelatin stock solution is prepared by dissolving 5 g gelatin in 100 ml water and stirring the solution over gentle heat on a stir plate until clear. Gela tin stock solution is aliquoted into smaller volumes (e.g. 15 ml centrifuge tubes) and refrigerated.
K	11		1.5.11 Blocking buffer is prepared by dissolving 1 pouch in 200 ml water. Blocking buffer solution is aliquoted into 50-ml centrifuge tubes and refrigerated.
K	11		1.5.12 PGT (PBS, 0.05% Tween, 0.5% gelatin) is made fresh daily by measuring 5 ml liquified gelatin stock solution into a 50-ml centrifuge tube and filling to 50 ml with PBS-Tween.

C	**11**		**1.5.13**	**Stock and working standard solutions are stored -10 ºC or below.**
C	**5**		**1.5.14**	**All standards used are within expiration date (or 1 year from open date if not provided).**
		1.6 Collection and Transportation of Samples		
O	4, 1		1.6.1	Shellstock are collected in clean, waterproof, puncture resistant containers.
K	4, 1		1.6.2	Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**4, 1**		**1.6.3**	**Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**
K	2, 10		1.6.4	~~Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur,~~When the time from collection to initiation of extraction is anticipated to exceed 24 hours, the sample submitter(s) ~~the~~and laboratory implement ~~has~~ an appropriate written contingency plan ~~in place~~ to handle these samples and protect sample integrity. ~~For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt.~~ The written contingency plan ~~also~~ must address~~es~~ field practices and~~/or laboratory~~ sample processing that ensure~~s~~ the ~~integrity of the~~ sample ~~or~~ and extract integrity until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5**	**Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
PART II – ASSAY OF SHELLFISH FOR NSP TOXINS				
		2.1 Preparation of Sample		
C	**4**		**2.1.1**	**At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish.**
O	4		2.1.2	The outside of the shell is thoroughly cleaned with fresh water.
O	4		2.1.3	Shellstock are opened by cutting the adductor muscles.
O	4		2.1.4	The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	4		2.1.5	Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**4**		**2.1.6**	**Damage to the body of the mollusk is minimized in the process of opening.**
O	4		2.1.7	Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	4		2.1.8	Pieces of shell and drainage are discarded.
C	**2, 4**		**2.1.9**	**Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction		
K	4		2.2.1	Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**11**		**2.2.2**	**One (1) gram of homogenized sample is weighed into a 15 ml or 50 ml polypropylene centrifuge tube and subsequently extracted.**

C	**11**		**2.2.3**	**The sample homogenate is extracted by adding 9 ml extraction solvent (80% aqueous methanol) and vortexing at highest speed for 2 minutes.**
C	**11**		**2.2.4**	**The homogenate/methanol mixture is centrifuged at a minimum of 3000*xg* for 10 minutes.**
C	**11**		**2.2.5**	**The supernatant is transferred to a clean, labeled graduated 15-ml centrifuge tube and brought to a final volume of 10 ml with extraction solvent.**
K	11		2.2.6	Crude extracts are sealed tightly in glass vials and stored at -10 ºC or below until analyzed.
		2.3 Analysis		
C	**11**		**2.3.1**	**Only high-binding flat-bottom plates no older than 1 year are used (e.g. Nunc Maxisorp Immunoplates).**
C	**11**		**2.3.2**	**When pipetting kit reagents that are pre-diluted in glycerol (A, C, and D):** **a. the pipet tip is not pre-rinsed,** **b. only the very tip of the pipet tip is inserted into the vial to withdraw the required amount,** **c. the tip is submerged into the buffer when dispensing and rinsed several times with buffer to ensure complete transfer**
K	2		2.3.3	The working standard solution and crude sample extracts are brought to room temperature and thoroughly mixed before use. TMB is brought to room temperature in the dark prior to Assay Step 7.
C	**11**		**2.3.4**	**Assay Step 1: Reagent A is diluted by 300 (or as specified in kit instructions) in PBS, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS (no Tween for this wash step).**
C	**11**		**2.3.5**	**Assay Step 2: Each well is filled with 250 µl of blocking buffer. The plate is incubated on a plate shaker for 30 minutes. After 30 minutes, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**
C	**11**		**2.3.6**	**Assay Step 3a: Crude sample extracts are diluted with PGT for analysis. The minimum dilution for shellfish extracts is 1:40 (25 µl + 975 µl PGT) (resulting in a sample dilution of 1:400). Serial dilutions (n=7) of each crude sample extract are prepared in PGT.**
C	**11**		**2.3.7**	**Assay Step 3b: A standard calibration curve of seven concentrations (0.078-5.0 ng PbTx-3/ml) is prepared in PGT and is included on each plate.**
C	**11**		**2.3.8**	**Assay Step 4: 100 µl of each sample or standard dilution is loaded on to the microplate as well as two reference wells (containing PGT only) adjacent to each set of sample dilutions. Each dilution of standard or sample is added to duplicate wells. Plate layout identifying locations of samples and standards on the plate is documented.**
C	**11**		**2.3.9**	**Assay Step 5: Reagent C is diluted by 300 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate (which contains samples or standards), and the plate is incubated on a plate shaker for 90 minutes. After 90 minutes, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**

C	**11**		**2.3.10 Assay Step 6: Reagent D is diluted by 800 (or as specified in kit instructions) in PGT, 100 µl is added to each well of the 96-well plate, and the plate is incubated on a plate shaker for 1 hour. After 1 hour, the liquid is poured from the plate, all wells are rinsed 3 times with 300 µl PBS-Tween, and one final time with 300 µl PBS only to ensure no Tween remains on the plate.**

C	**11**		**2.3.11 Assay Step 7: Each well is filled with 100 µl of room temperature TMB (3,3'5,5'-Tetramethylbenzidine) and incubated until a blue color develops in the reference wells. The reaction is stopped by adding 100 µl of 0.5M sulfuric acid solution to each well, and the absorbance in the wells at 450 nm is measured in a microplate reader.**
K	11		2.3.12 Plates are covered with microplate sealing film during all incubation steps (Steps 1-6 above).
C	**11**		**2.3.13 Plates are protected from light by covering with aluminum foil during color development (Step 7 above).**
K	11		2.3.14 The timing of the final step is reassessed with each new lot of kit reagents and each new lot of TMB to ensure that reference well absorbance values fall into an appropriate range.
		2.4 Quality Control	
C	**11**		**2.4.1 Acceptance of assay (plate) results is dependent on meeting the following criteria:** **a. Absorbance of standard reference wells (Amax) must be ≥ 0.6.** **b. CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (30-70% inhibition) must be < 20%.**
C	**11**		**2.4.2 Acceptance of individual sample results is dependent on meeting the following criteria:** **a. CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (30-70% inhibition) must be < 20%.** **b. CV of calculated concentrations of different sample dilutions within the linear range of the assay (30-70% inhibition) must be < 20%.**
		2.5 Calculation of Sample Toxin Concentration	
C	**11**		**2.5.1 Absorbance values are converted to % color inhibition:** **a. % inhibition = [1-(Avg of duplicate A/Amax)] x 100%where Amax is the average absorbance of the reference wells oriented below the sample or standard dilutions**
C	**11**		**2.5.2 Using the 4-parameter logistic (4PL) curve in an appropriate software program, a curve is fit to the positive control with ng toxin/ml on the x-axis (log scale) and % inhibition on the y-axis (linear scale).**
C	**11**		**2.5.3 The concentrations for sample dilutions falling within 30%-70% inhibition are interpolated from the standard curve.**
C	**11**		**2.5.4 Sample toxin concentration is calculated by multiplying the interpolated concentration by the sample dilution factor and dividing by 1000 to obtain PbTx-3 eq. results in ppm.**
C	**11**		**2.5.5 If more than one dilution of a sample falls within 30%-70%, the mean of the two calculated concentrations is used.**

C	**8**		**2.5.6**	**A result of ≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters is considered negative and can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.** **A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.8 ppm in oysters) requires additional testing by an Approved Method to support management actions.**
O	8		2.5.7	Laboratory reports to the Shellfish Management Authority detail sample date, location, species (matrix), date tested, analyst name, result of ELISA in ppm, and the actionable ELISA threshold for the species tested.

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice.
3. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
4. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
5. Consult reference standard product literature.
6. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
7. Consult pH electrode product literature.
8. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. *NSSP Guide for the Control of Molluscan Shellfish.* FDA/ISSC, Washington, D.C. and Columbia, S.C.
9. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
10. Compendium of Methods for the Microbiological Examination of Foods, 3rd Edition, pg. 901.
11. MARBIONC Enzyme-linked Immunosorbent Assay (ELISA) for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish. (ISSC proposal 17-107, supporting documents Appendix A)

LABORATORY:	DATE OF EVALUATION:

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
NEUROTOXIC SHELLFISH POISON (NSP or Brevetoxin) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	 _____ _____ _____

B. Criteria for Determining Laboratory Status of the brevetoxin (NSP) ELISA Component

1. **Conforms Status:** The NSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities.
   b. and <6 Key nonconformities.
   c. and <12 Total nonconformities**.**

2. **Provisionally Conforms Status:** The NSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. the number of critical nonconformities is **≥ 1** but **< 4**.
   b. and **< 6** Key nonconformities.
   c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The NSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is **≥ 4**.
   b. or the total # of Key nonconformities is **≥ 6.**
   c. or the total # of Critical, Key, or Other is **≥ 12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature: _____ Date: _____

LEO Signature: _____ Date: _____

NSSP Form *8 – MARBIONC Brevetoxin ELISA Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Diarrhetic Shellfish Poisoning Toxins (DSP) LC-MS/MS

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	**EMAIL:**
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "✓"**

C – Critical K - Key O - Other NA - Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 7, 8		1.1.1 Written Plan adequately covers all the following: (check those that apply)
			a. Organization of the laboratory
			b. Staff training requirements
			c. Standard operating procedures
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established
			e. Laboratory safety
			f. Internal performance assessment
			g. External performance assessment
C	**5**		**1.1.2 QA Plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, chemistry, or another appropriate discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst must have at least a high school diploma and shall have at least three (3) months of experience in laboratory sciences.
C	**3**		**1.2.5 LC-MS Operator must be trained in the operation and maintenance of the specific liquid chromatography-mass spectrometry system used.**
		1.3 Work Area	
O	1		1.3.1 Adequate for workload and storage.
O	1		1.3.2 Clean and well lighted.
O	1		1.3.3 Adequate temperature control.
O	8		1.3.4 All work surfaces are nonporous and easily cleaned.
		1.4 Laboratory Equipment	
C	**3**		**1.4.1 A heat block or water bath capable of heating samples to 76 ± 2 °C.**
K	2		1.4.2 Balances provide an appropriate sensitivity at the weights of use, at least 0.1 g for laboratory precision balances and 0.1 mg for analytical balances.
K	7, 8		1.4.3 The balance calibration is checked monthly using NIST class S, ASTM class 1 or 2 weights or equivalent. Results are recorded and records are maintained.
K	1		1.4.4 Refrigerator temperature is maintained between 0 and 4 ºC.
K	7		1.4.5 Refrigerator temperature is monitored at least once daily on working days. Results are recorded and records maintained.
K	2		1.4.6 Freezer temperature is maintained at -10 ºC or below.

K	7		1.4.7 Freezer temperature is monitored at least once daily on working days. Results are recorded and records maintained.
C	**10**		**1.4.8 All in-service thermometers are properly calibrated and immersed.**
K	4		1.4.9 All glassware is clean.
K	3, 12		1.4.10 An ultra-performance liquid chromatography system (UPLC) equipped with the following is used: a. mobile phase system capable of delivering a pulse-free flow of 0.12 mL/min b. solvent degasser (optional) c. autosampler (refrigerated preferred) with loop suitable for five (5) µL injections d. column compartment capable of controlling temperature at 40 ºC e. a data collection system (e.g., computer, integrator)
C	**3**		**1.4.11 A mass spectrometer equipped with the following is used:** **a. an electrospray ionization source operating in negative ion mode and** **b. multiple reaction monitoring scan mode capability.** **c. if a divert valve is used to divert LC flow at the beginning and end of each chromatographic run, the switching time should be at least one minute before the first peak elution and at least one minute after the last peak elution.**
K	2		1.4.12 Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.
K	3		1.4.13 A centrifuge capable of generating 2000 x g and holding 15 mL and 50 mL polypropylene tubes is used.
		1.5	**Reagents and Reference Solution Preparation and Storage**
C	**3**		**1.5.1 All solvents and reagents used are analytical or LC grade materials.**
O	7		1.5.2 Water contains < 100 CFU/ml determined monthly using the heterotrophic plate count method. Results are recorded and records are maintained. (Not required for bottled reagent grade or HPLC grade water when used immediately upon opening. If the bottle of water is not used entirely immediately, the water must be tested as above prior to continued use.)
K	7		1.5.3 Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**3**		**1.5.4 The mobile phase system used to analyze DSP toxins consists of: A: 2 mM ammonium formate and 50 mM formic acid in water B: 2 mM ammonium formate and 50 mM formic acid in 95% acetonitrile/5% water**
O	2		1.5.5 Mobile phase is degassed manually before use if the UPLC does not have a degasser or if the degasser is not in use.
C	**3**		**1.5.6 Only certified reference materials are used for standard solutions. Source of the reference standard: _____**
C	**6**		**1.5.7 All primary standards are stored appropriately as per supplier recommendations.**
C	**6**		**1.5.8 All standards used are within their expiration date.**

C	**2, 3**		**1.5.9 All standards are prepared either gravimetrically or using appropriate positive displacement pipettes or syringes.**
C	**3**		**1.5.10 Working standards are made up from primary standard by dilution with the toxin-free, extraction solvent (i.e., 100% methanol).**
		1.6 Collection and Transportation of Samples	
O	5, 1		1.6.1 Shellstock are collected in clean, waterproof, puncture resistant containers.
K	5, 1		1.6.2 Samples are appropriately labeled with the collector's name, type of shellstock, the harvest area, and time and date of collection.
C	**5, 1**		**1.6.3 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**
K	2		1.6.4 ~~Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur,~~When the time from collection to initation of extraction is anticipated to exceed 24 hours, the sample submitter(s) and laboratory ~~has~~ implement an appropriate written contingency plan ~~in place~~ to handle these samples. ~~For samples shipped live in accordance with 1.6.3, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt.~~ The written contingency plan ~~also~~ must address~~es~~ field practices and~~/or laboratory~~ sample processing that ensure~~s~~ the ~~integrity of the~~ sample ~~or~~and extract integrity until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
C	**2**		**1.6.5 Frozen shucked product or homogenates are allowed to thaw completely and all liquid is included as part of the sample before being processed further.**
		PART II – EXAMINATION OF SHELLFISH FOR DSP TOXINS	
		2.1 Preparation of Sample	
C	**2**		**2.1.1 At least 12 animals are used per sample or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish (e.g., three (3) geoduck gut balls).**
O	5		2.1.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.1.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.1.4 The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	5		2.1.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.1.6 Damage to the body of the mollusk is minimized in the process of opening.**

O	5		2.1.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for five (5) minutes.
K	5		2.1.8 Pieces of shell and drainage are discarded.

C	**2, 5**		**2.1.9 Drained meats or previously cooled/refrigerated shucked meats and their drip loss liquid or thawed homogenates with their freeze-thaw liquid are blended at high speed until homogenous (60-120 seconds).**
		2.2 Sample Extraction	
K	2		2.2.1 Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer at -10 ºC or below.
C	**3**		**2.2.2 Two (2.00) ± 0.05 g of homogenized sample is weighed into a 50 mL polypropylene centrifuge tube and subsequently extracted.**
C	**3**		**2.2.3 The sample homogenate is extracted with 9 mL of 100% methanol and vortexed to mix.**
K	3		2.2.4 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant decanted into a clean container (e.g. new polypropylene tube or glass vial).
C	**3**		**2.2.5 The tissue pellet is reextracted with nine (9) mL of 100% methanol and homogenized to mix.**
K	3		2.2.6 The sample homogenate/extract mixture is centrifuged for 10 minutes at 2000 x g and the supernatant combined with the supernatant in 2.2.4.
K	3		2.2.7 The total extract volume is adjusted to 20 mL with 100% methanol.
K	3		2.2.8 The crude extract is hydrolyzed or stored in the freezer at < -10 °C.
		2.3 Sample Hydrolysis and Cleanup	
K	3		2.3.1 A two (2) mL aliquot of the sample extract is transferred to an appropriately size* glass tube with a phenolic PTFE lined screw cap using a positive displacement pipette or syringe. *Note: A 16 x 100 mm tube will have sufficient volume to perform the hydrolysis and hexane wash steps and fit in a standard 15 mL centrifuge tube adaptor.
K	3		2.3.2 The sample extract is hydrolyzed by adding 250 µL of 2.5 M NaOH and the sample is homogenized with a vortex mixer for 30 seconds.
C	**3**		**2.3.3 Sample tube caps are securely fastened to prevent extract loss, and the weight of the sample tube is recorded. The sample tube is heated at 76 °C for 40 minutes, then allowed to cool to room temperature, dried, and re-weighed. If the weight has dropped by more than 0.1 g, lost volume is replaced using 100% MeOH.**
K	3		2.3.4 Samples are neutralized with 250 µL of 2.5 M HCL and vortexed to mix.
O	3		2.3.5 Nonpolar lipids are removed by adding seven (7) mL of hexanes to the hydrolyzed sample extract and vortexing to mix (2.3.5 – 2.3.7 Preferred).
K	3		2.3.6 The sample extract/hexane mixture is partitioned by centrifuging for 10 minutes at 2000 x g (2.3.5 – 2.3.7 Preferred).

K	3		2.3.7 The hexane layer is removed with a glass pipette and one (1) mL of the hydrolyzed methanolic extract is removed and filtered into an LC-MS glass autosampler vial using a 0.2 µm PTFE syringe tip filter (2.3.5 – 2.3.7 Preferred).

K	2, 3		2.3.8 The cleaned-up extract is loaded into the autosampler immediately for analysis.
		2.4 Analysis	
C	**3**		**2.4.1 Analytes are detected in standards and samples using the mass transitions in the table (negative ion mode).**
K	3		2.4.2 Other system parameters such as collision energy are optimized for the specific system using standards before analysis.
C	**3**		**2.4.3 A standard calibration curve of at least six (6) concentrations is performed at the beginning of each run. An additional curve is required if a run lasts longer than 24 hours.**
K	3		2.4.4 Five (5) µL of extract is injected for analysis.
K	2, 12		2.4.5 Samples are stored in the sample compartment of the autosampler at ≤ 10°C during analysis. Otherwise the samples must be analyzed within 24 hours if the autosampler is held at room temperature.
K	3		2.4.6 A column heater is used and the temperature is maintained at 40 °C, with a tolerance as specified by the manufacturer, during the analysis.
C	**3**		**2.4.7 An Acquity UPLC BEH C18 1.0 × 150 mm, 1.7 µm particle size (or equivalent) analytical column is used for analyte separation**
C	**3**		**2.4.8 Analytes are separated on the LC column using gradient elution.**
K	2		2.4.9 The column is stored following the manufacturer's instructions when not in use.
K	**2**		2.4.10 Dead volume in the system is minimized by the use of short lengths of connecting tubing of small internal diameter between the sample injector and the column and between the column and detector.

C	**3**		**2.4.11 A matrix or procedural blank (i.e. NRC CRM Zero-Mus or equivalent negative matrix, or methanol carried through sample preparation process at the same time as the samples) should be included in each analytical batch.**
		2.5 System Suitability	
C	**3**		**2.5.1 The retention time of analytes in all samples are within 3% that of one of the intermediate toxin standards, measured from the apex of the peak.**
C	**3**		**2.5.2 Chromatographic separation must be sufficient for resolving OA and DTX2. Peak resolution (Rs) of OA/DTX2 should ≥ 1 when calculated using the equation below (RT is retention time and W is peak width at baseline; Peak 1 is OA and Peak 2 is DTX2).**
K	2, 3		2.5.3 Each chromatographic peak must be defined by at least 10 data points.
C	**3**		**2.5.4 Reagent blanks (methanol) are analyzed after the high calibration standard, periodically (as determined by the laboratory's internal verification), and after fortified samples to ensure that analyte carryover is not occurring. Analyte carryover is defined as a confirmed peak > LOD.**
C	**3**		**2.5.5 To confirm the presence of each DST, two (2) mass transitions must be observed above the limit of detection (LOD).** **The transition yielding the highest signal-to-noise ratio (S/N) is used for quantitation (i.e. 817.5 → 255.2 for DTX-1, 803.5 → 255.2 for OA and DTX-2). The transition yielding the second highest S/N is used for confirmation. The S/N of the peak used for confirmation is ≥ 3.**
C	**3**		**2.5.6 The ratio of the abundance of the confirmation ion transition to the quantitation ion transition is calculated for each toxin. These ion ratios must be within ± 20% of that of the toxin standards in order to confirm toxin identity.**
C	**3**		**2.5.7 When ≥ 10 samples are analyzed, analysts must show that the calibration has not significantly drifted using an option provided below:**
		OPTION 1 – USE OF BRACKETING CALIBRATION CURVES	
C	**3**		**2.5.7.1 A second standard curve is analyzed at the end of the analytical sequence and the averaged peak areas are used for the linear regression.**
C	**3**		**2.5.7.2 The linear regression of the averaged calibration curves must yield an $R^2 \geq 0.98$. Results are recorded and records are maintained.**

Compound	Q1 (m/z)	Q3 (m/z)
OA	-803.5	-255.2
OA	-803.5	-113.1
OA	-803.5	-151.1
DTX2	-803.5	-255.2
DTX2	-803.5	-113.1
DTX2	-803.5	-151.1
DTX1	-817.5	-255.2
DTX1	-817.5	-113.1
DTX1	-817.5	-151.1

$$RRRR = 22 \times (RRRR22 - RRRRRR)/(WWRR + WW22)$$

C	**3**		**2.5.7.3 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The average of bracketing calibration curves yields an $R^2 < 0.98$.** **b) The difference in the slope between bracketing calibration curves exceeds 25%.** **c) The difference in retention times of the standards in the bracketing standard curves exceeds 3%.**

		OPTION 2 – USE OF A CONTINUING CALIBRATION VERIFICATION (CCV) STANDARD	
C	**3**		**2.5.7.4 The linear regression of the single calibration curve must yield an $R^2 \geq 0.99$.**
C	**3**		**2.5.7.5 A continuing calibration verification (CCV) standard, matching on of the intermediate standards from the calibration curve, is analyzed after every 10 samples and at the end of a run.**
C	**3**		**2.5.7.6 A new calibration curve is prepared, and samples are reanalyzed, if any of the following are observed:** **a) The calibration curve yields an $R^2 < 0.99$.** **b) The retention time of a CCV sample exceeds 3% of the corresponding standard.*** **c) The peak area of a CCV sample exceeds ± 15% compared to the corresponding standard in the calibration curve.*** **** Samples immediately preceding and post the failed CCV shall be reanalyzed with a new standard curve.***
C	**3**		**2.5.8 Repeated injections of calibration or control samples at a concentration near the action level agree within ± 10% (as determined through the use of the coefficient of variation).**
		2.6 Calculation of Sample Toxicity	

<table>
<tr>
<td>C</td>
<td>3, 11</td>
<td></td>
<td>
2.6.1 The toxicity of the individual toxins is calculated as follows:

$\frac{uuuu}{uu}\,ttttttttt = CC \times \frac{VV}{WW}$ X Hyd X ReTx

where:

C = the concentration in µg/ml of the extract injected, determined using the standard curve

V = total volume of homogenate and extraction solvent (mL)

W = weight (g) of tissue homogenate extracted

Hyd = dilution factor for hydrolysis (1.25)

ReTx = relative toxicity of toxin vs. Okadaic Acid

Relative Toxicity Values
<table>
<tr><th>Toxin</th><th>ReTx</th></tr>
<tr><td>OA</td><td>1</td></tr>
<tr><td>DTX1</td><td>1</td></tr>
<tr><td>DTX2</td><td>0.6</td></tr>
</table>
The individual toxicities for each toxin are summed to obtain the overall sample toxicity in ppm OA equivalents.
</td>
</tr>
<tr>
<td>C</td>
<td>9</td>
<td></td>
<td>2.6.2 Any value at or above 0.16 ppm OA equivalents (mg/kg or µg/g) of the sum of any analytes present is actionable. Shellfish Program Management is made aware of positive result. Laboratory action is_____ .</td>
</tr>
</table>

REFERENCES

1. American Public Health Association. 1984. *Compendium for the Microbiological Examination of foods,* 2nd Edition. APHA. Washington D.C.
2. Good Laboratory Practice. 21 CFR 58.
3. Interstate Shellfish Sanitation Conference (ISSC), Proposal 17-103 Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Method for the Determination of Diarrhetic Shellfish Poisoning (DSP) Toxins in Shellfish
4. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
5. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
6. Consult reference standard product literature.
7. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
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   D.C. and Columbia, S.C.
10. U.S. Department of Commerce. 1976. NBS Monograph 150. U.S. Department of Commerce, Washington, D.C.
11. The EFSA Journal. 2009. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Summary on regulated marine biotoxins. 1306, 1-23.
12. Deeds, J.R. and M.D. Celiz, Personal Communication, Addendum to proposal 19-136 DSP LC MS/MS Additional Ruggedness Testing; effect of the sample storage temperature during analysis (24 hrs), Email Received by ISSC Checklist Subcommittee 2/18/2021.

LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
DIARRHETIC SHELLFISH POISON (DSP) COMPONENT: PARTS I AND II	
A. Results Total # of **Critical (C)** Nonconformities Total # of Key (K) Nonconformities Total # of Critical, Key, and Other (O) Nonconformities	_____ _____ _____

B. Criteria for Determining Laboratory Status of the DSP Component

1. **Conforms Status: The DSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.**
   - **a. No Critical nonconformities.**
   - **b. and <6 Key nonconformities.**
   - **c. and <12 Total nonconformities.**

2. **Provisionally Conforms Status:** The DSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   - a. the number of critical nonconformities is ≥ 1 but **< 4**.
   - b. and **< 6** Key nonconformities.
   - c. and **< 12** Total nonconformities.

3. **Does Not Conform Status**: The DSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   - a. The total # of Critical nonconformities is **≥4**.
   - b. or the total # of Key nonconformities is **≥6.**
   - c. or the total # of Critical, Key, or Other is **≥12.**

C. Laboratory Status (*circle appropriate)*

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgement by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before_____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

NSSP Form *10 – DSP LC-MS/MS Checklist*, Rev. June 2024

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**

LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE			
CODE	**REF**		**ITEM**
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3		1.1.1 Written Plan (Check √ those items which apply).
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance and rejection criteria established.
			e. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			f. Internal performance assessment.
			g. External performance assessment.
C	**2**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analysts meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial laboratories, the analysts must have at least a high school diploma and at least three months of experience in laboratory sciences.
C	**6**		**1.2.5 Training regarding radiation laboratory safety, handling and disposal practices is documented and records are maintained.**
C	**15**		**1.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitoxin in this assay. Alternatively, the laboratory uses less than 50 µCi per year and adheres to the American Radiolabeled Chemical (ARC) exemption status.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**

		1.4 Laboratory Equipment	
C	**4**		**1.4.1 Any lab equipment that may come into contact with [^{3}H]-STX at any point in the preparation or assay procedures must be specially labelled and must remain in the work area designated for low-level radiation work.**
O	5		1.4.2 The pH meter has a standard accuracy of 0.1 pH units.
K	7		1.4.3 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8		1.4.4 The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1		1.4.5 The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.
K	1		1.4.6 The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	9		1.4.7 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.
K	6		1.4.8 pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6		1.4.9 The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3		1.4.10 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
			1.4.11 Balances must be calibrated by an exte[illegible] year. Results are recorded and records
K	2		1.4.12 Refrigerator temperatures are maintain[illegible] Freezer securityfor ^{3}HSTX and cold S[illegible] federal requirements for these material
K	1		1.4.13 Refrigerator temperatures are monitor[illegible] workdays. Results are recorded and re[illegible]
C	**4, 6, 10**		**1.4.14 Freezer temperature used to store [^{3}H] STX standard, rat brain membrane tissue preparation, interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -80 °C or below. Freezer security for ^{3}HSTX and cold STX must meet state and federal requirements for these materials.**

FDA LEOs
2024-05-02 03:32:00

No criticality code or reference determined in LC

K	6, 10		1.4.15 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.16 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
O	8		1.4.17 All glassware is clean.
C	**3**		**1.4.18 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.19 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**
C	**6**		**1.4.20 Micropipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.**
C	**11**		**1.4.21 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.22 Minimum radiation safety equipment and protocols include the following: A wipe-test is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
		1.5 Reference Solution Reagent Storage, Preparation and Security	
C	**12**		**1.5.1 [^{3}H] STX standard is stored in a freezer at -80 °C or below.**
C	**10**		**1.5.2 Concentration of [^{3}H] STX standard is calculated from the lot information provided by the supplier with each batch.**
K	6		1.5.3 Unopened diHCl STX standard may be stored at room temperature or refrigerated.
C	**10**		**1.5.4 Preparation of MOPS assay buffer includes the following:** **a. 100 mM MOPS/L.** **b. 100 mM choline chloride/L.** **c. pH adjustment to 7.4 with NaOH.** **e. refrigerated storage at 4 °C.** **d. Maintained ice cold while in use.**
C	**10**		**1.5.6 Bulk standard curve dilutions are stored at 4 °C for up to one (1) month.**
K	1		1.5.7 Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice).* b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____ c. Water contains <100 CFU/mL using the heterotrophic plate count method.

		1.6 Rat Brain Membrane Tissue Preparation and Storage	
C	**10**		**1.6.1 MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to MOPS/choline chloride fresh on the day of use.**
C	**10**		**1.6.2 The cerebral cortex of 6-week old Sprague-Dawley rats is used in membrane tissue preparations, placed in iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL) and homogenized with no visible chunks remaining in the homogenate. This procedure is repeated until twenty (20) rat brains have been processed.**
C	**10**		**1.6.3 The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**
K	10		1.6.4 The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).
K	10		1.6.5 The resuspended rat brain tissue preparations are pooled and the centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue**.**
K	10		1.6.6 The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.
K	10		1.6.7 The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed for 20 seconds to obtain a homogeneous membrane tissue preparation.
C	**10**		**1.6.8 Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months.**
		1.7 Rat Brain Membrane Tissue Protein Receptor Determination	
C	**10**		**1.7.1 The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch using a Pierce Micro BCA Protein Assay Reagent Kit No. 23235 (micro plate method) or No. 23225 (tube method) or equivalent.**
C	**10**		**1.7.2 The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined.**
K	10		1.7.3 Dilutions of the protein/receptor concentration of the rat brain membrane tissue preparation of less than 1:4 are not used as they may be too viscous.
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS - RBA			
		2.1 Collection and Transportation of Samples	
C	**5**		**2.1.1 A representative sample of shellfish is collected.**
K	5		2.1.2 Shellfish samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	5		2.1.3 Shellfish samples are labeled with the collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**5**		**2.1.4 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10 °C with ice or cold packs for transport to the laboratory.**

K	6, 13		2.1.5 ~~Time from collection to initiation of the extraction should not exceed 24 hours. However, if significant delays are anticipated or if they occur,~~When the time from collection to initiation of extraction is anticipated to exceed 24 hours, the sample submitter(s) and ~~the~~ laboratory ~~has~~ implement an appropriate written contingency plan ~~in place~~ to handle these samples and protect sample integrity. ~~For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt~~. The written contingency plan ~~also~~ must address~~es~~ field practices and ~~/or laboratory~~ sample processing that ensur~~es~~ the ~~integrity of the~~ sample ~~or~~and extract integrity until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
		2.2 Preparation of Samples for Analysis – Homogenization	
C	**5, 6**		**2.2.1 At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8 Pieces of shell and drainage are discarded.
C	**5, 6**		**2.2.4 Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender with all freeze-thaw liquid forhomogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender with all freeze-thaw liquid for homogenization.**
K	6, 13		2.2.5 Homogenates should be extracted immediately. If homogenates must be stored, they should be frozen.
		2.3 Preparation of Samples for Analysis – Extraction	
K	5, 10		2.3.1 0.1 M HCl is used for extractions.
K	5, 10		2.3.2 At least five (5) grams of tissue +/- 0.1g is extracted using a 1:1 mass to volume ratio of 0.1 M HCl.

C	**10**		**2.3.3 The pH of the sample is checked and adjusted as necessary to between 3.0– 4.0.**
C	**10**		**2.3.4 Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**

C	**6**		**2.3.5 The sample is promptly brought to a boil at 99.0 +/- 1.0 °C and gently boiled for 5 minutes.**
O	6		2.3.6 The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.7 The sample is allowed to cool to room temperature.
C	**10**		**2.3.8 The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted if necessary, with the dropwise addition of 5 M HCl to lower the pH or 0.1 M NaOH to raise the pH, as appropriate, while constantly stirring the mixture.**
K	5, 10		2.3.9 The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water).
K	10		2.3.10 The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; then b. an aliquot of the sample is centrifuged at 3000 x g for 10 minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.11 The sample extract is analyzed immediately, refrigerated at 4 °C in a sealed container for up to 24 hours, or frozen at -20 °C.
		2.4 Sample Assay	
K	6		2.4.1 One analyst performs the entire plate set-up for the assay.
K	6		2.4.2 Microtubes containing dilutions and samples are vortexed immediately before dispensing.
K	10		2.4.3 The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).
C	**10**		**2.4.4 The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension.**
K	10		2.4.5 Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.
C	**10**		**2.4.6 The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.7 Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane.
K	10		2.4.8 All wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, in order to ensure even pressure and filtration across the plate.
C	**10**		**2.4.9 Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate).**
K	10		2.4.10 [^{3}H] STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.
C	10		**2.4.11 An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).**

K	10		2.4.12 Standard curve fitting is calculated using appropriate software program.
C	**10**		**2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.**

C	**10**		**2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.15 The IC_{50} is in acceptable range (2.0 nM +/- 30%). If the IC_{50} is outside this range, the assay results are rejected and the assay must be repeated**
C	**10**		**2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.**
C	**10**		**2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.**
K	4		2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.
		2.5 Calculation of Sample Toxicity	
C	**10**		**2.5.1 When more than one dilution falls within B/B_0 of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**
C	**10**		**2.5.2 Sample toxicity is calculated as follows:** **(nM STX equiv.) x (sample dilution) x (210 µL total volume/35 µL sample = mM STX equivalent in extract** **(nM STX diHCl equiv. in extract) x 1L/1000 mL x 372 ng/nmol x1 µg/1000 ng =µg STX diHCl equiv./mL** **µg STX diHCl equiv./mL x mL extract/g shellfish x 1000 g/kg =µg STX diHCl equiv./kg**
C	**14**		**2.5.3 Any value equal to or greater than 80 µg STX diHCl equiv./100 g) of sample is actionable.**
C	**14**		**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:**

REFERENCES

1. American Public Health Association (APHA). 1992. *Standard Methods for Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
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5. American Public Health Association (APHA). 1970. Recommended Procedures for the Examination of Sea Water and Shellfish, Fourth Edition. APHA, Washington, D.C.
6. Good Laboratory Practice.
7. Fisher J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories*. AOAC, Arlington, VA.
9. Consult pH electrode product literature.
10. Association of Official Analytical Chemists (AOAC). 2016. Official Method 2011.27 Paralytic Shellfish Toxins (PSTs) in Shellfish Receptor Binding Assay.
11. Consult instrument manufacturer instructions.
12. Technical Data Sheet, American Radiolabeled Chemicals, Inc. 101 Arc Drive, St. Louis, MO 63146.
13. Wilt, d. s. (ed). 1974. Proceedings of the 8th National Shellfish Sanitation Workshop. U. S. Food and Drug Administration, Washington, D.C.
14. U. S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 2017. NSSP *Guide for the Control of Molluscan Shellfish*. FDA/ISSC, Washington D.C. and Columbia, S.C.
15. U. S. Nuclear Regulatory Commission Materials, Section 30.18, 10 CFR Part 30, and American Radiolabeled Chemicals Licenses.

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting *(Tab to go to next field)*	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	US Food & Drug Administration (FDA)
3. Affiliation	US Food & Drug Administration (FDA)
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Email	Melissa.Abbott@fda.hhs.gov
9. Proposal Subject	Checklist – Microbiology Update MSC (with MA DMF)
10. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists
11. Text of Proposal/ Requested Action	The requested action is to consolidate the MSC sections (Shellfish, Part III; Wastewater, Part IV) into Part IV. The proposed section is titled "MSC Analysis for Shellfish Meats and Wastewater". The proposal addresses redundancy for laboratories performing analysis in shellfish and wastewater, and clarifies quality control line items. Summary of proposed changes: Removal of Parts 3.10 through 3.15; line items are incorporated into updated Part IV Part 4.1: Combined shellfish and wastewater sample collection, defined matrix specific line items; clarified use of sodium thiosulfate; addition of collection of temperature control for wastewater analysis Part 4.2: Defined matrix specific line items; updated balance requirement to align with application Part 4.3: No proposed changes Part 4.4: No proposed changes Part 4.5: Updated shellfish preparation for shellfish analysis; edited sterile growth broth dilution Part 4.6: Defined matrix specific line items; clarified wastewater plating, added reference; combined analysis QC line items; defined sample set Part 4.7: Defined plaques; updated variable to "supernatant extracted"; included full equation to account for less than 25 grams plated Part 4.8: Defined plaques; added language for calculating further dilutions See specific edits to all line items in the supporting documentation.

	Editorial re-numbering and formatting will be addressed, as applicable .
12. Public Health Significance	The proposed checklist removes duplicate line items between the shellfish and wastewater sections. Laboratories running both MSC methods for both will reduce the amount of potential nonconformities. The quality control line items will be better understood, increasing likelihood to conform to requirements.
13. Cost Information	None
14. Changes Needed to Guidance (if proposed change is adopted)	N/A

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SHELLFISH LABORATORY EVALUATION CHECKLIST
Microbiology

LABORATORY:		
ADDRESS:		
TELEPHONE:	**FAX:**	
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**

LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	**SHELLFISH SPECIALIST:**

OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: **Conformity is noted by a "√"**

C- Critical K - Key O - Other N/A- Not Applicable

Check the applicable analytical methods:

	Multiple Tube Fermentation Technique for Seawater (APHA) [PART II]
	Multiple Tube Fermentation Technique for Seawater using MA-1 [PART II]
	Membrane Filtration Technique for Seawater using mTEC [PART II]
	Membrane Filtration Technique for UV Treated Process Water using mEndo Agar LES [Part II]
	Multiple Tube Fermentation Technique for Shellfish Meats (APHA) [PART III]
	Standard Plate Count for Shellfish Meats [PART III]
	Elevated Temperature Coliform Plate Method for Shellfish Meats [PART III]
	Male Specific Coliphage for Shellfish Meats [PART III]
	Male Specific Coliphage for Wastewater [PART IV]

PART 1 - QUALITY ASSURANCE				
CODE	**REF**		**ITEM**	
		1.1 Quality Assurance (QA) Plan		
K	8, 11		1.1.1	Written Plan (Check those items which apply.)
				a. Organization of the laboratory.
				b. Staff training requirements.
				c. Standard operating procedures.
				d. Internal quality control measures for equipment, their calibration, maintenance, repair, performance, and rejection criteria established.
				e. Laboratory safety.
				f. Internal performance assessment.
				g. External performance assessment.
C	**8**		**1.1.2**	**QA Plan Implemented.**
K	11		1.1.3	The Laboratory participates in a proficiency testing program annually. Specify Program(s):
		1.2 Educational/Experience Requirements		
C	**State's Human Resources Department**		**1.2.1**	**In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2	In state/county laboratories, the analyst(s) meets the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3**	**In commercial laboratories, the supervisor must have at least a bachelor's degree or equivalent in microbiology, biology, or equivalent discipline with at least two (2) years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4	In commercial laboratories, the analyst(s) must have at least a high school diploma and shall have at least three (3) months of experience in laboratory science.
		1.3 Work Area		
O	8,11		1.3.1	Adequate for workload and storage.
K	11		1.3.2	Clean, well-lighted.
K	11		1.3.3	Adequate temperature control.
O	11		1.3.4	All work surfaces are nonporous, easily cleaned and disinfected.
K	11		1.3.5	Microbiological quality of the air is fewer than 15 colonies for a 15 minute exposure and determined monthly. The results are recorded and records maintained.
		1.4 Laboratory Equipment		
O	9		1.4.1	To determine the pH of prepared media, the pH meter has a standard accuracy of 0.1 units.
O	14		1.4.2	pH electrodes consisting of pH half-cell and reference half-cell or equivalent combination electrode free from (Ag/AgCl) or contains an ion exchange barrier preventing passage of Ag ions into the medium which may affect the accuracy of the pH reading.
K	11		1.4.3	The effect of temperature on the pH is compensated for by an ATC probe or by manual adjustment.
K	8		1.4.4	pH meter is calibrated daily or with each use. Results are recorded and records maintained.

K	11		1.4.5	The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the electrode isopotential point (pH 7) and the second near the expected sample pH (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	8,15		1.4.6	Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope. (*Circle the method used.)*
K	9		1.4.7	Balance provides a sensitivity of at least 0.1 g at weights of use.
K	11,13		1.4.8	Balance calibrations are verified monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use. Results are recorded and records maintained.
K	11		1.4.9	Refrigerator temperature(s) are monitored at least once daily on workdays. Results are recorded and records maintained.
K	1		1.4.10	Refrigerator temperature is maintained at 0 to 4°C.
C	**9**		**1.4.11**	**The temperature of the incubator is maintained at 35 ± 0.5°C.**
C	**11**		**1.4.12**	**Thermometers used in the air incubator(s) are graduated in at least 0.1°C increments.**
K	9		1.4.13	Working thermometers are located on top and bottom shelves or appropriately placed based on the results of spatial temperature checks.
C	**11**		**1.4.14**	**Temperature of the waterbath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15**	**The thermometers used in the waterbath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16**	**The waterbath has adequate capacity for workload.**
K	9		1.4.17	The level of water in the waterbath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18	Air incubator/waterbath temperatures are taken twice daily on workdays. The results are recorded and records maintained.
C	**4**		**1.4.19**	**All working thermometers are appropriately immersed.**
C	**9, 36**		**1.4.20**	**Working thermometers are either: calibrated mercury-in-glass thermometers, calibrated non-mercury-in-glass thermometers, or appropriately calibrated electronic devices, including Resistance Temperature Devices (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy and tolerance appropriate for the application.**
C	**11**		**1.4.21**	**A standards thermometer has been calibrated by NIST or a qualified calibration laboratory using a standard traceable to NIST or an equivalent authority at the points 0, 35 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22	Standards thermometers are verified annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination _____

C	**9**		**1.4.23 Either mercury-in-glass thermometers, non-mercury-in-glass thermometers having the accuracy (uncertainty), tolerance and response time of mercury or low drift electronic resistance thermometers with an accuracy and tolerance appropriate for the application are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 32		1.4.24 The accuracy of working thermometers are verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1°C from the reference/ standards device. Results are recorded and records maintained.
O	11		1.4.25 Appropriate pipet aids are available and used to inoculate samples. Mouth pipetting is not permitted.
		1.5 Labware and Glassware Washing	
O	9		1.5.1 Utensils and containers are clean borosilicate glass, stainless steel or other noncorroding materials.
K	9		1.5.2 Culture tubes are of a suitable size to accommodate the volume for nutritive ingredients and samples.
K	9		1.5.3 Sample containers are made of glass or some other inert material.
O	9		1.5.4 Dilution bottles and tubes are made of borosilicate glass or plastic and closed with rubber stoppers, caps or screw caps with nontoxic liners.
K	9		1.5.5 Graduations are indelibly marked on dilution bottles and tubes or an acceptable alternative method is used to ensure appropriate volumes.
C	**9**		**1.5.6 Pipettes used to inoculate the sample deliver accurate aliquots, have unbroken tips and are appropriately graduated. Pipettes larger than 10 mL are not used to deliver 1mL aliquots; nor, are pipets larger than 1.1mL used to deliver 0.1 mL aliquots.**
K	9		1.5.7 Reusable sample containers are capable of being properly washed and sterilized.
K	9		1.5.8 In washing reusable pipettes, a succession of at least three (3) fresh water rinses plus a final rinse of distilled/deionized water is used to thoroughly rinse off all the detergent.
C	**2**		**1.5.9 An alkaline or acidic detergent is used for washing glassware/labware.**
C	**11**		**1.5.10 With each load of labware/glassware washed the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali) with aqueous 0.04% bromothymol blue. Results are recorded and records maintained.**
		1.6 Sterilization and Decontamination	
K	9		1.6.1 Autoclave(s) are of sufficient size to accommodate the workload.
O	8		1.6.2 Routine autoclave maintenance is performed and the records are maintained.
C	**29, 32, 33**		**1.6.3 The autoclave provides sterilization conditions suitable to the load contents. Sterilization temperature range may be 119ºC - 124ºC as determined by the lab's equipment Quality Assurance Verification Testing and recommended practices from the media manufacturer. Sterilization is determined for each load using a working maximum registering thermometer or an appropriate working temperature monitoring device.**

K	11		1.6.4	An autoclave standards thermometer has been calibrated using a device traceable to NIST or an equivalent authority at 121°C.
K	16		1.6.5	The autoclave standards thermometer is recalibrated every five (5) years for accuracy at 121°C by a qualified calibration laboratory; or, is verified in-house at the steam point (100°C) if it was previously calibrated at both 100°C and 121°C. Any change in temperature at the steam point changes the calibrated temperature at 121°C by the same magnitude. Date of most recent determination _____
K	1		1.6.6	Working autoclave thermometers are verified against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	32		1.6.7	Spore strips/suspensions with a kill time appropriate for use in an autoclave liquid media cycle are used monthly according to manufacturer's instructions to evaluate the effectiveness of the sterilization process. Results are recorded and the records maintained.
O	11		1.6.8	Heat sensitive tape is used with each autoclave batch.
K	11, 13		1.6.9	Autoclave sterilization records including length of sterilization, total heat exposure time and chamber temperature are maintained. Type of record: Autoclave log, computer printout or chart recorder tracings. (*Circle appropriate type or types.*)
K	11		1.6.10	For dry heat sterilized material, the hot-air sterilizing oven provides heating and sterilizing temperatures ≥160°C.
K	9		1.6.11	A thermometer capable of determining temperatures ≥160°C accurately is used to monitor the operation of the hot-air sterilizing oven.
K	13		1.6.12	Records of temperatures and exposure times are maintained for the operation of the hot-air sterilizing oven during use.
K	11		1.6.13	Spore strips/suspensions are used quarterly to evaluate the effectiveness of the sterilization process in the hot-air oven. Records are maintained.
K	11		1.6.14	Reusable sample containers are sterilized for 60 minutes at 170°C in a hot-air oven or autoclaved for 15 minutes at 121°C.
C	**1**		**1.6.15**	**The sterility of reusable sample containers is determined for each load sterilized. The results are recorded and the records maintained.**
C	**1**		**1.6.16**	**The sterility of pre-sterilized disposable sample containers is determined for each lot received. Results are recorded and the records maintained.**
K	9		1.6.17	Reusable pipettes are stored and sterilized in aluminum or stainless steel canisters.
K	9		1.6.18	Reusable pipettes (in canisters) are sterilized in a hot-air oven at 170°C for two (2) hours.
C	**2**		**1.6.19**	**The sterility of reusable pipettes is determined with each load sterilized. Results are recorded and records maintained.**
C	**2**		**1.6.20**	**The sterility of pre-sterilized disposable pipettes is determined with each lot received. Results are recorded and the records maintained.**
K	18		1.6.21	Hardwood applicator transfer sticks or reusable loops are properly sterilized. Alternatively, presterilized loops are used for transfers. Method of sterilization _____

C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks/presterilized loops is checked routinely. Results are recorded and the records maintained.**
O	13		1.6.23 Spent broth cultures and agar plates are decontaminated by autoclaving for at least 30 minutes before conventional disposal.
		1.7 Media Preparation	
K	3, 5		1.7.1 Media is commercially dehydrated except in the case of A-1 medium which must be prepared from the individual components and modified MacConkey agar which may be prepared from its components.
K	11		1.7.2 Media is prepared and sterilized according to manufacturer's instructions.
O	11		1.7.3 Dehydrated media and media components are properly stored in a cool, clean, dry place.
O	11		1.7.4 Dehydrated media are labeled with date of receipt and date opened.
C	**12**		**1.7.5 Caked or expired media or media components are discarded.**
C	**11**		**1.7.6 Reagent water is distilled or deionized (circle appropriate choice), tested monthly and exceeds 0.5 megohm-cm resistance (2 megohms-cm in-line) or is less than 2.0 µSiemens/cm conductivity at 25°C. (*Circle the appropriate water quality descriptor determined.)* Results are recorded and the records maintained.**
C	**11**		**1.7.7 Reagent water is analyzed for residual chlorine monthly and is at a non- detectable level (< 0.1 mg/L). Results are recorded and the records maintained.** **Specify method of determination _____**
K	11		1.7.8 Reagent water contains<100 CFU/mL as determined monthly using the heterotrophic plate count method. Results are recorded and the records maintained.
K	9		1.7.9 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**		**1.7.10 Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.11 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**40**		**1.7.12 Media productivity is determined using media-appropriate, properly diluted positive and negative control cultures for each lot of dehydrated media received or with each batch of media prepared when the medium is made from its individual components.** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____** **Media: _____ Positive Control: _____ Negative Control: _____**

O	9		1.7.13	Sterile phosphate buffered dilution water is used as the sample diluent, unless otherwise specified in the method specific sections of this checklist.
K	11		1.7.14	The pH of the prepared media is determined after sterilization to ensure that it is consistent with the manufacturer's requirements, unless otherwise specified in the original method documentation. Results are recorded and records are maintained.
		1.8 Storage of Prepared Culture Media		
K	9		1.8.1	Prepared culture media are stored in a cool, clean, dry space where excessive evaporation and the danger of contamination are minimized.
K	5,11		1.8.2	Brilliant green bile 2% broth and A-1 media are stored in the dark.
K	13		1.8.3	Stored media are labeled with the storage expiration date or the sterilization date.
K	9		1.8.4	Storage of prepared culture media at room temperature does not exceed seven (7) days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed one (1) month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed three (3) months.
K	17		1.8.7	All prepared MPN broth media stored under refrigeration must reach room temperature prior to use. Culture tubes containing any type of precipitate or Durham tubes containing air bubbles are discarded.
		PART II – SEAWATER SAMPLES		
		2.1 Collection and Transportation of Samples		
C	**11**		**2.1.1**	**Sample containers are of a suitable size to contain at least 110 mL of sample and to allow adequate headspace for proper shaking. Seawater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
K	1		2.1.2	Samples are identified with collector's name, harvest area, sampling station, time and date of collection.
C	**9**		**2.1.3**	**Immediately after collection, seawater samples are placed in dry storage (ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.**
O	1		2.1.4	A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.
C	**9**		**2.1.5**	**Analysis of the sample is initiated as soon as possible after collection. Seawater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		2.2 Bacteriological Examination of Seawater by the APHA MPN		
C	**9**		**2.2.1**	**Lactose broth or lauryl tryptose broth is used as the presumptive medium. (*Circle appropriate one.*)**
C	**9, 34**		**2.2.2**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**

C	**9**		**2.2.3**	**In a multiple dilution series not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.2.4**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.5**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
K	9		2.2.6	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**		**2.2.7**	**Appropriately diluted process control cultures accompany the samples *throughout both the presumptive and confirmed phases of incubation.* Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
K	9		2.2.8	Inoculated tubes are read after 24 ± 2 hours and 48 ± 3 hours of incubation and transferred at both time interval if positive for growth (the presence of turbidity) and gas or effervescence in the culture tube. These tubes are considered presumptive positive requiring further confirmatory testing.
		2.3 Confirmed Test for Seawater by APHA MPN		
C	**9**		**2.3.1**	**Brilliant green bile 2% broth (BGB) is used as the confirmatory medium for total coliforms.**
C	**9**		**2.3.2**	**EC medium is used as the confirmatory medium for fecal coliforms.**
K	9, 11		2.3.3	Transfers are made to BGB/EC by either sterile loop or sterile hardwood transfer stick from positive presumptive tubes incubated for 24 and 48 hours as appropriate. (*Circle the method of transfer.*)
C	**9**		**2.3.4**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.5	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.6**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.7**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.8**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.4 Computation of Results – APHA MPN		
K	9		2.4.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
K	7		2.4.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.4.3**	**Results are reported as MPN/100 mL of sample.**
		2.5 Bacteriological Examination of Seawater by the MA-1 Method		
C	**5**		**2.5.1**	**A-1 medium complete is used in the analysis.**
C	**2, 30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1 medium without salicin. Study records are maintained and are available upon request.**

C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**9, 34**		**2.5.4**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before inoculation.**
C	**9**		**2.5.5**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**
C	**6**		**2.5.6**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.7**	**In a single dilution series, the volumes analyzed are adequate to meet the needs of routine monitoring.** **Sample volume inoculated _____** **Range of MPN_____** **Strength of media used_____**
C	**2**		**2.5.8**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and waterbath incubation*. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**2,5**		**2.5.9**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.10**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating waterbath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.11**	**The presence of turbidity and any amount of gas or effervescence in the culture tube constitutes a positive test.**
		2.6 Computation of Results – APHA MPN		
K	9		2.6.1	Results of multiple dilution tests are read from tables in *Recommended Procedures for the Examination of Sea Water and Shellfish,* 4th Edition.
K	7		2.6.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**7, 9**		**2.6.3**	**Results are reported as MPN/100 mL of sample.**
		2.7 Bacteriological Analysis of Seawater by Membrane Filtration (MF) using mTEC Agar - Materials and Equipment		
C	**23, 24**		**2.7.1**	**When used for elevated temperature incubation in conjunction with ethafoam resuscitation, the temperature of the hot air incubator is maintained at 44.5 ± 0.5°C under any loading capacity.**
C	**23**		**2.7.2**	**When using a waterbath for elevated temperature incubation, the level of the water completely covers the plates.**
C	**23**		**2.7.3**	**Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches with tight fitting lids are used.**
C	**2**		**2.7.4**	**The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	11		2.7.5	Colonies are counted with the aid of magnification.
C	**11, 23**		**2.7.6**	**Membrane filters are made from cellulose ester material, white, grid marked, 47 mm in diameter with a pore size of 0.45 µm and certified by the manufacturer for fecal coliform analyses.**

C	**2**		**2.7.7**	**Lot number, date of receipt and if provided the expiration date of the membrane filters are recorded and records maintained.**
C	**2**		**2.7.8**	**When initiating monitoring by mTEC or switching brands or types of membrane filters used and no previous lots of filters are available for comparing acceptable performance, an appropriate method for determining the suitability of the lot is developed and the comparison testing implemented. The results are recorded and the record is maintained.**
K	2, 11		2.7.9	New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.
C	**2**		**2.7.10**	**The sterility of each lot or autoclave batch of membrane filters are checked before use.**
K	2		2.7.11	Membrane filters which are beyond their expiration date are not used.
O	11		2.7.12	Forceps tips are clean.
O	11		2.7.13	Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	11		2.7.14	Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11		2.7.15	If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically or with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
K	11		2.7.16	Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**11**		**2.7.17**	**Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series.**
O	11, 23, 26		2.7.18	A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.7.19	The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2		2.7.20	Maintenance of the UV sterilization unit is performed as needed. This maintenance is documented and the records maintained.
		2.8 Media Preparation and Storage – MF using mTEC Agar		
K	11		2.8.1	Phosphate buffered saline is used as the sample diluent and filter funnel rinse.
C	**11**		**2.8.2**	**The phosphate buffered saline is properly sterilized.**
K	23		2.8.3	A sufficient amount of medium (4-5 mL) is used in each plate.
O	11		2.8.4	Refrigerated prepared plates are stored for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
		2.9 Sample Analyses - MF using mTEC Agar		
C	**24**		**2.9.1**	**mTEC agar is used.**
C	**23, 34**		**2.9.2**	**The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**23**		**2.9.3**	**The membrane is placed grid side up within the sterile filter apparatus.**

C	**23, 25**		**2.9.4 Sample volumes tested are consistent with the sampling regime employed (i.e., half log or other appropriate dilutions are used with systematic random sampling).**
C	**23**		**2.9.5 Sample volumes are filtered under vacuum.**
K	26		2.9.6 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.7 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered saline after sample filtration.**
C	**23**		**2.9.8 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mTEC agar so that no bubbles form between the filter and the agar.**
C	**11**		**2.9.9 Blanks are run at the beginning of filtration, after every 10th aliquot and at the end of the filtration run to check the sterility of the testing system (phosphate buffered saline, filter funnel, forceps, membrane filter, media and culture plate).**
C	**2, 11**		**2.9.10 Appropriately diluted process control cultures accompany the samples throughout both resuscitation and elevated temperature incubation. Results are recorded and the records maintained.** **Positive process control _____ Negative process control _____**
C	**11, 23, 24**		**2.9.11 Inoculated plates are placed inverted into a watertight, tightly sealed container prior to being placed in the air incubator and incubated at 35 ± 0.5°C for two (2) hours of resuscitation. Alternatively inoculated plates may be placed in ethafoam prior to air incubation at 44.5 ± 0.5°C for 24 ± 2 hours.**
C	**11, 23, 24**		**2.9.12 After 2 hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating waterbath at 44.5 ± 0.2°C, submerged completely and incubated for 22-24 hours.**
		2.10 Computation of Results - MF using mTEC Agar	
C	**23**		**2.10.1 All yellow, yellow-green or yellow-brown colonies are counted.**
C	**23**		**2.10.2 Only plates having 80 or fewer colonies are counted. If it is unavoidable to use plates having more than 80 colonies, counts are given as >80 x 100/the volume of sample filtered.**
C	**2, 11, 23**		**2.10.3 When multiple dilutions are filtered, the laboratory has developed a procedure for assessing the contribution of all positive dilutions to the final count.**
C	**23, 11**		**2.10.4 The number of fecal coliforms is calculated by the following equation:** **Number of fecal coliforms per 100 mL = [number of colonies counted per plate used in the count / volume (s) of sample filtered in ml] x 100.**
C	**23, 11**		**2.10.5 Results are reported as CFU/100 mL of sample.**
		2.11 Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES – Materials and Equipment	
C	**9, 11, 21**		**2.11.1 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**		**2.11.2 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**

C	**11, 19, 21**		**2.11.3 Membrane filters are made from cellulose ester material, white, grid marked, 47 mm diameter with a pore size of 0.45µm and certified by the manufacturer for total coliform analysis.**
C	**2**		**2.11.4 Membrane filter lot numbers, dates of receipt and expiration dates are recorded and the records maintained.**
C	**2**		**2.11.5 If previous lots of agar or membrane filters are not available for comparability testing, an appropriate method for determining lot suitability has been developed and comparison testing implemented when the following has occurred:** **a. initiating monitoring by mEndo Agar LES;** **b. changing agar manufacturers; or** **c. changing brands of membrane filters used.** **The results are recorded and the records are maintained.**
K	2, 11		2.11.6 Recovery of total coliforms from new lots of membrane filters and mEndo Agar LES is compared against the recovery from the previously acceptable lot.
C	**2**		**2.11.7 The sterility of each lot and autoclave batch of membrane filters is verified before use.**
K	2		2.11.8 Expired membrane filters are not used.
K	9, 11, 19, 21		2.11.9 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
K	11		2.11.10 If indelible graduation marks are used on clear glass or plastic funnels to measure sample volumes, their accuracy is checked gravimetrically with a Class A graduated cylinder before use and periodically rechecked. Funnels having a tolerance greater than 2.5% are not used. Checks are recorded and records maintained.
C	**9, 11**		**2.11.11 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121+/- 2°C prior to the start of a filtration run. A new run occurs when there is a break of 30 minutes or more between the previous filtration run.**
O	11, 19, 26, 35		2.11.12 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11		2.11.13 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and the records are maintained.
K	2		2.11.14 Maintenance of the UV sterilization unit is performed as needed. Maintenance is documented and the records maintained.
O	9, 11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	**9, 11, 19, 21, 35**		**2.12.1 mEndo Agar LES is used.**
K	11, 21, 35		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	9, 11, 35		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	**9, 11, 35**		**2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 44-50°C before dispensing.**
C	**9, 11, 35**		**2.12.5 mEndo Agar LES is never autoclaved.**
K	9, 11, 35		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.

O	9, 11, 35		2.12.7 Prepared plates of mEndo Agar LES are stored at 4°C in the dark for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation.
K	9, 11, 21, 35		2.12.8 Sterile phosphate buffered water or sterile phosphate buffered saline is used as a sample blank, filter funnel rinse and process and productivity control diluent for UV treated process water samples.
C	**11**		**2.12.9 The phosphate buffered water/saline is properly sterilized and the sterility is tested before being placed in service. Results are recorded and records maintained**
		2.13 Sample Analysis	
C	**9, 11, 35**		**2.13.1 The sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) before filtration.**
C	**11, 21, 35**		**2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.**
C	**11, 26, 35**		**2.13.3 A 100 mL quantity of sample is filtered under vacuum.**
K	26		2.13.4 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 26, 35**		**2.13.5 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water/saline as appropriate after filtration.**
C	**9, 11, 35**		**2.13.6 The membrane filter is removed from the filtering apparatus with sterile forceps and rolled onto mEndo Agar LES so that no bubbles form between the filter and the agar.**
K	9, 11, 35		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.
C	**11, 35**		**2.13.8 Blanks are run at the beginning and at the end of the filtration run to check the sterility of the testing system (phosphate buffered water/saline, filter funnels, forceps, membrane filters, media and culture plates).**
C	**2, 35**		**2.13.9 An appropriate properly diluted positive process control culture accompanies the sample throughout incubation. Results are recorded and the records are maintained.** **Positive process control**
C	**9, 11, 35**		**2.13.10 Inoculated plates are incubated inverted at 35 ± 0.5°C for 22 to 24 hours.**
K	2, 9, 11		2.13.11 An appropriate level of humidity is maintained in the incubator to prevent the plates from drying out.
		2.14 Computation of Results	
K	9, 11		2.14.1 Colonies are counted with the aid of magnification.
C	**9, 11, 19, 21, 23**		**2.14.2 All metallic sheen colonies are counted as total coliforms.**
C	**9, 11, 21, 35**		**2.14.3 Results are reported as total coliforms/100mL.**
C	**11, 20, 35**		**2.14.4 When no colonies are observed, results are reported as <1.0 coliform/100mL (nondetectable)**

PART III – SHELLFISH SAMPLES				
		3.1 Collection and Transportation of Samples		
C	**9**		**3.1.1**	**A representative sample of shellstock is collected.**
K	9		3.1.2	Shellstock samples are collected in clean, waterproof, puncture resistant containers loosely sealed.
K	9		3.1.3	Shellstock samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**9**		**3.1.4**	**Immediately after collection, shellfish samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed under refrigeration unless processed immediately.**
C	**1**		**3.1.5**	**Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
		3.2 Preparation of Shellfish for Examination		
K	2,32		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2, 32		3.2.2	Blades of shucking knives are not corroded.
O	9, 32		3.2.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2, 32		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9, 32		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9, 32		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9, 32		3.2.7	Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**, 32		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**, 32		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	2, 9		3.2.10	At least 200 grams of shellfish meat or a quantity of meat sufficient to cover the blender blades is used for the analysis.
K	9		3.2.11	A representative sample of at least 12 shellfish is used for the analysis.
K	2		3.2.12	The sample is weighed to the nearest 0.1 gram and an equal amount by weight of diluent is added.
C	**9**		**3.2.13**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.14	APHA *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition is followed for the analysis of previously shucked and frozen shellfish meats.
		3.3 MPN Analysis for Fecal Coliform Organisms, Presumptive Test, APHA		
C	**9**		**3.3.1**	**Appropriate strength lactose or lauryl tryptose broth is used as presumptive media in the analysis. (*Circle the medium used.*)**
K	9		3.3.2	Immediately (within two (2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.

C	**9**		**3.3.3**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.4**	**Allowing for the initial 1:1 dilution of the sample, appropriate portions are inoculated (i.e., 2 ml of original 1:1 dilution for the 1 g portion) and diluted for subsequent inoculation (i.e., 20 ml of 1:1 diluted sample to 80 ml of diluent or the equivalent for 0.1 g portion). All successive dilutions are prepared conventionally.**
K	6		3.3.5	In a single dilution series, the volumes examined are adequate to meet the needs of routine monitoring. Sample volume inoculated _____ Range of MPN _____ Strength of media used _____
C	**2**		**3.3.6**	**Appropriately diluted process control cultures accompany the samples throughout both the presumptive and confirmed phases of incubation. Results are recorded and the records maintained.** **Positive Process control _____ Negative Process control _____**
K	9		3.3.7	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.8	Tubes are read after 24 ± 2 hours of incubation and transferred if positive for growth (the presence of turbidity and gas or effervescence in the culture tube). These tubes are considered presumptive requiring further confirmatory testing.
		3.4 Confirmed Test for Fecal Coliforms - APHA		
C	**9**		**3.4.1**	**EC medium is used as the confirmatory medium.**
K	9, 11		3.4.2	Transfers are made to EC medium by either sterile loop or hardwood sterile transfer sticks from positive presumptives. (*Circle the method of transfer.*)
C	**9**		**3.4.3**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.4	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.5**	**The presence of turbidity and any amount of gas and/or effervescence in the Durham tube constitutes a positive test.**
		3.5 Computation of Results for MPN Analyses		
K	9		3.5.1	Results of multiple dilution tests are read from tables in *Recommended Procedure for the Examination of Sea Water and Shellfish,* 4th Edition and multiplied by the appropriate dilution factor.
K	7		3.5.2	Results from single dilution series are calculated from Hoskins' equation or interpolated from Figure 1, Public Health Report 1621 entitled "Most Probable Numbers for Evaluation of Coli aerogenes Tests by Fermentation Tube Method".
C	**9**		**3.5.3**	**Results are reported as MPN/100 grams of sample.**
		3.6 Standard Plate Count Method		
O	20		3.6.1	A standard plate count (SPC) analysis maybe performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four (4) plates are used, duplicates of two (2) dilutions. One (1) of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	15 to 20 mL of tempered sterile plate count agar is used per plate.
C	**9**		**3.6.4**	**Agar tempering bath maintains the agar at 44-46°C.**

C	**9**		**3.6.5 An agar based temperature control having a similar volume and shape as the tempering plate count agar is used in the tempering bath.**
K	9		3.6.6 Samples or sample dilutions to be plated are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before plating.
C	**9**		**3.6.7 Not more than 1 mL nor less than 0.1 mL of sample or sample dilution is plated.**
K	11		3.6.8 Control plates are used to check air quality and the sterility of the agar and the diluent.
K	9, 21		3.6.9 Solidified plates are incubated at 35 ± 0.5°C for 48 ± 3 hours inverted and stacked no more than four (4) high.
K	9		3.6.10 Quebec Colony Counter or its equivalent is used to provide the necessary magnification and visibility for counting plates.
K	1		3.6.11 A hand tally or its equivalent is used for accuracy in counting.
		3.7 Computation of Results -SPC	
K	9		3.7.1 Colony counts determined in accordance with Pa rt III, A, Sections 4.31 through 4.33 in *Recommended Procedures for the Examination of Sea Water and Shellfish,* Fourth Edition.
C	**19**		**3.7.2 Colony counts are reported as CFU/grams of sample.**
		3.8 Bacteriological Analysis of Shellfish Using the ETCP	
C	**2,3**		**3.8.1 Prepared modified MacConkey agar is used on the day that it is made.**
K	3		3.8.2 Double strength modified MacConkey agar is used.
C	**3**		**3.8.3 Prepared double strength modified MacConkey agar is heated to boiling, removed from the heat, and boiled again. This agar is never autoclaved.**
K	2, 3		3.8.4 Twice boiled, double strength modified MacConkey agar and is maintained in a tempering bath at 45 to 50°C until used.
K	2, 3		3.8.5 Phosphate buffered saline is used as the sample diluent in the ETCP.
C	**2, 3**		**3.8.6 The phosphate buffered saline is tempered at 45 - 50°C to prevent premature solidification of the agar.**
C	**9**		**3.8.7 The sample homogenate is cultured within two (2) minutes of blending.**
C	**2,3**		**3.8.8 Six (6) grams of shellfish (12 grams of homogenate if initially diluted 1:1) is placed into a sterile container and the contents brought up to 60 mL with sterile, tempered phosphate buffered saline.**
K	3		3.8.9 Sixty (60) mL of tempered, twice boiled double strength Modified MacConkey Agar is added.
K	2,3, 22		3.8.10 The container is gently swirled or slowly inverted once to mix the contents, which are subsequently distributed uniformly over six (6) plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**3, 13**		**3.8.12 When solidified, the plates are placed inverted into an air incubator at 45.5 ± 0.5°C for 18 to 30 hours of incubation.**
C	**2**		**3.8.13 Plates are stacked no more than three (3) high in the incubator.**

C	**2**		**3.8.14 Appropriately diluted pour plated process control cultures *accompany each set of samples throughout incubation.* The results are recorded and the records maintained.** **Positive Process Control: _____ Negative Process Control:_____**
		3.9 Computation of Results - ETCP	
K	11		3.9.1 Quebec Colony counter or its equivalent is used to provide the necessary magnification and visibility for counting.
O	1		3.9.2 A hand tally or its equivalent is used to aid in counting.
C	**3, 6**		**3.9.3 All brick red colonies greater than 0.5 mm in diameter are totaled over all the plates and multiplied by a factor of 16.7.**
C	**3**		**3.9.4 Results are reported as CFU/100 grams of sample.**
~~Bacteriological Examination of Shellfish Meats for Male Specific Coliphage (MSC)~~			
		~~3.10 MSC Equipment and Supplies~~	
~~K~~	~~2~~		~~3.10.1 Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene) and hold at least 100mL.~~
~~K~~	~~2~~		~~3.10.2 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.~~
~~K~~	~~1~~		~~3.10.3 The sterility of each batch/ lot of pre-sterilized or reusable syringes, filters and/or filter units is determined. Results are recorded and records maintained.~~
~~**C**~~	~~**2**~~		~~**3.10.4 The balance used provides a sensitivity of at least mg (0.01g.).**~~
~~**C**~~	~~**27, 28, 31**~~		~~**3.10.5 The temperature of the incubator used is maintained at 36 ± 1°C.**~~
~~K~~	~~2~~		~~3.10.6 The temperature of the freezer is maintained at ≤ -15°C.~~
~~**C**~~	~~**1**~~		~~**3.10.7 The sterility of disposable 50 mL centrifuge tubes is determined with each lot. Results are recorded and records maintained.**~~
		~~3.11 MSC Media Preparation~~	
~~K~~	~~28, 31~~		~~3.11.1 Media preparation and sterilization is according to the validated method.~~
~~K~~	~~27, 28, 31~~		~~3.11.2 Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.~~
~~O~~	~~27, 28, 31~~		~~3.11.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.~~
~~K~~	~~2~~		~~3.11.4 Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.~~
~~K~~	~~27, 28, 31~~		~~3.11.5 The soft agar is sterilized for 15 minutes at 121°C before use.~~
~~K~~	~~28, 31~~		~~3.11.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months and with loose fitting closures shall not exceed one (1) month.~~
~~K~~	~~2, 27, 28, 31~~		~~3.11.7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.~~
		~~3.12 Preparation of Host Culture for MSC Analysis~~	
~~**C**~~	~~**28, 31**~~		~~**3.12.1 *E. coli* Famp ATCC 700891 is the bacterial host strain.**~~
~~K~~	~~27, 28, 31~~		~~3.12.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.~~

K	27, 28, 31		3.12.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.
C	27, 28, 31		3.12.4 After inoculation, the host cell growth broth culture is not shaken.
		3.13 Preparation of the Shellfish for MSC Analysis	
K	2,37		3.13.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13.2 The blades of shucking knives are not corroded.
O	9		3.13.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.13.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	9		3.13.8 Shellfish are not shucked through the hinge.
C	9		3.13.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.
K	9		3.13.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13.11 The sample is weighed to the nearest 0.1 gram.
C	28, 31		3.13.12 Two (2) times the weight of the sample sterile growth broth, by volume, is added.
C	28, 31		3.13.13 Samples are blended at high speed for 180 seconds.
		3.14 MSC Sample Analysis	
C	28, 31		3.14.1 Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.
C	28, 31		3.14.2 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.
C	27, 28, 31		3.14.3 The supernatant is transferred to a new sterile tube, weighed and the weight recorded.
C	27, 28, 31		3.14.4 The supernatant is allowed to warm to room temperature prior to analysis.
K	27, 28, 31		3.14.5 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28, 31		3.14.6 200 microliters (0.2 mL) of log phase host strain E coli is added to the tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.7 The sample supernatant is shaken or vortexed before being added to the tempered soft agar.
C	27, 28, 31		3.14.8 2.5 mL of sample supernatant (avoiding bubbles where necessary) is added to a tube of tempered soft agar.
C	27, 28, 31		3.14.9 The tube of soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.
C	27, 28, 31		3.14.10 The tube contents is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.

~~C~~	~~28, 31~~		~~3.14.11 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.~~
~~K~~	~~27, 28, 31~~		~~3.14.12 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are recorded and records maintained.~~ ~~Positive Control: _____~~
~~K~~	~~27, 28, 31~~		~~3.14.13 Room temperature growth broth is used as the negative control or blank.~~
~~K~~	~~27, 28, 31~~		~~3.14.14 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.~~
~~K~~	~~2~~		~~3.14.15 A negative control plate is plated at the end of each set of samples analyzed.~~
~~K~~	~~27, 28, 31~~		~~3.14.16 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.~~
~~C~~	~~27, 28, 31~~		~~3.14.17 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.~~
		~~3.15 Computation of Results - MSC~~	
~~C~~	~~27~~		~~3.15.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.~~
~~C~~	~~28, 31, 37~~		~~3.15.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.~~
~~K~~	~~28, 31~~		~~3.15.3 The formula used for determining the density of MSC in PFU/100 grams is:~~ ~~(0.364) (N) (Ws), where N = total number of plaques counted on all 10 plates and Ws = weight of the supernatant used.~~
~~O~~	~~2~~		~~3.15.4 The MSC count is rounded off conventionally to give a whole number.~~
PART IV – MSC ~~WASTEWATER~~ ANALYSIS FOR SHELLFISH MEATS AND WASTEWATER			
		4.1 Collection and Transportation of Samples	
C	**9**		**4.1.1 [Shellfish Only] A representative sample of shellstock is collected**
C	**11**		**4.1.2~~1~~ Immediately after collection, samples are placed in dry storage ice chest or equivalent) with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.~~ ~~Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.~~**
K	9		**4.1.3** [Shellfish Only] Samples are collected in clean, waterproof, puncture resistant containers and loosely sealed. Samples are labeled with collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.

K	1		4.1.4 [Shellfish Only] The temperature of the shellstock samples or a seawater temperature control (TC) is taken upon receipt in the laboratory. Results are recorded and maintained. a. When a seawater TC is used to document temperature at receipt it must be: i. between 0-10°C or ii. less than the temperature of the growing area at the time of collection. b. When the temperature of shellstock is used to document temperature at receipt it must be: i. between 0-10°C or ii. less than the temperature of the shellstock or growing area at the time of collection.
C	**1**		**4.1.5 [Shellfish Only] Analysis of the samples is initiated as soon as possible after collection. Shellfish samples are not tested if the time interval between collection and analysis exceeds 24 hours.**
C	**11**		**4.1.6 [Wastewater Only] Sample containers are of a suitable size to contain sample and to allow adequate headspace for proper shaking. Wastewater samples are collected in clean, sterile, watertight, properly labeled sample containers.**
C	**39**		**4.1.72 [Wastewater Only] Effluent samples collected from wastewater treatment facilities employing chlorine disinfection ~~bottles~~ must contain 0.2 mL of 10% sodium thiosulfate solution ~~for every~~per 200 mL of sample to inactivate any residual chlorine.**
K	1		4.1.83 [Wastewater Only] Samples are identified with collector's name, sampling location, time and date of collection.
~~C~~K	9		4.1.94 [Wastewater Only] A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature must be between 0-10°C or less than temperature at collection if collected at the time of sampling. Results are recorded and maintained. ~~Immediately after collection, wastewater samples are placed in dry storage ice chest or equivalent) capable of maintaining a temperature of 0 to 10°C with ice or cold packs for transport to the laboratory. Once received, the samples are placed in the refrigerator unless processed immediately.~~

~~O~~	~~1~~		~~4.1.5 A temperature blank is used to represent the temperature of samples upon receipt at the laboratory. Temperature should be equivalent or less than that of the growing area waters. Results are recorded and maintained.~~
C	**9**		**4.1.11~~6~~ [Wastewater Only] Analysis of the sample is initiated as soon as possible after collection. Wastewater samples are not tested if they have been held for more than 30 hours from the time of collection.**
		4.2 MSC Equipment and Supplies	
~~K~~	~~38~~		~~4.2.1 Wastewater samples or appropriately diluted samples are transferred into a sterile vessel.~~
K	2		4.2.1 [Shellfish Only] Sample containers used for the shucked sample are sterile, made of glass or some other inert material (i.e. polypropylene)
K	9		4.2.2 The level of water in the tempering bath covers the level of ~~liquid and~~ agar ~~in the container or~~ culture tubes.
K	1		4.2.3 Sterile ~~The sterility of each batch/lot of pre-sterilized or reusable~~ syringes, filters, and/or filter units are used.~~is determined. Results are recorded and records maintained.~~
C	**27, 28**		**4.2.4 The balance used for antibiotic preparation provides a sensitivity of at least 0.05~~1~~g.**
C	**27, 28**		**4.2.5 The temperature of the incubator used is maintained at 36 ±1°C.**
K	2		4.2.6 The temperature of the freezer is maintained at ≤ -15°C.
C	**1, 28**		**4.2.7 ~~The sterility of the transfer vessel utilized is determined with each lot. Results are recorded and records maintained.~~Sterile 50 mL centrifuge tubes and/or transfer vessels are used.**
		4.3 MSC Media Preparation	
K	28		4.3.1 Media preparation and sterilization is according to the validated method.
K	27, 28		4.3.2. Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.
~~O~~K	27, 28		4.3.3 Storage of the bottom agar under refrigeration does not exceed six (6) weeks.
K	27, 28		4.3.4 Unsterilized soft agar is stored at ≤ -15°C for up to three (3) months.
~~K~~C	**27, 28**		**4.3.5 The soft agar is sterilized for 15 minutes at 119°C to 124°C before use.**
K	27, 28		4.3.6 Storage under refrigeration of prepared growth broth with screw-cap closures shall not exceed three (3) months, and with loose fitting closures shall not exceed one (1) month.
K	27, 28		4.3.7 Bottom agar plates stored under refrigeration are allowed to reach room temperature before use.
		4.4 Preparation of Host Culture for MSC Analysis	
C	**28**		**4.4.1 *E.coli* Famp ATCC 700891 is the bacterial host strain**
K	27, 28		4.4.2 Host cell growth broth is tempered at 36 ± 1°C prior to inoculation with host cells.
K	27, 28		4.4.3 Several host cell colonies are transferred to a tube of tempered growth broth and incubated at 36 ± 1°C for 4-6 hours to provide host cells in log phase growth for sample analysis.

C	**27, 28**		**4.4.4 After inoculation, the host cell growth broth culture is not shaken.**

		4.5 Preparation of Shellfish for MSC ~~Sample~~ Analysis	
K	2, 37		4.5.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		4.5.2 The blades of shucking knives are not corroded.
O	9		4.5.3 Immediately prior to cleaning the shells of debris, ~~T~~the hands of the analyst are thoroughly washed with soap and water or ~~new~~clean gloves are donned. ~~immediately prior to cleaning the shells of debris.~~
O	2		4.5.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		4.5.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		4.5.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		4.5.7 Immediately prior to shucking, the hands of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**4.5.8 Shellfish are not shucked through the hinge.**
C	**9**		**4.5.9 The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9		4.5.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		4.5.11 The sample is weighed to the nearest 0.1 gram.
C	**28, 31**		**4.5.12 Sterile growth broth, two (2) times the weight of the sample, is added volumetrically or gravimetrically.**
C	**28, 31**		**4.5.13 Samples are blended at high speed for 180 seconds.**
		4.6 MSC Sample Analysis	
C	**28, 31**		**4.6.1 [Shellfish Only] Immediately after blending, 33 grams of the homogenate is weighed into a centrifuge tube.**
C	**28, 31**		**4.6.2 [Shellfish Only] The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28, 31**		**4.6.3 [Shellfish Only] The supernatant is transferred to a new sterile tube.~~,~~ The total ~~volume~~amount of shellfish supernatant ~~plated~~ extracted is weighed and the weight recorded.**
C	**27, 28, 31**		**4.6.4 [Shellfish Only] The supernatant is allowed to warm to room temperature prior to analysis.**

C	38		4.6.5 [Wastewater Only] Wastewater sample is allowed to warm to room temperature prior to analysis. The sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then transferred into a sterile 50 mL centrifuge tube. High range dilutions are prepared using sterile room temperature growth broth and sterile vessels.
~~C~~	~~38~~		~~4.5.1 Wastewater sample is shaken vigorously (25 times in a 12" arc in 7 seconds by hand or for at least 15 seconds when using a mechanical shaker) and then diluted to the high range (10-2) and/or low range (no dilution) with sterile room temperature growth broth based on sample type.~~
K	27, 28		4.6.6~~5.2~~The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		4.~~5.3~~6.7Two hundred microliters (0.2 mL) of log phase host strain E. coli is added to the tempered soft agar immediately prior to adding the sample.
K	27, 28, 31		4.6.8 The sample supernatant/wastewater sample is gently mixed to avoid the introduction of air bubbles before being added to the tempered soft agar.
C	**27, 28**		**4.6.9~~5.4~~2.5 mL of wastewater sample or shellfish supernatant is added to a tube of tempered soft agar.**
C	**27, 28**		**4.6.10~~5.5~~ ~~———~~The tube contents are~~is~~ gently rolled between the palms of the hands to mix.**
C	**27, 28**		**4.6.11~~5.6~~ The tube contents are~~is~~ overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	28, 31		4.6.12 [Shellfish Only] 10 plates are used for analysis of each sample with 2.5 mL of sample supernatant per plate for a total of 25 mL of supernatant analyzed per sample, unless fewer than 25 mL of supernatant is obtained from the sample in which all supernatant is plated.
C	**38**		**4.6.13~~5.7~~ [Wastewater Only] Four (4) plates are used for the analysis of high range samples. Eight (8) plates are used for the analysis of low range samples. ~~Four (4) plates are used for the analysis of Influent samples and high range Pretreated Effluent samples. Eight (8) plates are used for the analysis low range Pretreated Effluent and Effluent samples.~~**
K	27, 28, 31		~~4.5.8~~6.14 ~~Negative and positive control plates are run with MSC analyses.~~prepared and accompany each set of samples analyzed. The results are recorded and records maintained.
K	27, 28, 31		4.6.14~~5.9~~ Negative and positive control plates are run with MSC analysis. Room temperature growth broth is used as the negative control. Type strain MS2 male specific bacteriophage (ATCC 15597-B1), appropriately diluted to provide countable low levels of phage, is used as the positive control. The results are recorded, and records are maintained.~~Room Temperature Growth broth is used as the negative control or blank.~~
~~K~~	~~27, 28~~		~~4.5.10 Type strain MS2 (ATCC 15597-B1) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.~~

K	27, 28, 31		4.6.15~~65.11~~ The positive control is plated after all the samples are inoculated and immediately prior to a final negative control. ~~A negative control plate is plated at the end of each set of samples analyzed.~~
K	~~27, 28~~2		4.6.16 ~~7 5.12~~ A negative control plate is plated at the end of each sample set (i.e. 10 plates for shellfish sample, or influent/effluent wastewater sample set) analyzed and at the end of sample analysis. ~~The positive control is plated after all the samples are inoculated and immediately prior to the final negative control~~.
C	**27, 28**		**4.6~~5~~.17~~3~~ All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		4.7~~6~~ Computation of Results ~~–~~ MSC in Shellfish Meats	
C	**27**		**4.7.1 Circular zones of clearing (referred to as plaques) of any diameter in the lawn of host bacteria are counted.**
C	**28, 31, 37**		**4.7.2 The countable plaque range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the reported value is <6 PFU/100 grams for soft-shelled clams, <7 PFU/ 100 grams for American oysters, and <5 PFU/ 100 grams for quahog (hard) clams. If the density exceeds 200 PFU per plate on all plates, the count is given as > 20,000 PFU/100 grams.**
C	**28, 31**		**4.7.3 When at least 25 grams of supernatant is recovered, the formula used for determining the density of MSC in PFU/100 grams is:** $0.364 \times N \times W_s = PFU/100\ grams$ **where N = total plaques counted on all 10 plates** **W_s = weight of the supernatant ~~plated~~extracted.** **When less than 25 grams of supernatant is recovered/plated, the formula used for determining the density of MSC in PFU/100 grams is:** $\frac{N}{S_p} \times \frac{W_s}{11\ grams} \times 100 = PFU/100\ grams$ **where N = total plaques counted on all plates** **S_p = total amount of supernatant plated** **W_s = weight of supernatant extracted**
O	2		4.7.4 The MSC count is rounded off conventionally to give a whole number.
		4.8 Computation of Results – MSC in Wastewater	
C	**38**		**4.8~~6~~.1 Circular zones of clearing (referred to as plaques) of any diameter in the lawn of host bacteria are counted.~~Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.~~** **The countable plaque range of the method is 1 to 300 PFU per plate.**
C	**38**		**4.8~~6~~.2 When there are no plaques on all 4 plates of high range, the count is <1000 PFU/100 mL. When there are no plaques on all 8 plates of low range, the count is <5 PFU/100 mL.**
~~K~~C	**28**		**4.8~~6~~.3 The formula used for determining the density of MSC in PFU/100 mL is:** **High Range: N * 1000 = PFU/100mL;** **Low Range: N * 5 = PFU/100mL;** **Where N is the total number of plaques counted on all plates.**

C	**38**		**4.8.4 When high range dilutions other than 10^{-2} are analyzed, the high range formula is adjusted appropriately. If no plaques are present, the reported value (< PFU/100mL) is also adjusted appropriately.**
O	9		4.8~~6~~.5~~4~~ The MSC count is rounded off conventionally to give a whole number.

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36. NIST Monograph 150 states "the accuracy attainable is principally limited by the characteristics of the thermometer itself."
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Proposal No. 27-153

Proposal for Task Force Consideration at the ISSC 2025 Biennial Meeting *(Tab to go to next field)*	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Laboratory Committee
3. Affiliation	
4. Address Line 1	4801 Hermitage Rd. Ste 102
5. Address Line 2	
6. City, State, Zip	Richmond, VA 23227
7. Phone	804-330-6380
8. Fax	
9. Email	issc@issc.org
10. Proposal Subject	Modify Laboratory Chapter of Model Ordinance to Add Clarity, To Account For New Lab Method, and To Correct For Changes In Guidance Documents
11. Specific NSSP Guide Reference	Section II, Chapter III
12. Text of Proposal/ Requested Action	Chapter III. Laboratory Requirements for the Authority [Note: The Authority must meet the requirements of this section even if the Authority does not formally adopt this section in regulation.] For the purposes of this chapter, "Regulatory Decision" is defined as: a. the determination of classification(s) within a growing area, with the exception of the Prohibited classification; or b. the determination of the open status when reopening a growing area or portion of a growing area previously placed in the closed status due to association with illnesses or hazardous substances, as described in Section II. Chapter II. @.01F and @.02A(10)(a); or c. when determining pathogen concentrations as part of an investigation or determining growing area status after finding human pathogens present in shellfish meats, as described in Section II. Chapter II. @.04; or d. the determination of the open status of a growing area or portion of a growing area, unless alternative processes for determining open status are defined within the Model Ordinance, as described in Section II. Chapter IV. @.03 and @.04; or e. the determination of controls for Vibrio management as described in Section II. Chapter II. @.06E(1)(b)(iv), @.06E(1)(e), @.07B(4)(b)(vi), and @.07B(5); or f. the release of restricted shellstock from an area in the controlled access status; or g. the determination of the microbiological suitability of water used in land-based aquaculture, wet storage, or depuration, as described in Section II. Chapter VI. .05, Chapter VII. .04C, and Chapter XV. .02A(4); or h. cleansing or contaminant reduction studies, as described in Section II. Chapter IV. @.03A(5)(d), @.03C(2)(c), or Chapter V.; or

	i. validation or verification of other processes for pathogen reduction, as described in Section II. Chapter XVI. .01A and .02D. @.01 Quality Assurance … @.02 Methods A. Microbiological. ~~Methods for~~Microbiological laboratory methods generating data to support regulatory decisions~~the analysis of shellfish and shellfish growing or harvest waters~~ shall be: (1) The Approved NSSP Methods validated for use in the NSSP under Procedure XV. of the Constitution, Bylaws, and Procedures of the ISSC and/or cited in the Guidance Documents ~~Chapter II. Growing Areas .14 Approved NSSP Laboratory Tests.~~Chapter III Laboratory @.02 Methods .01 Approved NSSP Laboratory Tests. (2) When there is an immediate or ongoing critical need for a method and no Approved NSSP Method exists, the following may be used: (a) A validated AOAC, Bacteriological Analytical Manual (BAM), or EPA method; (b) An Emergency Use Method pursuant to @.02 D. (1) and (2) below. B. Chemical and Physical. ~~Methods for~~Chemical and physical laboratory methods ~~the analysis of shellfish and shellfish growing or harvest waters~~generating data to support regulatory decisions shall be: (1) The Approved NSSP Methods validated for use in the NSSP under Procedure XV. of the Constitution, Bylaws, and Procedures of the ISSC and/or cited in the Guidance Documents Chapter III Laboratory @.02 Methods .01 Approved NSSP Laboratory Tests. ~~Chapter II. Growing Areas .14 Approved NSSP Laboratory Tests.~~ (2) Results shall be expressed for chemical and physical measurements in standard units and not instrument readings. (3) When there is an immediate or ongoing critical need for a method and no Approved NSSP Method exists, the following may be used: (a) A validated AOAC, BAM, or EPA method; (b) An Emergency Use Method pursuant to @.02 D. (1) and (2) below. C. Biotoxin. ~~Methods~~ Biotoxin laboratory methods ~~for the analysis of shellfish and shellfish harvest waters~~generating data to support regulatory decisions shall be: (1) The Approved NSSP Methods validated for use in the NSSP under Procedure XV. of the Constitution, Bylaws, and Procedures of the ISSC and/or cited in the Guidance Documents Chapter III Laboratory @.02 Methods .01 Approved NSSP Laboratory Tests.~~Chapter II. Growing Areas .14 Approved NSSP Laboratory Tests.~~
13. Public Health Significance	The phrase "generating data to support regulatory decisions…" was introduced to the Laboratory Chapter of the Model Ordinance as part of amendments to @.01A adopted by the Conference at the 2023 Biennial Meeting. During followup discussions, it was pointed out that what constitutes a "regulatory decision" might not be clear to all users of the Model Ordinance, and especially Laboratory Method

	Developers or other users new to the NSSP. In order to add clarity to this Chapter, a definition for the term "Regulatory Decision" is proposed. This definition is intended to be applicable only to the Laboratory Chapter of the Model Ordinance, and is not intended to create any new requirements. Instead, it is intended to serve as a roadmap pointing the user towards all instances already defined within the Model Ordinance where an Approved or Approved Limited Use Laboratory Method is required to be used. Also at the 2023 Biennial Meeting, a new laboratory method, the Male-Specific Coliphage Enumeration in Wastewater by Direct Double-Agar Overlay Method, was adopted into the program. The current text of the Microbiological Lab Methods section in the Model Ordinance only includes language referencing the testing of shellfish and shellfish growing or harvest waters. Proposed modifications are intended to expand the language of these methods sections to include this method for wastewater analysis, and to allow for the analysis of other items besides shellfish and shellfish growing or harvest waters if/when other methods are adopted into the program. Lastly, the Guidance Documents were rearranged following the 2023 Biennial Meeting, and the references to the Methods Table need to be updated to point to the right location within the Guidance Documents.
14. Cost Information	N/A

Proposal No. 27-154

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative

Submitter	Tod Leighfield and Kelly Cusick
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Proposal Subject	NSSP Receptor Binding Assay Paralytic Shellfish Poisoning Checklist
Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter III. Laboratory, .01 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists, 11. Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP) Checklist
Text of Proposal/ Requested Action	The requested action is to update the receptor binding assay (RBA) for paralytic shellfish poisoning (PSP) lab evaluation checklist.
Public Health Significance	A comprehensive review of the RBA for PSP checklist was conducted using data from both the RBA matrix extension study and workflow feedback from the Washington State Department of Health. Data from 107 assay runs were analyzed to identify procedural elements relevant to the existing checklist and provided the basis for targeted revisions to the checklist, including removing redundant requirements, clarifying evaluation criteria, and adding necessary procedural detail. The proposed revisions are based on AOAC Official Method 2011.27. In addition, supplemental validation data from the ISSC submitted matrix extension study which demonstrated the applicability of the RBA across multiple shellfish species, support updating the current NSSP RBA lab evaluation checklist to reflect current laboratory practices. Adoption of the revised checklist will improve the facilitate uniform implementation across participating laboratories while maintaining rigorous standards for analytical quality, regulatory oversight, and protection of public health.
Cost Information	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240- 402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
Receptor Binding Assay for Paralytic Shellfish Poisoning (RBA PSP)

LABORATORY:		
ADDRESS:		
TELEPHONE:		**FAX:**
EMAIL:		
DATE OF EVALUATION:	**DATE OF REPORT:**	**LAST EVALUATION:**
LABORATORY REPRESENTED BY:		**TITLE:**
LABORATORY EVALUATION OFFICER:		**SHELLFISH SPECIALIST:**
OTHER OFFICIALS PRESENT:		**TITLE:**

Items which do not conform are noted by: Conformity is noted by a "√"

C- Critical K - Key O - Other N/A- Not Applicable

PART I – QUALITY ASSURANCE

CODE	REF		ITEM
		1.1 Quality Assurance (QA) Plan	
K	1, 2, 3		1.1.1 Written Plan: (check √ those items which apply).
			a. Organization of the Laboratory.
			b. Staff training requirements. Training must include radiation lab safety.
			c. Standard operating procedures (SOPs).
			b. Internal quality control measures for equipment, calibration, maintenance, repair, performance and rejection criteria established.
			c. Laboratory safety. Radiation safety practices (e.g., handling and disposal) must be included.
			d. Internal performance assessment.
			e. External performance assessment.
C	**2**		**1.1.2 The QA plan is implemented.**
		1.2 Educational/Experience Requirements	
C	**State's Human Resources Department**		**1.2.1 In state/county laboratories, the supervisor meets the state/county educational and experience requirements for managing a public health laboratory.**
K	State's Human Resources Department		1.2.2 In state/county laboratories, the analyst(s) meet the state/county educational and experience requirements for processing samples in a public health laboratory.
C	**USDA Microbiology & EELAP**		**1.2.3 In commercial/private laboratories, the supervisor must have at least a bachelor's degree in microbiology, biology or other appropriate discipline with at least two years of laboratory experience.**
K	USDA Microbiology & EELAP		1.2.4 In commercial/private laboratories, the analyst(s) must have at least a high school diploma and at least three months of experience in laboratory sciences.
C	**6**		**1.2.5 Training regarding radiation laboratory safety, handling and disposal practices is documented and records are maintained.**

C	**15**		**1.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) or equivalent state license for the use of tritiated saxitoxin in this assay. Alternatively, the laboratory ~~uses less than 50 µCi per year and~~ adheres to the American Radiolabeled Chemicals (ARC) NRC exemption status requirements.**
		1.3 Work Area	
O	2		1.3.1 The work area is adequate for the workload and storage.
K	2		1.3.2 The work area is clean and well lighted.
K	2		1.3.3 The work area has adequate temperature control.
O	3		1.3.4 All work surfaces are nonporous, easily cleaned and disinfected.
C	**3,4**		**1.3.5 The work area is located in an appropriate space designated for low-level radiation work. Radioactive materials are only handled and manipulated in designated areas which are clearly identified and labeled accordingly.**
		1.4 Laboratory Equipment	
C	**4**		**1.4.1 Any lab equipment that ~~may~~ come into contact with ~~[~~3H-~~H~~ STX at any point in the preparation or assay procedures must be specially labelled and must remain in the work area designated for low-level radiation work.**
O	5		1.4.2 The pH meter has a standard accuracy of 0.1 pH units.
K	7		1.4.3 The pH electrodes being used consist of a pH half cell and reference half cell or equivalent combination electrode/triode free from silver/silver chloride (Ag/AgCl) or contains an ion exchange barrier to prevent the passage of silver (Ag) ions into the substance being measured.
K	3, 8		1.4.4 The pH meter is calibrated daily when in use. Results are recorded and records maintained.
K	1		1.4.5 The effect of temperature on the pH has been compensated for by an ATC probe, use of a triode, or by manual adjustment.
K	1		1.4.6 The pH meter manufacturer instructions are followed for calibration, or a minimum of two (2) standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, the first must be near the isopotential point (pH 7) and the second near the expected sample (i.e., pH 4 or pH 10). Standard buffer solutions are used once and discarded.
O	9		1.4.7 Electrode acceptability is determined daily or with each use by the millivolt procedure or through determination of the slope.

K	6		1.4.8 pH paper in the appropriate pH range (i.e., 1-5), if used, measures accurately to a minimum of 0.5 pH units over the covered pH range.
K	6		1.4.9 The differing sensitivities in weight measurements required by the various steps in the assay are met by the balance(s) being used. a. To prepare Phenyl methylsulfonyl fluoride solution (PMSF), the balance used must have a sensitivity of at least 0.001 gram at a load of 1 gram. b. For sample extraction, the balance used must have a sensitivity of at least 0.1 gram at a load of 100 grams. c. For MOPS buffer preparation, the balance used must have a sensitivity of at least 0.01 gram at a load of 100 grams.
K	1, 3		1.4.10 Balance calibrations are checked monthly according to manufacturer's specifications using NIST Class S or ASTM Class 1 or 2 weights or equivalent. The accuracy of the balance is verified at the weight range of use.
K	2		1.4.12 Refrigerator temperatures are maintained between 0 and 4 °C. Freezer security for [^{3}H~~H~~3H-]~~]~~STX and cold STX must meet state and federal requirements for these materials.
K	1		1.4.13 Refrigerator temperatures are monitored at least once daily on workdays. Results are recorded and records maintained.
C	**4, 6, 10**		**1.4.14 Freezer temperature used to store [3H-^{3}H]~~]~~ STX standard and~~,~~ rat brain membrane tissue preparation is at -~~8~~70 °C or below. The freezer temperature used to store~~,~~ interassay calibration standard (QC check) and archived shellfish tissue homogenate is maintained at -~~80~~ 20 °C or below. Freezer security for [3H-^{3}H]~~]~~STX and cold STX must meet state and federal requirements for these materials.**
K	6, 10		1.4.15 Freezer temperature used for all other purposes is maintained at -20 °C or below.
O	1		1.4.16 Freezer temperature is monitored at least once daily on workdays. Results are recorded and records maintained.
O	8		1.4.17 All glassware is clean.
C	**3**		**1.4.18 An alkaline or acid-based detergent is used for washing glassware/labware.**
C	**1**		**1.4.19 With each load of labware/glassware washed, the contact surface of several dry pieces from each load are tested for residual detergent (acid or alkali as appropriate) with aqueous 0.04% bromothymol blue (BTB) solution. Results are recorded and records maintained.**

C	**6**		**1.4.20 Micropipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded and records are maintained.**
C	**11**		**1.4.21 Scintillation counter is serviced according to manufacturer specifications and calibrated annually. Results are recorded and records maintained.**
C	**4**		**1.4.22 Minimum radiation safety equipment and protocols include the following: An area survey ~~wipe test~~ is conducted in the radiation work area as described in the QA plan. Results are recorded and records maintained.**
		1.5 Reference Solution Reagent Storage, Preparation and Security	
C	**12**		**1.5.1 ~~[~~^{3}~~H~~3H-~~]~~ STX standard is stored in a freezer at -~~8~~70 °C or below.**
C	**10**		**1.5.2 ~~Concentration of ^{3}[H]STX standard is calculated from the lot information provided by the supplier with each batch.~~ A working standard of tritiated saxitoxin is prepared such that the highest concentration ('top' of the standard curve) produces a response of at least 1,000 CPM.**
K	6		1.5.3 Unopened diHCl STX standard ~~may be~~is stored either at room temperature or refrigerated.
C	**10**		**1.5.4 ~~Preparation~~ Prepare~~of~~ 100 mM MOPS/100 mM choline chloride, pH 7.4 as the assay buffer. Store assay buffer at 4 °C and must be maintained ice cold while in use for the duration of the assay. ~~includes the following:~~** **~~a. 100 mM MOPS/L.~~** **~~b. 100 mM choline chloride/L.~~** **~~c. pH adjustment to 7.4 with NaOH.~~** **~~d. Refrigerated storage at 4 °C.~~** **~~e.~~a. ~~Maintained ice cold while in use.~~**
C	**10**		**1.5.6 ~~1.5.6~~ Bulk standard curve dilutions are prepared and stored at 4~~ °C for up to one (1) month.**
C	**10**		**1.5.7 Interassay calibration standard (QC check): Reference standard STX (1.8 x 10^{-8} M STX). For long-term storage, keep at -~~8~~70°C or below; for routine use (up to one month), store at 4°C.**

K	1		1.5.8~~7~~ Reagent water is distilled or deionized *(circle appropriate choice)* and is analyzed monthly for the following criteria, with all results recorded and records maintained: a. Exceeds 0.5 megohm-cm resistivity (2 megohm-cm in-line) or less than 2.0 µSiemens/cm conductivity at 25 °C *(circle appropriate choice).* b. Residual chlorine is at a non-detectable level (<0.1 ppm). Specify method of determination_____ c. Water contains <100 CFU/mL using the heterotrophic plate count method.
	1.6 Rat Brain Membrane Tissue Preparation and Storage		
C	**10**		**1.6.1 MOPS/choline chloride/phenyl methylsulfonyl floride (PMSF), pH 7.4 is used in preparing rat brain membrane tissue. PMSF is added to 100 mM MOPS/ 100 mM choline chloride assay buffer fresh on the day of use**.
C	**10**		**1.6.2 The cerebral cortex of 6-week old Sprague-Dawley rats is homogenized in ~~used in membrane tissue preparations, placed in~~ iced MOPS/choline chloride/PMSF buffer (pH 7.4; 1 brain/12.5 mL). No ~~and homogenized with no~~ visible chunks remained~~ing~~ in the homogenate.** a. **This procedure is repeated ~~This procedure is repeated~~ until twenty (20) rat brains have been processed.** b. The homogenized tissue from the twenty (20) brain cortices are centrifuged at 20,000xg for 15 minutes at 4°C.
~~C~~	~~10~~		~~**1.6.3 The homogenized cerebral cortex tissue from the twenty (20) rat brain cortices is pooled and centrifuged at 20000 x g for 15 minutes at 4 °C.**~~
K	10		~~1.6.4 The pellet of the centrifuged rat brain tissue preparation is fully resuspended in ice cold MOPS/choline chloride/PMSF buffer (up to 10 mL/brain).~~
K	10		1.6.3~~5~~ All rat cerebral cortex pellets ~~tissue~~ ~~The resuspended rat brain tissue preparations~~ are resuspended~~,~~ pooled, ~~centrifuged,~~ and blended to obtain ~~and the~~a homogenous membrane preparation. ~~centrifuge tubes used for these preparations are rinsed with a small amount of MOPS/choline chloride/PMSF buffer to recover all the rat brain tissue.~~
~~K~~	~~10~~		~~1.6.6 The total volume of the pooled rat brain tissue is adjusted to 200 mL with MOPS/choline chloride/PMSF buffer while iced.~~
~~K~~	~~10~~		~~1.6.7 The iced contents of the pooled rat brain tissue are blended using a Polytron at 70% power or a small hand- held blender at low speed~~

			~~for 20 seconds to obtain a homogeneous membrane tissue preparation.~~
C	10		1.6.4~~8~~ The pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -70 °C. ~~Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -870 °C for up to six (6) months~~
	1.7 Rat Brain Membrane Tissue Protein Receptor Determination		
C	**1**		**1.7.1 The protein/receptor concentration of the rat brain membrane tissue preparation is determined for each new batch. Analyst must refer to the protocol in RBA for PSP Single Laboratory Validation study (13-114).**
C	**10**		**1.7.2 The dilution of the protein/receptor concentration of the rat brain membrane tissue preparation needed to obtain a working stock of 1 mg/mL is determined with the final working concentration determined by either titration to meet the required 'top' value or by generating a tissue linearity curve.~~.~~**
K	10		1.7.3 Dilutions of the protein/receptor concentration of the rat brain ~~.~~membrane tissue preparation of less than 1:4 ~~are~~is not used as they will~~ may be too viscous (filtration wells may become~~ clog ~~ge~~during the final filtration step of the assay~~).~~ ~~.~~
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS - RBA			
	2.1 Collection and Transportation of Samples		
C	**5**		**2.1.1 A representative sample of shellfish is collected.**
K	5		2.1.2 Shellfish samples are collected in clean, waterproof, puncture resistant containers, loosely sealed.
K	5		2.1.3 Shellfish samples are labeled with the collector's name, type of shellstock, the source or harvest area, sampling station, time, date and place (if applicable) of collection.
C	**5**		**2.1.4 Immediately after collection, shellstock samples are placed in dry storage (ice chest or equivalent) which is maintained between 0 and 10°C with ice or cold packs for transport to the laboratory.**

K	6, 13		2.1.5 Time from collection to initiation of the extraction must ~~should~~ not exceed 24 hours. However, if significant delays are anticipated or if they occur, the laboratory has an appropriate contingency plan in place to handle these samples. For samples shipped live in accordance with 2.1.4, the contingency plan ensures samples remain within allowable temperature tolerances and animals are alive upon receipt. The contingency plan also addresses field and/or laboratory processing that ensures the integrity of the sample or extract until initiation of the assay. For example, samples are washed, shucked, drained and processed as follows: a. refrigerated or frozen until extracted; b. homogenized and frozen until extracted; or c. extracted, the supernatant decanted, and refrigerated or frozen until assayed.
	2.2 Preparation of Samples for Analysis – Homogenization		
C	**5, 6**		**2.2.1 At least 12 animals are used per sample, or the laboratory has an appropriate contingency plan for dealing with non-typical species of shellfish or collection conditions.**
O	5		2.2.2 The outside of the shell is thoroughly cleaned with fresh water.
O	5		2.2.3 Shellstock are opened by cutting the adductor muscles.
O	5		2.2.4 The inside surfaces of the shells and meats are rinsed with fresh water to remove sand or other foreign material.
O	5		2.2.5 Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	**5**		**2.2.6 Damage to the body of the mollusk is minimized in the process of opening.**
O	5		2.2.7 Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.
K	5		2.2.8 Pieces of shell and drainage are discarded.

C	**5, 6**		**2.2.9 Meats are blended at high speed until homogenous (60 – 120 seconds), using the following criteria:** **a. Freshly drained/air dried meats are placed into the blender for homogenization.** **b. Previously frozen shucked, rinsed, and drained meats are completely thawed, then placed in the blender with all freeze thaw liquid for homogenization.** **c. Previously frozen homogenates are completely thawed then placed in the blender with all freeze-thaw liquid for homogenization.**
K	6, 13		2.2.10 Homogenates are ~~should be~~ extracted immediately. If homogenates must be stored, ~~they should be frozen~~freeze the homogenates.
	2.3 Preparation of Samples for Analysis – Extraction		
K	5, 10		2.3.1 0.1 ~~M~~ N HCl is used for extractions.
K	5, 10		2.3.2 At least five (5) grams of tissue +/- 0.1g is extracted using a 1:1 mass to volume ratio of 0.1 M HCl. a. Homogenate/acid mixture is stirred thoroughly before boiling to completely mix the contents.
C	**10**		**2.3.3 The pH of the sample is checked and adjusted as necessary to between 3.0– 4.0. If needed, the adjustment of the pH is accomplished by dropwise addition of either 1 N HCl or 0.1 N NaOH, as appropriate.**
~~**C**~~	~~**10**~~		~~**2.3.4 Adjustment of the pH is accomplished by dropwise addition of either 5 N HCl or 0.1 N NaOH, as appropriate, while constantly stirring the sample.**~~
C	**6**		**2.3.4~~5~~ The sample is promptly brought to a boil at 100~~99.0~~ +/- 1.0 °C ~~-~~and gently boiled for 5 minutes.**
O	6		2.3.5~~6~~ The sample is boiled under adequate ventilation (e.g., fume hood).
O	10		2.3.6~~7~~ The sample is allowed to cool to room temperature.
C	**10**		**2.3.~~8~~ 7 The pH of the cooled mixture after boiling is between 3.0 - 4.0, adjusted, if necessary, with the dropwise addition of ~~5~~ 1 ~~M~~ N HCl to lower the pH or 0.1 ~~M~~ N NaOH to raise the pH, as appropriate.~~ ~~while constantly stirring the mixture.~~**
K	5, 10		2.3.~~9~~ 8 The volume of the sample is adjusted to the original (pre-boiling) volume, by adding 0.001N HCl (pH 3 water)..

K	10		2.3.~~10~~ 9 The sample is stirred gently to homogeneity, then treated as follows: a. The sample is allowed to settle to remove particulates, then the supernatant is carefully decanted into a clean container; ~~then~~ or, b. t~~an aliquot of t~~he sample is centrifuged at 3,000 ~~x~~ g for 10 minutes, then the supernatant is carefully decanted into a clean container.
K	6, 10		2.3.~~11~~ 10 The sample extract is analyzed immediately, refrigerated at 4°C in a sealed container for up to 24 hours, or frozen at -20°C.
	2.4 Sample Assay		
K	6		2.4.1 One analyst performs the entire plate set-up for the assay.
K	6		2.4.2 Microtubes containing dilutions and samples are vortexed immediately before dispensing into multiple dilutions prepared in assay buffer. Sample extracts are diluted at least 1:10 prior to performing the assay.~~.~~
K	10		2.4.3 The standard curve consists of at least 7 concentrations (minimum 6 x 10^{-10} M and maximum 6 x 10^{-6} M).~~).~~
			2.4.4 Prepare 3H-STX working solution daily in assay buffer based on the calculation of 3H-STX stock provided by the supplier or titration to reach acceptable 'top' values, which vary between lots.
K	10		2.4.5 3H-STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.
K	10		**2.4.6**~~4~~ The rat brain membrane tissue preparation is diluted from frozen stock daily and is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension. Do not freeze and reuse thawed rat brain membrane preparation after setting up the assay. ~~**The rat brain membrane tissue preparation is diluted from frozen stock daily and is kept on ice and mixed often during addition to the plate to maintain a homogenous suspensionsetting up the**~~
K	10		2.4.7 ~~5~~ Each day an assay is conducted, a standard curve is required for each plate. An inter-assay QC calibration and reference blank are required for each plate analyzed. ~~Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate~~

			~~lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed.~~
C	**10**		**2.4.8~~6~~ The standard curve, reference blank, interassay QC calibration standard, and test samples are all run in triplicate.**
K	10		2.4.9~~7~~ Assay buffer is added to the plate before any other components of the assay, in order to properly wet the filter membrane. Add the solution into the wells: a. 100 mM MOPS/100 mM choline chloride assay buffer b. STX diHCl standard/QC check/sample extract c. 3H-STX d. Rat brain membrane preparation (ensure the solution is homogeneous) e. Incubate at 4°C
K	10		2.4.10~~8~~ All empty wells of the plate (including any unused wells) are filled with MOPS/choline chloride buffer during vacuum filtration, ~~in order~~ to ensure even pressure ~~and filtration~~ across the plate during filtration. a. Use the vacuum manifold attached to the vacuum pump with an in-line side arm flask to catch filtrate from the filtration process.
C	**10**		**2.4.11 Preparation of the assay for counting: Appropriate scintillation cocktail is used, depending on the type of scintillation counter. Incubate for at least 30 minutes at room temperature in the dark to allow the scintillation cocktail to be evenly dispersed in the standard/sample mixture.** **~~9 Appropriate scintillation cocktail is used, depending on the~~** **a. ~~type of scintillation counter (traditional or microplate).~~**
K~~K~~	4~~10~~		2.4.12 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements. ~~2.4.10 [^{3}H] STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value.~~
~~C~~	**2.5 Data Analysis~~2.4.11 An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate).~~**		
K	10		2.5~~4~~.1~~2~~ ~~Standard curve fitting is calculated using appropriate software program.~~ Perform standard curve fitting using a four-parameter logistic fit (sigmoidal dose response curve with variable slope) with an appropriate software program.

C	10		**2.5.2 Acceptance of Assay Results Criteria:** • **Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0).** • **The relative standard deviation (RSD) of triplicate CPM for samples must be less than 30%.** • **The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM ± 30%).** • **The IC_{50} is in acceptable range (1.75 nM +/- 30%).** • **The calculated 'top' value must be greater than 1,000 CPM.** **If the slope, standard RSD, IC_{50}, QC, and 'top' values falls outside the accepted range (including recounts within 8 hours), assay results are rejected and the assay must be repeated.** • ~~atat the estshould be assessed according to the performing lab's acceptance criteria.2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.~~
C	10		**2.5.3 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample is further diluted and repeated if a quantification is needed otherwise, it can be reported as greater than the calculated 0.2 concentration value.** ~~2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.~~
~~C~~	~~10~~		~~2.4.15 The IC50 is in acceptable range (2.0 nM +/- 30%). If the IC50 is outside this range, the assay results are rejected and the assay must be repeated~~
~~C~~	~~10~~		~~2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated.~~
~~C~~	~~10~~		~~2.4.17 Sample dilutions are quantified only if B/B_0 is between 0.2 – 0.7. If B/B_0 is greater than 0.7, then the sample is reported as below the limit of detection. If B/B_0 is less than 0.2, then the sample should be further diluted and repeated if a quantification is needed.~~

K	4		**2.5.4 Sample toxicity is calculated as follows:** **(nM STX eq) x (sample dilution) x [(210 µL total volume/35 µL sample)] = mM STX eq in extract** **(nM STX diHCl eq in extract) x (1L/1000 mL) x (372 ng/nmol) x (1 µg/1000 ng) = µg STX diHCl eq/mL** **µg STX diHCl equiv./mL x (mL extract/g shellfish) x (1000 g/kg) = µg STX diHCl equiv./kg** ~~2.4.18 Assay materials are cleaned and disposed of in accordance with federal, state, and local requirements.~~
	~~**2.5 Calculation of Sample Toxicity**~~		
C	**10**		**2.5.5 Any value equal to or greater than 80 µg STX diHCl eq/100 g shellfish of sample is actionable.**~~**2.5.1 When more than one dilution falls within B/Bo of 0.2 – 0.7, all wells corresponding to these dilutions are used to calculate sample toxicity.**~~
C	**10**		**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:** ~~**2.5.2 Sample toxicity is calculated as follows:**~~ ~~**(nM STX eq) x (sample dilution) x [(210 µL total volume/35 µL sample] = mM STX eq in extract**~~ ~~**(nM STX diHCl eq in extract) x (1L/1000 mL) x (372 ng/nmol) x (1 µg/1000 ng) = µg STX diHCl eq/mL**~~ ~~**µg STX diHCl equiv./mL x (mL extract/g shellfish) x (1000 g/kg) = µg STX diHCl equiv./kg**~~
~~**C**~~	~~**14**~~		~~**2.5.3 Any value equal to or greater than 80 µg STX diHCl eq/100 g) of sample is actionable.**~~
~~**C**~~	~~**14**~~		~~**2.5.4 Shellfish Program Management is made aware of positive result. Laboratory action to identify positive result is:**~~

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1. American Public Health Association (APHA). 1992. *Standard Methods for Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.

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15. U. S. Nuclear Regulatory Commission Materials, Section 30.18, 10 CFR Part 30, and American Radiolabeled Chemicals Licenses.

16. Interstate Shellfish Sanitation Conference (ISSC). 2013. Single Lab Validation (SLV) Proposal 13-114: *Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination*, Columbia, SC.

LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
RECEPTOR BINDING ASSAY FOR PSP COMPONENT: (Part I-II)	
A. Results	____
Total # of Critical (C) Nonconformities	_____
Total # of Key (K) Nonconformities	_____
Total # of Critical (C), Key (K), and Other (O) Nonconformities	_____

B. Criteria for Determining Laboratory Status of the RBA for PSP:

1. **Conforms Status:** The RBA for PSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a.
   b.
   c.

2. **Provisionally Conforms Status:** The RBA for PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a.
   b.
   c.

3. **Does Not Conform Status**: The RBA for PSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a.
   b
   c.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:
Date:_____

LEO Signature:_____ Date:_____

Appendix A: Data Support for NSSP RBA for PSP Checklist Modifications

A comprehensive review of the protocol components included in the National Shellfish Sanitation Program (NSSP)-approved Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) checklist was conducted using data from the RBA matrix extension study and workflow feedback from the Washington State Department of Health. Data from 107 assay evaluations were analyzed to identify procedural elements that contributed to inconsistencies and delays during laboratory evaluations. The results of this review provided the basis for targeted revisions to the checklist, including removing redundant requirements, clarifying evaluation criteria, and adding necessary procedural detail. The proposed revisions are based on AOAC Official Method (OM) 2011.27 because the protocol used in the Single Laboratory Validation Study for the RBA is identical to the protocol specified in AOAC OM 2011.27. In addition, advances in instrumentation and supplemental validation data from the matrix extension study, which demonstrated the applicability of the RBA across multiple shellfish species, support updating the current NSSP RBA evaluation checklist to reflect current laboratory practices. For instance, the traditional scintillation counter is outdated and laboratories are using microplate scintillation counters to complete the assays. Adoption of the revised checklist will improve the consistency and efficiency of laboratory evaluations, reduce unnecessary administrative burden, and facilitate uniform implementation across participating laboratories while maintaining rigorous standards for analytical quality, regulatory oversight, and protection of public health.

In Section 1.6.8 in the current NSSP RBA for PSP checklist:

> *"1.6.8. Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -80 °C for up to six (6) months."*

Response:

The current NSSP RBA for PSP evaluation checklist does not include sufficient procedural detail in several areas, requiring laboratories to routinely consult AOAC OM 2011.27 to ensure proper implementation of the assay. One area of inconsistency is the recommended storage duration for rat brain membrane preparations. While AOAC OM 2011.27 states, *"This preparation is stable for at least six (6) months,"* the NSSP RBA checklist contains different wording, *"stored at -80 °C for up to six (6) months,"* creating uncertainty regarding the acceptable storage period for membrane preparations.

To evaluate whether extended storage affects membrane preparation quality, six rat brain membrane preparations stored at −80 °C and representing preparation dates from 2005 through 2024 were evaluated in 2026 using the RBA. The assay was performed on two 96-well plates using a standard curve ranging from 0 to 1,000 nM saxitoxin. The membrane preparations evaluated were from September 2024 (analyzed on both plates to assess inter-plate reproducibility), May 2023, June 2022, November 2018, January 2017, and July 2005.

Parameters generated from the four-parameter sigmoidal dose-response curve, including the 'bottom', 'top', $LogIC_{50}$, Hill slope, and IC50, were consistent across all membrane preparations regardless of storage duration (Table 1). Counts per minute (CPM) for the three replicates at each standard concentration also showed no statistically significant differences among preparations (two-factor ANOVA, $p = 0.92$), indicating that extended storage at −80 °C did not adversely affect membrane preparation performance (Figure 1).

All membrane preparations produced IC_{50} values within the current acceptance criterion of 2.0 nM ± 30%, with the exception of the May 2023 preparation (1.32 nM). However, evaluation of 107 assay plates generated during the Washington State Department of Health Matrix Extension Study across multiple shellfish species demonstrated that assays with IC_{50} values as low as 1.25 nM continued to produce acceptable analytical performance (Figure 2, and revisions will additionally be proposed for section 2.4.15). Within the context of this larger dataset, the May 2023 membrane preparation remained suitable for use.

These data demonstrate that rat brain membrane preparations stored at −70 °C remain stable and suitable for use well beyond six months. Accordingly, the proposed revision removes the six-month storage limitation from the NSSP RBA for PSP evaluation checklist. If an expiration date is needed, up to ten years is adequate for the checklist. This change aligns the checklist with available performance data, eliminates ambiguity between the NSSP checklist and AOAC Official Method 2011.27, provides laboratories with greater operational flexibility, and maintains assay reliability, data quality, and regulatory confidence.

Suggested revision:

"1.6.4. Two (2) mL/tube of the pooled, homogeneous rat brain membrane tissue preparation is aliquoted into cryovials, frozen and stored at -70 °C."

Table 1. Summary of dose–response parameters across six time points. Values for 'bottom', 'top', $LogIC_{50}$, Hill Slope, and IC_{50} (nM) are shown for September 2024 (twice), May 2023, June 2022, November 2018, January 2017, and July 2005, along with their average, standard deviation, and percent coefficient of variation (%CV).

	Bottom	**Top**	**$LogIC_{50}$**	**Hill Slope**	**IC_{50} (nM)**
2024, September	28.33	2,733	-8.79	0.89	1.60
2024, September	41.18	2,430	-8.66	1.05	2.19
2023, May	48.85	2,326	-8.88	0.91	1.32
2022, June	65.06	2,449	-8.72	1.00	1.89
2018, November	62.75	2,423	-8.69	1.05	2.05

2017, January	70.17	2,502	-8.62	1.03	2.4
2005, July	68.77	1,808	-8.73	1.08	1.85
Average	55.02	2,381.57	-8.73	1.00	1.90
Standard Deviation	15.92	282.46	0.09	0.07	0.36
%CV	28.94	11.86	-0.99	7.47	19.04

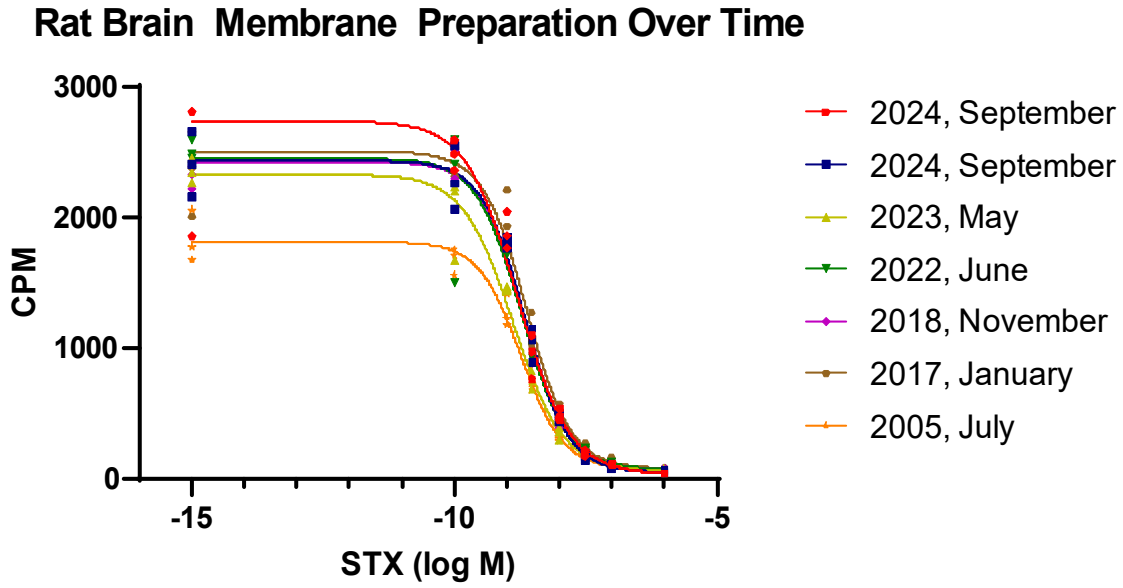


Figure 1. Standard curves for receptor binding assay across six rat brain membrane preparations. Curves were generated using three replicates at concentrations of 1,000, 100, 30, 10, 3, 1, 0.1, and 0 nM on a single plate for each preparation: September 2024 plate 1 (red circles), September 2024 plate 2 (blue squares), May 2023 (yellow triangles), June 2022 (green triangles), November 2018 (purple diamonds), January 2017 (brown circles), and July 2005 (orange stars).

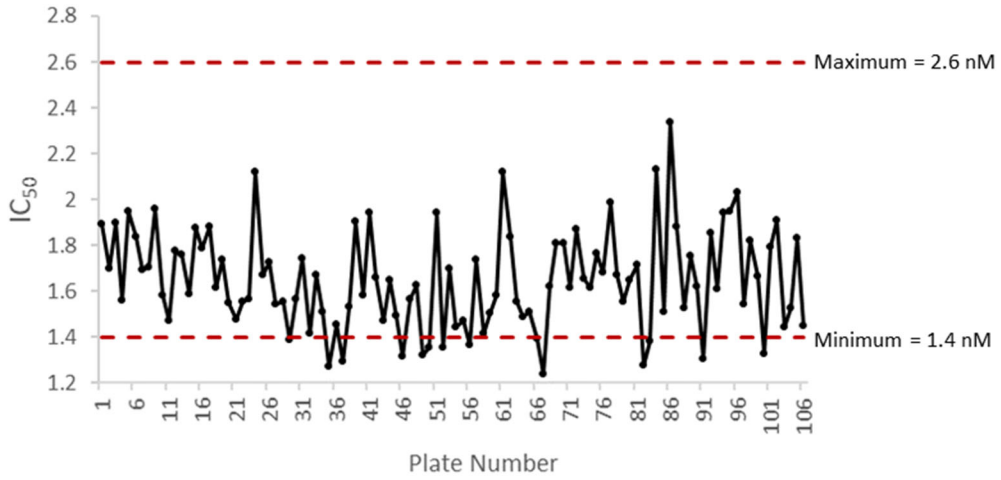


Figure 2. IC_{50} results (nM) for the assay calibration standards performed on 107 plates. IC_{50}: 1.65 ± 0.22 nM (mean and standard deviation (SD)). The minimum and maximum concentration for IC_{50} is at 1.4 and 2.6 nM, respectively.

In Section 2.4.4 in the current NSSP RBA for PSP checklist:

"2.4.4 The rat brain membrane tissue preparation is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension

Response:

The current NSSP RBA for PSP checklist does not specify the handling of the rat brain membrane. To improve consistency in the interpretation to optimize assay performance, additional details greater clarity and consistency without altering the approved method or its established performance requirements.

Suggested Revision:

"2.4.6 The rat brain membrane tissue preparation is diluted from frozen stock daily and is kept on ice and mixed often during addition to the plate to maintain a homogenous suspension. Do not freeze and reuse thawed rat brain membrane preparation after setting up the assay."

In Section 2.4.5 in the current NSSP RBA for PSP checklist:

"2.4.5 Each day an assay is conducted, a standard curve is required. However, filter plates of the same lot must be used if the assay requires multiple plates to accommodate all samples. If the filter plate lot changes over the course of a day, a new standard curve must be performed for the new lot of filter plates. An inter-assay QC calibration and reference blank are required for each plate analyzed."

Response:

The current NSSP RBA for PSP evaluation checklist requires a single standard curve per day, provided the same lot of filter plates is used. However, analysis of assay performance demonstrated substantial variability in standard curve characteristics between individual plates,

even when the same reagents, filter plate lot, and assay conditions were used (Figure 2). This plate-to-plate variability can influence calculated assay parameters, including IC_{50}, 'top', and 'bottom' (Figure 3 and 4), if results are normalized to a standard curve generated on a different plate. Requiring a standard curve on every plate to account for this variability by ensuring that each set of samples is calibrated using plate-specific assay performance. This revision improves the accuracy and reliability of sample quantification, aligns with our long-standing laboratory practices, and provides a more robust assessment of assay validity without increasing analytical complexity.

Suggested Revision:

"2.4.7 Each day an assay is conducted, a standard curve is required for each plate. An inter-assay QC calibration and reference blank are required for each plate analyzed."

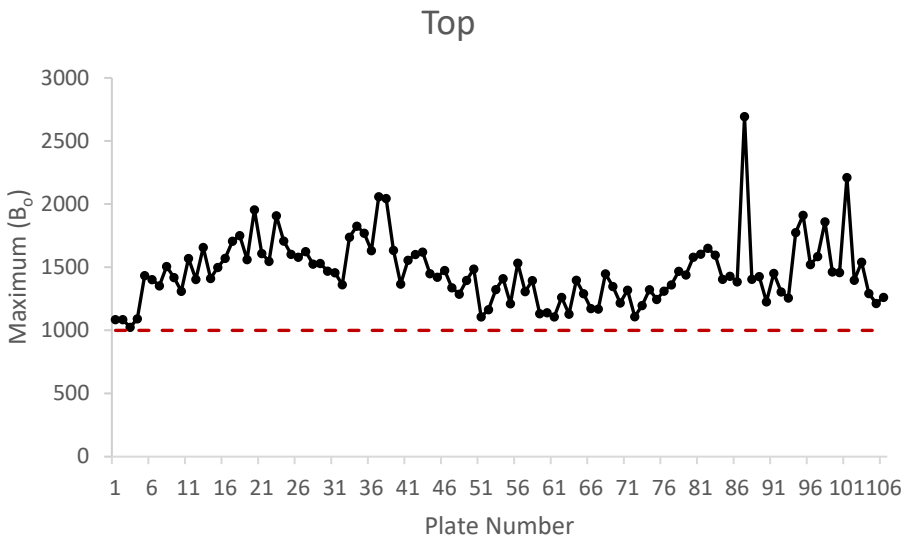


Figure 3. 'Top' value results in counts per minute (CPM) for the assay calibration standards performed on 107 plates. 'Top': 1,464 ± 257 CPM (mean and SD). The minimum criteria for 'top' are at 1,000 CPM as indicated by the red dashed line.

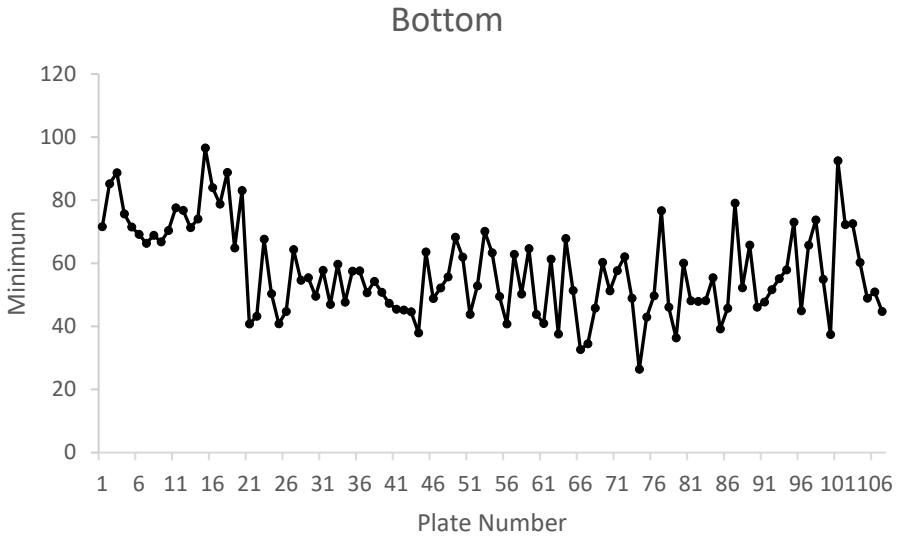


Figure 4. Bottom value results in counts per minute (CPM) for the assay calibration standards performed on 107 plates. Bottom: 57.5 ± 14.3 CPM (mean and SD).

In Section 2.4.10 in the current NSSP RBA for PSP checklist:

"2.4.10. [3H]STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value."

This requirement was established when laboratories routinely used tube-based liquid scintillation counters. However, many laboratories now use MicroBeta plate readers exclusively, and not all MicroBeta configurations include a standalone CPM measurement function. Radioligand performance is already verified through the assay acceptance criteria specified in AOAC OM 2011.27, including standard curve performance and other quality control parameters. Therefore, a separate CPM verification is unnecessary and may create compliance issues for laboratories using the most common instrumentation.

Suggested Revision:

"2.4.5. 3H-STX working solution is checked for counts per minute (CPM) and is consistent within 15% of the expected value."

In Section 2.4.9 and 2.4.11 in the current NSSP RBA for PSP checklist:

"2.4.9 Appropriate scintillation cocktail is used, depending on the type of scintillation counter (traditional or microplate)."

"2.4.11. An appropriate dark adaptation interval is employed, based on type of scintillation counter (traditional or microplate)."

Response:

For Section 2.4.9, this requirement was established when laboratories routinely used tube-based liquid scintillation counters for assay plate counting. However, many laboratories now use MicroBeta plate readers exclusively. Because the appropriate scintillation cocktail may

vary depending on the counting instrument, laboratories should use the scintillation cocktail recommended for the specific instrument being used. Revising this requirement would better reflect current laboratory practices and ensure compatibility between the scintillation cocktail and counting instrument without altering the approved analytical method or its performance requirements.

For Section 2.4.11, the current NSSP RBA for PSP checklist is inconsistent with AOAC OM 2011.27 regarding the incubation period prior to plate counting. While AOAC OM 2011.27 states, *" Allow to incubate 30 min at room temperature. Place the plate in a counting cassette…,"* the NSSP RBA checklist contains different wording, *"an appropriate dark adaptation interval is employed…,"* Incorporating the 30-minute incubation period specified in AOAC OM 2011.27 into the NSSP checklist would eliminate this ambiguity and promote consistent implementation of the approved analytical method. A 30-minute incubation period provides sufficient time for the scintillation cocktail to become uniformly dispersed with the samples and standards in the wells. Therefore, specifying the incubation time would provide a clear and consistent procedural requirement while maintaining the established assay performance requirements.

To assess the effect of read time on assay performance, a standard curve (0 – 1000 nM, triplicate) was analyzed repeatedly on a MicroBeta plate reader for every 10 minutes over 17.5 hours. The 'top' value (0 nM), which reflects total binding and serves as a key assay validity parameter, was compared to AOAC-recommended 30-minute reference. An incubation time of 30 minutes is sufficient time for the scintillant cocktail to become uniformly dispersed with samples/standards in the wells, therefore time points at 0 - 20 minutes were excluded from the statistical analysis. The remaining data were assessed for normality at each time point using the Shapiro–Wilk test. As the data were found to be normally distributed, parametric testing was applied. A one-sample t-test was conducted at each time point to determine whether the mean difference in CPM significantly deviated from zero (i.e., from the 30-minute reference). The resulting p-values were adjusted for multiple comparisons using the Benjamini–Hochberg (BH) method. Statistical analysis showed no significant change in the 'top' value until 590 minutes (9 hours and 50 minutes) after incubation ($p_{adj} < 0.05$, Figure 5). Although the mean 'top' value decreased from 1,543 CPM at the 30-minute to 1,289 CPM at 590 minutes, it remained above the above minimum criterion of 1,000 CPM, indicating that the assay performance was maintained.

Suggested Revision: ***(Section 2.4.9 and 2.4.11 are combined.)***
"2.4.11 Preparation of the assay for counting: Appropriate scintillation cocktail is used, depending on the type of scintillation counter. Incubate for at least 30 minutes at room temperature in the dark to allow the scintillation cocktail to be evenly dispersed in the standard/sample mixture."

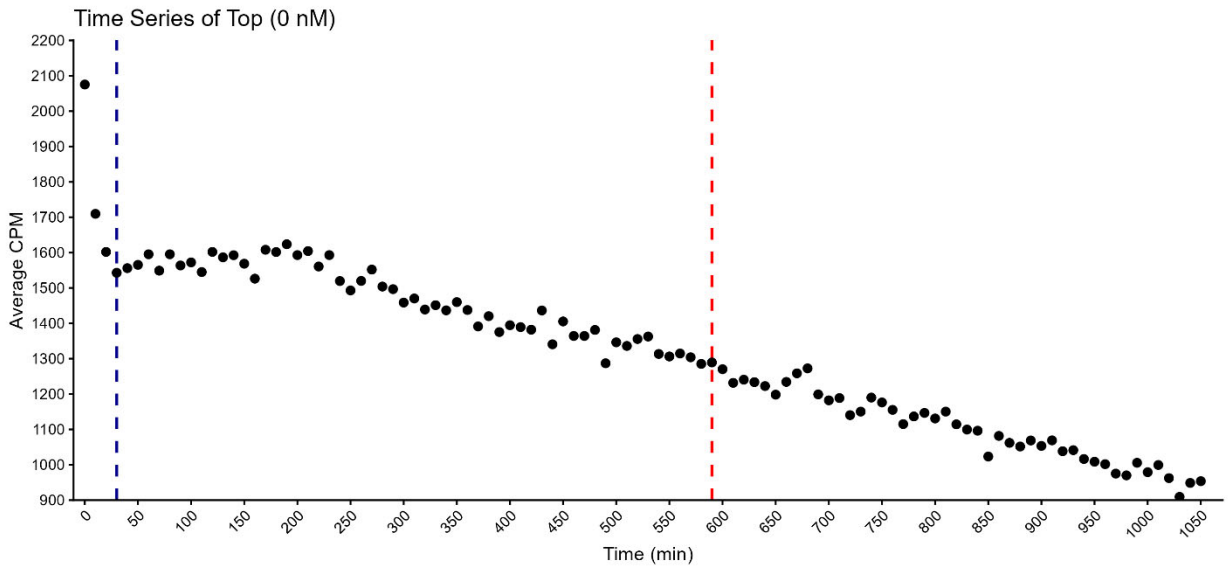


Figure 5. A time series plot of the 'top' value (0 nM) of a 96-wells plate over 1,050 minutes period with the averaged CPM recorded in 10 minutes intervals. The blue dashed line indicates the baseline reference at 30 minutes (1,543 CPM), while the red dashed line marks the 590 minutes time point (1,289 CPM) at which the 'top' value significantly deviated from baseline (1543 CPM; $p_{adj} < 0.05$; t-test with Benjamini-Hochberg adjustment).

In Sections 2.4.13 through 2.4.16, the current NSSP RBA for PSP Checklist states:

> *"2.4.13 Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0). If the slope falls outside these criteria, the assay results are rejected and the assay must be repeated.*
>
> *2.4.14 The relative standard deviation of triplicate CPM for standards and samples must be less than 30%. If greater than 30%, the assay results are rejected and the assay must be repeated.*
>
> *2.4.15 The IC_{50} is in acceptable range (2.0 nM +/- 30%). If the IC_{50} is outside this range, the assay results are rejected and the assay must be repeated*
>
> *2.4.16 The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM +/- 30%). If the QC check sample is outside this range, the assay results are rejected and the assay must be repeated."*

Response:

For section 2.4.15, the current NSSP RBA for PSP checklist's acceptance criterion for IC_{50} is currently defined as 2.0 nM ± 30% (1.40 nM to 2.60 nM). The proposal requests an update to the IC_{50} acceptance criterion to shift the range to 1.75 nM ± 30% (1.23 nM to 2.28 nM). From 107 completed assays in WADoH laboratory as a part of the matrix extension study,

valid assay plates frequently yield IC_{50} values as low as 1.25 nM, while other valid runs reach up to 2.33 nM. The runs across this entire window consistently pass all internal quality criterions (e.g., 'top', slope, RSD), including QC check sample evaluations and matrix spike recoveries well within the required ± 30% tolerance. Maintaining the historical 2.00 nM target forces an artificial lower cutoff at 1.40 nM, resulting in the false rejection of the assay results. Adjusting the IC_{50} criterion to 1.75 nM ± 30% tolerance structure resolves these unnecessary rejections without expanding or relaxing statistical quality control limits.

The proposed 1.75 nM ± 30% specification represents a scientifically balanced midpoint between our internal method capability and published validation literature. A statistical evaluation of our dataset (n = 107 independent assay runs) demonstrated a mean IC_{50} of 1.65 nM (SD = 0.22 nM), showing that 1.75 nM serves as an ideal operational bridge to the AOAC OM 2011.27 collaborative study system mean of 1.90 ± 0.45 nM (Van Dolah et al., 2012). This range is further supported by foundational binding studies demonstrating that saxitoxin interacts with rat brain synaptosomes with equilibrium dissociation constants (K_d) of 0.8 nM to 2.0 nM (Weigele & Barchi, 1978; Baumgold et al., 1983). Because sample toxicity is quantified on a relative basis against the standard curve processed on that identical 96-well plate, adjusting the target to 1.75 nM maintains full analytical rigor and eliminates false rejections.

While the acceptable threshold for the 'top' value was not included in either the NSSP RBA for PSP checklist or AOAC OM 2011.27, but the 'top' value in a receptor binding assay reflects the maximum signal (CPM) and is critical for ensuring data quality. A minimum threshold of 1,000 CPM is required to maintain an adequate signal-to-noise ratio and a sufficient dynamic range, both of which are essential for accurate curve fitting and reliable IC_{50} determination. This threshold ensures consistent and reproducible results across assays.

The time series of 'top' also demonstrate that plates may be read well beyond 30 minutes, provided that all established assay criteria, including standard curve, IC_{50}, slope, QC, and 'top' value, continue to be met (Figure 5). Laboratories must recognize that assays with lower initial 'top' values may have a shorter acceptable read window and show evaluate assay validity using the established performance criteria. For example, the average 'top' value at the 30-minute reference is 1,200 CPM or lower, the assay may become unreliable in less than 590 minutes, as the 'top' value could fall below the 1,000 CPM threshold. Adding the buffer statement "(including recounts within 8 hours)" helps protect data quality, though analysts must verify that all validity criteria are fully met prior to data acceptance.

Suggested revision:

It is recommended to combine all of the criteria into one section for clarity as they all contribute to the requirements of accepting the assay results.

"2.5.2 Acceptance of Assay Results Criteria:

- *Slope of standard curve is between -0.8 and -1.2 (the theoretical slope is - 1.0).*
- *The relative standard deviation (RSD) of triplicate CPM for samples must be less than 30%.*
- *The inter-assay QC calibration standard (QC check) sample is in the acceptable range (3 nM ± 30%).*

- *The IC_{50} is in acceptable range (1.75 nM +/- 30%).*
- *The calculated 'top' value must be greater than 1,000 CPM.*

If the slope, standard RSD, IC_{50}, QC, and 'top' values falls outside the accepted range (including recounts within 8 hours), assay results are rejected and the assay must be repeated."

References

Weigele, J. B., & Barchi, R. L. (1978). Analysis of saxitoxin binding in isolated rat synaptosomes using a rapid filtration assay. FEBS Letters, 91(2), 310–314. https://doi.org/10.1016/0014-5793(78)81200-2

Baumgold, J., Zimmerman, I., & Bambrick, L. (1983). Appearance of [3H]saxitoxin binding sites in developing rat brain. Developmental Brain Research, 9(3), 405–407. https://doi.org/10.1016/0165-3806(83)90022-8

Van Dolah, F. M., Fire, S. E., Leighfield, T. A., Mikulski, R. J., & Doucette, G. J. (2012). Determination of paralytic shellfish toxins in shellfish by receptor binding assay: Collaborative study. *Journal of AOAC International*, *95*(3), 795–812. https://doi.org/10.5740/jaoacint.CS2011_27

Proposal No. 27-155

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative

Submitter	Hosseinali Safavieh
Affiliation	Sensoreal Inc.
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Fax	Not applicable
Email	roozbeh@sensoreal.com
Proposal Subject	ASP Alert™: Qualitative Detection of Domoic Acid in Mussels and Scallops
Specific NSSP Guide Reference	Section IV Guidance Documents, Chapter III. Laboratory, @.02 Methods, .01 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	Request for approving ASP Alert™ as a qualitative screening tool for the detection of domoic acid in mussels and scallops
Public Health Significance	Domoic acid (DA) is a heat-stable marine biotoxin produced by certain species of diatoms, particularly those in the genus *Pseudo-nitzschia*. It can accumulate in various species of shellfish, especially filter-feeding bivalve molluscs. Domoic acid causes Amnesic Shellfish Poisoning (ASP), which is characterized by symptoms such as nausea, vomiting, diarrhea, abdominal cramps, headache, dizziness, confusion, and in severe cases, short-term memory loss, seizures, or death. These symptoms may occur in humans after consumption of contaminated shellfish such as mussels, clams, scallops, or oysters. The toxic effects of domoic acid are primarily associated with its action as an excitatory neurotoxin that binds to glutamate receptors and causes neuronal damage. Recently, elevated levels of domoic acid have been detected in North American coastlines leading to beach closures and intoxication of several marine mammals such as dolphins and sea lions.
Cost Information	Refer to "ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP" document attached for Sensoreal Inc.

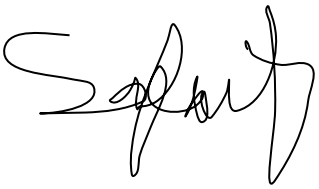
ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	ASP Alert™
Name of the Method Developer	Sensoreal Inc.
Developer Contact Information	Hosseinali Safavieh 6674 Esplanade Avenue (Suite B), Montreal QC H2V 4L5

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for a simple, rapid domoic acid (DA) screening method for shellfish that can be used both in the field and in the laboratory.
2. What is the intended purpose of the method?		Qualitative screening of domoic acid in mussels and scallops
3. Is there an acknowledged need for this method in the NSSP?		**There is a need for simple assays that deliver rapid and accurate results for limited-use screening in marine biotoxin testing.**
4. What type of method? i.e. chemical, molecular, culture, etc.		Lateral flow immunoassays
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		ASP Alert™
Method Scope		Qualitative screening and detection of domoic acid in mussels and scallops
References		Study Report, kit insert/IFU and MSDS included in submission
Principle		Competitive lateral flow immunoassay for detection of domoic acid using methanol-based extraction
Any Proprietary Aspects		Yes, commercial test kit
Equipment Required		Centrifuge, shucking tools, conical tubes, blender, timer, pipettes, Sensoreal Sensoread™ scanner.
Reagents Required		ASP Alert™ test devices and provided reagents inside the kit, 100% methanol, distilled water.
Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2–8°C prior to testing.
Safety Requirements		Used test devices, concical tubes containing slurry or methanol extracts, and pipettes should be handled as though contaminated with domoic acid and disposed of accordingly. Gloves and lab coats should be worn during testing.

Clear and Easy to Follow Step-by-Step Procedure		Step-by-step procedure card included in kit insert and submission
Quality Control Steps Specific for this Method		Each test device includes an internal control line to confirm proper function. A mussel certified reference material (CRM-DA-h; domoic acid concentration = 35.6mg DA/kg) from the National Research Council Canada can be used as positive control.
C. Validation Criteria		
1. Accuracy / Trueness (Probability of Detection)		100% accurate detection of domoic acid was achieved for the following conditions: • Mussel and scallop samples with DA levels at or above the regulatory limit of 20mg DA/kg. • Mussel and scallop samples with DA levels at or less than 7mg DA/kg were all accurately detected as negative.
2. Measurement Uncertainty		Not applicable
3. Precision Characteristics (repeatability and reproducibility)		Not applicable.
4. Recovery		Not applicable
5. Specificity		Not applicable
6. Working and Linear Ranges		Not applicable
7. Limit of Detection		The positive threshold of the ASP Alert™ has been set at 15mg DA/kg.
8. Limit of Quantitation / Sensitivity		Not applicable
9. Ruggedness		No impact on performance was observed across different lots or with a ±2 minute variation in LFA run time.
10. Matrix Effects		No significant matrix effects are observed for mussel and scallop samples.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		The assay demonstrated comparability to the LC-UV reference method when testing naturally incurred domoic acid samples.
D. Other Information		
1. Cost of the Method		Approximately $32/test
2. Special Technical Skills Required to Perform the Method		None. Training on assay operation is provided by Sensoreal Inc..
3. Special Equipment Required and Associated Cost		Centrifuge. Sensoreal Sensoread™ scanner (approximately $4000)
4. Abbreviations and Acronyms Defined		All relative abbreviations and acronyms have been defined in the study report.
5. Details of Turn Around Times (time involved to complete the method)		The test can be operated in approximately 45 minutes, including sample preparation.
6. Provide Brief Overview of the Quality Systems Used in the Lab		
Submitters Signature 	Date: April 14, 2026	
Submission of Validation Data and Draft Method to Committee	Date:	
Reviewing Members	Date:	
Accepted	Date:	
Recommendations for Further Work	Date:	
Comments:		

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery** – The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range** – the range of analyte or measurand concentration over which the method is applied.

REFERENCES:

1. Eurachem Guide, 1998. The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. LGC Ltd. Teddington, Middlesex, United Kingdom.
2. IUPAC Technical Report, 2002. Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Pure Appl. Chem., Vol. 74, (5): 835-855.
3. Joint FAO/IAEA Expert Consultation, 1999. Guidelines for Single-Laboratory Validation of Anilytical Methods for Trace-Level Concentrations of Organic Chemicals.
4. MAF Food Assurance Authority, 2002. A Guide for the Validation and Approval of New Marine Biotoxin Test Methods. Wellington, New Zealand.
5. National Environmental Laboratory Accreditation. , 2003. Standards. June 5.
6. EPA. 2004. EPA Microbiological Alternate Procedure Test Procedure (ATP) Protocol for Drinking Water, Ambient Water, and Wastewater Monitoring Methods: Guidance. U.S. Environmental Protection Agency (EPA), Office of Water Engineering and Analysis Division, 1200 Pennsylvania Avenue, NW, (4303T), Washington, DC 20460. April.

Material Safety Data Sheet (MSDS) for Toxin Alert: ASP Alert Kit

I. Identification

Product name: ASP Alert Test Kit
Product number: ALRT-ASP
Supplier: Sensoreal Inc.
Address: 6674 Esplanade Avenue (Suite B)
Montréal, Quebec, H2V 4L5
Customer Service: sales@sensoreal.com
Web: www.sensoreal.com

II. Composition, Information on Ingredients

Buffer Solution: Phosphate Buffer Saline (PBS)
Diluent: Triton X-100 (CAS# 9036-19-5): Acute toxicity, Oral (Category 4), H302; Skin irritation (Category 2), H315; Serious eye damage (Category 1), H318; Short-term (acute) aquatic hazard (Category 1), H400; Long-term (chronic) aquatic hazard (Category 1), H410
Conjugate: Lyophilized Mouse antibody
Cassettes: nitrocellulose lateral flow dipsticks encased in plastic cassettes

III. Health Hazard Data

Emergency overview: Some reagents are considered hazardous with direct contact or ingested, aquatic toxicity, or corrosive to metals.
HMIS rating:
Health: 4 Flammability: 1 Reactivity: 1
NFPA rating:
Health: 4 Flammability: 1 Reactivity: 1
Routes of entry: Avoid ingestion and inhalation, avoid contact with skin, eyes, and dispose of properly.
Threshold Limit Value (TLV) and source: Data N/A. To the best of our knowledge, the chemical, physical, and toxicological properties of the components of this kit have not been thoroughly investigated.
Potential acute health effects: May cause skin, eye, or respiratory irritation. To the best of our knowledge, the chemical, physical, and toxicological properties of the components of this kit have not been thoroughly investigated. Allergy-prone individuals should be particularly cautious with antibodies or other materials of biologic origin.
Carcinogenic effects data: N/A
Chronic effects of overexposure: Triton X-100: aquatic chronic toxicity
Vapor pressure: Triton X-100: < 0.01 hPa at 20 °C (68 °F)
Solubility in water: All solutions and chemicals readily soluble in water

IV. Emergency and First Aid Procedures

First aid personnel should ensure self-protection, PPE including latex gloves and eye protection.

Swallowing: If any component of this kit is swallowed, drink water (two glasses at most), provided person is conscious. Seek medical attention.
Skin: If any component of this kit contacts the skin, immediately remove contaminated clothing and wash skin with soap and water.
Inhalation: If any component of this kit is inhaled, remove to fresh air. If not breathing, give artificial respiration with medical device and immediately seek medical attention.
Eyes: If any component of this contacts the eyes, rinse the eyes with plenty of water for several minutes. Keep eyes wide open while rinsing and remove contact lenses. Seek medical attention.

V. Fire and Explosion Hazard Data

Flammability: For reagents development of hazardous combustion gases or vapours possible in the event of fire. Storage class (TRGS 510): 11: Combustible liquids (Triton X-100)
Flash points: Triton X-100 (251 °C (484 °F) - closed cup - c.c
Extinguishing media: Use dry chemical. Do not use a solid water stream as it may scatter and spread fire.
Special firefighting procedures: wear self-contained breathing apparatus pressure-demand, MSHA/NIOSH (approved or equivalent) and full protective gear.
Unusual fire and explosion hazards: None known.

VI. Accidental Release Measures

Use appropriate personal protective equipment. Avoid inhalation of dust, vapors, and aerosols. Use appropriate methods to clean up spilled substances and dispose of all waste in accordance with applicable laws. Evacuate the danger area, observe emergency procedures, consult an expert.

VII. Handling and Storage

Storage with desiccant (included) recommended for cassettes. Conjugates and cassettes should be stored at 2-8℃ upon delivery. Other components can be stored at room temperature.

VIII. Exposure Controls / Personal Protection

Wear appropriate gloves, protective clothing and eyewear and follow safe laboratory practices. ACGIH/OSHA Permissible Exposure Limit Data: Not determined

IX. Physical Data

Phosphate Buffer Saline (PBS):
Physical State: Liquids, Solutions
Boiling point: 100.0 °C 212.0 °F at 1,013 hPa
Vapor pressure: N/A
Vapor density: N/A
Specific gravity: N/A
Melting/Freezing point: 0.0 °C (32.0 °F)

Evaporation rate: N/A
Solubility in water: All solutions soluble in water
Conjugates:
Physical State: Dry solutes
Boiling point: CS1 (1390 °C / 2534 °F @ 760 mmHg); DS(> 200 °C > 392 °F at 1,013 hPa)
Vapor pressure: CS1 (1 mbar @ 700 °C); DS (< 0.01 hPa at 20 °C (68 °F))
Vapor density: N/A
Specific gravity: N/A
Melting point: CS1 (318°C 604.4°F); CS2 (176°C(349°F)); DS (Solidification point: 6°C (43°F))
Evaporation rate: N/A
Solubility in water: all solutes soluble in water
Plastic tubes:
Physical State: plastic
Cassettes:
Physical State: plastic, nitrocellulose

X. Stability/Reactivity

Stability and Reactivity: All reagents are stable.
Conditions to avoid: high temperature (>60°C)
Materials to avoid: N/A
Hazardous decomposition products: Triton X-100 (Carbon oxides

XI. Toxicological Information

Irritability: Triton X-100 (skin);
Mutagenicity: N/A
Reproduction toxicity: N/A
Other toxic effects on humans: Triton can cause irritation in case of eye contact, inhalation, or ingestion.

XII. Ecological Information

Do not flush any reagents into surface waters or sewage system.

XIII. Disposal Considerations

Consult local, state, or national regulations for proper disposal.

XIV. Transportation information

Triton X-100:
TDG
UN number: 3082 Class: 9 Packing group: III
Proper shipping name: ENVIRONMENTALLY HAZARDOUS SUBSTANCE, LIQUID, N.O.S. (p-tertiary-Octylphenoxy polyethyl alcohol)
Labels: 9

ERG Code: 171
Marine pollutant: no

IMDG

UN number: 3082 Class: 9 Packing group: III EMS-No: F-A, S-F Proper shipping name: ENVIRONMENTALLY HAZARDOUS SUBSTANCE, LIQUID, N.O.S. (p- tertiary-Octylphenoxy polyethyl alcohol)
Marine pollutant : yes
Marine pollutant : no

IATA

UN number: 3082 Class: 9 Packing group: III
Proper shipping name: Environmentally hazardous substance, liquid, n.o.s. (p-tertiary-Octylphenoxy polyethyl alcohol)

Further information

EHS-Mark required (ADR 2.2.9.1.10, IMDG code 2.10.3) for single packagings and combination packagings containing inner packagings with Dangerous Goods > 5L for liquids or > 5kg for solids.Packages smaller than or equal to 5 kg / L , not dangerous goods of Class 9

XV. Regulatory information

Observe the general safety regulations when handling chemicals. Use CAS# to identify proper handling.

XVI. Other information

This material is sold for in vitro use or research purposes only. It is not for any human or animal therapeutic or clinical diagnostic use.
Read protocol before using the product. Observe the general safety regulations when handling chemicals. Good laboratory practice is the best preventive measure to avoid hazards. The information shall not be taken as being all inclusive and is to be used only as a guide. All materials and mixtures may present unknown hazards and should be used with caution. Since our corporation cannot control the actual methods, volumes, or conditions of use, the Company shall not be held liable for any damages or losses resulting from the handling or from contact with the product as described herein.
This information is prepared with our present knowledge. However, this shall not constitute a guarantee for any specific product features and shall not establish a legally valid contractual relationship.

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Date: 14-April 2025

Laboratory Methods Review & Quality Assurance Committee
Interstate Shellfish Sanitation Conference
209-2 Dawson Road
Columbia, SC 29223-1740

Dear Members of the Committee:

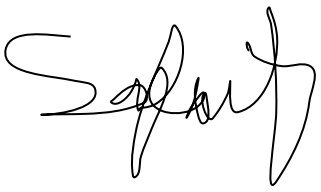
Please find enclosed the validation study report and supporting documentation for ASP Alert™, Sensoreal's rapid test for the detection of domoic acid in shellfish, the causative toxin associated with Amnesic Shellfish Poisoning (ASP) following ingestion.

We respectfully request your review of this submission and consideration of ASP Alert™ for acceptance as an ISSC-approved method for the determination of domoic acid in mussels and scallops. We believe this assay offers important advantages in terms of time-to-result, operational simplicity, and ease of implementation in laboratory and field-support settings, and that its acceptance by ISSC would benefit both the shellfish industry and public health authorities.

ASP Alert™ was developed by Sensoreal Inc. to support rapid screening and timely decision-making in shellfish safety monitoring. We believe the method can help improve response time while maintaining a practical workflow for laboratories processing shellfish samples.

We would be pleased to answer any questions or provide any additional information that may assist the Committee in its review. Thank you very much for your time and consideration.

Sincerely,



Hosseinali Safavieh
CEO, Sensoreal Inc.

Enclosures:
Validation study report
Kit insert
MSDS
Single lab validation checklist
Laboratory Evaluation Checklist – ASP

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Single Laboratory Validation Study of the Sensoreal ASP Alert™ Lateral Flow Assay for the Qualitative Detection of Domoic Acid in Shellfish

Daud Khan[1], Andrew D. Turner[2], Ryan Alexander[2], Shamina Page[2], Karl Dean[2], Maarij Baig[1], Hosseinali Safavieh[1*]

[2]Centre for Environment, Fisheries and aquaculture Sciences. The Nothe, Barrack Road, Weymouth, Dorset DT4 8UB UK

[1]Sensoreal Inc. 6674 Esplanade Avenue (Suite B), Montreal QC H2V 4L5

Corresponding author:
- Hosseinali Safavieh
- Email: roozbeh@sensoreal.com; Phone: +1-4387281243
- Address: 6674 Esplanade Avenue (Suite B), Montreal QC H2V 4L5 Canada
- Submitted: March 2026

1. Introduction

Domoic acid (DA) is a marine toxin produced by certain Pseudo-nitzschia diatoms during harmful algal blooms (HABs) and is the cause of Amnesic Shellfish Poisoning (ASP) in humans[1,2]. ASP became a recognized public health threat after the 1987 outbreak in Canada linked to contaminated mussels, which caused gastrointestinal and serious neurological symptoms, including memory loss[1] and recent outbreak in California has led to intoxication of sea lions and dolphins[3]. Because DA poses significant health risks, regulatory authorities in Canada, the U.S., Europe, and other regions require routine monitoring of bivalve shellfish and generally apply a maximum limit of 20 mg/kg, with harvest closures and market restrictions when levels exceed this threshold[4–7].

Official regulatory testing is typically performed by laboratories using LC-UV-based methods[7]. While suitable for confirmatory and regulatory testing, these methods are often impractical for food business operators because they require specialized equipment, trained personnel, strong QA/QC systems, and relatively long turnaround times. This creates a clear need for simple, rapid, and reliable screening tools that can be used with minimal infrastructure. Lateral flow immunoassays (LFAs) are well suited for this purpose, but many previously marketed rapid tests lacked comprehensive, publicly available validation data, making fitness-for-purpose difficult to assess.

This study addresses that gap by evaluating ASP Alert™, a Canadian-developed LFA by Sensoreal, for the qualitative detection of domoic acid in bivalve mollusks. The validation was

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conducted solely in accordance with the Interstate Shellfish Sanitation Conference (ISSC) single-laboratory validation (SLV) study requirements and assessed key performance characteristics including Probability of Detection (POD), ruggedness, and comparability to the LC-UV reference method[8]. The shellfish species chosen are mussels and scallops. Together, these evaluations provide a comprehensive assessment of ASP Alert™ for routine shellfish safety monitoring.

2. Principle of the Method

Sensoreal ASP Alert™ is a rapid lateral flow immunoassay (LFA) for the qualitative detection of domoic acid (DA) in bivalve mollusks. The assay uses a competitive binding format. DA is extracted from homogenized shellfish tissue using 50% methanol, then the extract is diluted and incubated with a lyophilized antibody–gold nanoparticle conjugate.

If DA is present, it binds to the conjugate antibodies and reduces the amount of free antibody–gold complex available to bind at the test line. The mixture is then added to the lateral flow cassette, where it migrates across a membrane containing immobilized DA-protein at the test line. As a result, test line intensity is inversely related to DA concentration (higher DA = fainter test line). A control line appears in all valid tests to confirm proper assay function.

Results are read using the Sensoreal SensoRead™ scanner, which reports a numerical T/C ratio and a qualitative classification (High, Medium, or Low). In this validation study, High results were considered positive, while Low and Medium were considered negative unless otherwise specified. The assay is designed for users with basic laboratory skills (shellfish handling, homogenization, and simple pipetting) and is intended as a screening tool to support harvest-area closure decisions and help prevent contaminated shellfish from reaching the market.

3. Intended Use

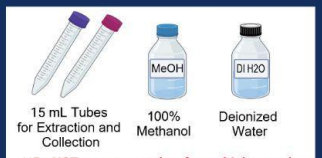
ASP Alert™ is intended for the qualitative screening of shellfish for domoic acid (DA), with a positive result indicating samples at or above the toxic/regulatory threshold of 20 mg/kg (20 ppm). The test kit is designed for use by quality control personnel, laboratory staff, and other trained users familiar with handling shellfish samples and performing basic sample preparation and pipetting procedures.

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4. ASP Alert™ Method

ASP Alert
Rapid detection of Domoic Acid in Shellfish and Molluscs

KIT COMPONENTS SUPPLIED BY CUSTOMER



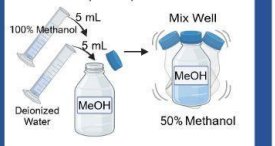
Do NOT re-use any tubes for multiple samples due to a high risk of toxicity and contamination. Dispose of the tubes after single use

COMPONENTS SUPPLIED BY SENSOREAL



PREPARING 50% METHANOL

Label an bottle "MeOH". Add 5 mL DI water, then add 5 mL Methanol (100%). Mix well.

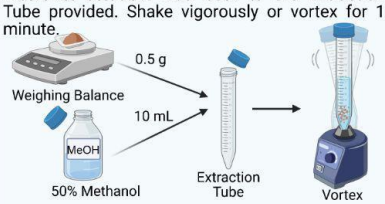


PART A: TOXIN EXTRACTION

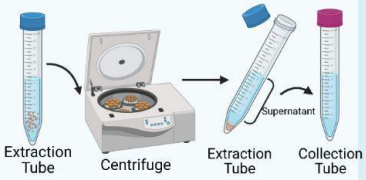
1. Shuck the shellfish. Use at least 100 g of meat as a representative sample. Strain excess liquid from shellfish. Blend the meat with a blender.



2. Weigh out **0.5 g of sample** and **10 mL of 50% methanol solution.** Add both to the Extraction Tube provided. Shake vigorously or vortex for 1 minute.

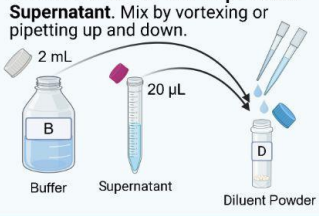


3. Centrifuge at 5000 rpm for 15 minutes. Transfer the supernatant into a Collection Tube.

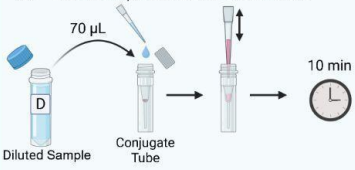


PART B: USING THE LFA

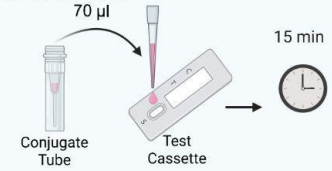
4. To the diluent powder tube "D", add **2 mL of Buffer "B"** and **20 µL of the Supernatant**. Mix by vortexing or pipetting up and down.



5. Add **70 µL of the Diluted Sample** to the Conjugate tube. Pipette up and down to dissolve. Let sit uninterrupted at room temperature for 10 minutes.

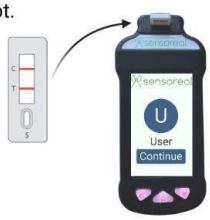


6. Add **70 µl of the Conjugate mix** to the test cassette. Leave the cassette on a horizontal surface and set a timer for 15 minutes.

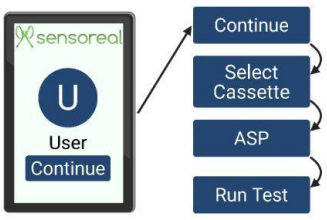


SENSOREAD - INTERPRETING THE RESULTS

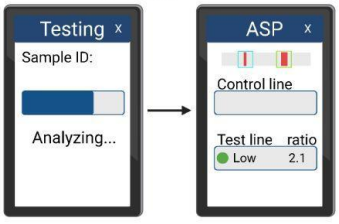
7. After 15 minutes, insert the cassette into the reader slot.



8. Follow the steps on the screen.



9. Test results will be displayed after a few seconds.



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4.1 Materials Provided

The ASP Alert™ kit includes the following:

- Lateral Flow Test Strips (Cassettes)
- Conjugate vials (lyophilized antibody-gold nanoparticles)
- Diluent powder vials (“D”)
- Buffer solution (“B”)

4.2 Additional materials required, but not provided

Equipment and consumables not provided with the kit but necessary for operation include:

- Sensoreal’s SensoRead™ Scanner (required for result interpretation)
- Extraction Solvent: 50% Methanol (Prepared as 1:1 reagent-grade 100% methanol and deionized water)
- Shellfish shucking tools (knife, etc.)
- Immersion blender or equivalent laboratory blender for tissue homogenization
- Analytical weighing balance
- Centrifuge (compatible with 15 mL conical tubes and capable of 5000 rpm)
- Electronic timer
- Adjustable precision pipettes covering volumes of 20–200 µL and 100–1000 µL
- Disposable pipette tips
- 15 mL conical tubes (for extraction and collection)
- The use of test tube racks and a vortex mixer is recommended for operational ease; however, these components are not mandatory for the methodology.

4.3 Precautions

- Do not open cassette or conjugate components until ready to use. Keep all test components in their original packaging/containers until the time of testing.
- Do not use kit contents beyond the expiration date.
- Treat all samples, extracts, and used test components as potentially contaminated with domoic acid and biological material. Wear appropriate PPE (e.g., gloves, lab coat, eye protection) during testing.
- ASP Alert™ uses 50% methanol solvent (1:1 reagent-grade methanol: deionized water, v/v), which is flammable and hazardous. Handle in accordance with laboratory chemical safety procedures, use in a well-ventilated area, and keep away from ignition sources.
- To avoid cross-contamination, use clean pipette tips/tubes and fresh extraction/dilution materials for each sample
- Follow the specified extraction, dilution, incubation, and read times exactly; deviations may affect test performance.
- Use only the designated Sensoreal SensoRead™ scanner/method settings for interpretation, as applicable.
- Dispose of sample extracts, methanol-containing waste, and used kit components in accordance with institutional, local, and regulatory requirements.
- Safety documentation (SDS/MSDS) for relevant reagents/solutions should be available to users upon request / maintained by the laboratory.

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4.4 Storage Requirements

- Store ASP Alert™ kit components according to the product labeling (controlled storage conditions as specified by Sensoreal).
- Do not freeze kit components, except the antibody conjugate.
- Keep components in their original packaging until use.
- Protect kit components from excess heat, moisture, and direct sunlight.
- Do not use components that are expired or show signs of damage/deterioration.

4.5 SensoRead™ scanner set-up

- Turn on the scanner and scan the QR code to select the correct test for the lot number.

4.6 ASP Alert™ Method Protocol

4.6.1 Toxin Extraction

1. To prepare the 50% methanol extraction solvent, combine 5 mL of deionized (DI) water with 5 mL of 100% methanol in a labeled container and agitate thoroughly to ensure a homogeneous 1:1 (v/v) mixture.
2. Using a clean spatula and weighing balance, weigh exactly 0.5 g of the homogenized shellfish sample and transfer it into a 15 mL conical tube.
3. Add 10 mL of 50% methanol extraction solvent to the conical tube containing the sample.
4. Cap the conical tube tightly and shake vigorously (or utilize a vortex mixer) for 1 minute.
5. Transfer the tube to a centrifuge and spin at 5000 rpm for 15 minutes to separate the particulate matter from the toxin-containing solvent.
6. Following centrifugation, carefully transfer the supernatant (the clear liquid) into a clean 15 mL conical tube.

4.6.2 Test Strip Analysis Procedure

Ensure the Sensoreal SensoRead™ scanner is turned on and the correct lot QR code has been scanned prior to initiating the test.

1. Locate the Diluent powder tube ("D"). Using a pipette fitted with a new tip, add 2 mL of Buffer "B" and 20 µL of the sample supernatant to Tube "D". Mix thoroughly by vortexing or pipetting up and down.
2. Using a pipette fitted with a new tip, transfer 70 µL of the Diluted Sample (from Tube “D”) into the Conjugate vial. Pipette the mixture up and down to achieve complete dissolution of the lyophilized pellet, ensuring that no red specks remain adhered to the interior walls of the tube. Once the pellet is fully dissolved and the solution is homogeneous, let the vial sit uninterrupted at room temperature for exactly 10 minutes.
3. After the 10-minute incubation, transfer 70 µL of the Conjugate mix into the sample port of the test cassette.

4. Leave the cassette on a flat, horizontal surface. Set a timer and allow the assay to develop for exactly 15 minutes.

4.6.3 SensoRead™ Scanner Analysis

1. After exactly 15 minutes of development, immediately insert the test cassette into the slot of the SensoRead™ Scanner. Follow the on-screen prompts: Select Cassette -> ASP Alert™
2. You will be prompted to identify the Lot being used. Either select an existing Lot from the on-screen list, or for a new Lot, press “Add New Lot” and scan the Lot-specific QR code located on the LFA test pouch or the Certificate of Analysis. This QR code provides the system with integral calibration parameters required for accurate interpretation. (Note: Each Lot only needs to be scanned once; for subsequent tests with the same Lot, simply select it from the saved list).
3. Once the Lot is confirmed, enter the unique Sample ID using the on-screen keypad and press “Run Test”. The device will immediately initiate the analysis process to receive the qualitative result.

4.6.4 Interpretation of Results

The SensoRead™ scanner provides an objective determination of line intensities for consistent interpretation, as well as a digital photographic record of all test strips.

- Positive: The scanner has detected Domoic Acid at or above the 15 mg/kg threshold, indicating a potentially toxic sample.
- Negative: The scanner has detected Domoic Acid concentrations are below the 15 mg/kg threshold, indicating a potentially compliant sample.

4.7 LC-UV Method Analysis

- For LC-UV analysis, 2.00 ± 0.01 g of homogenized tissue was weighed for extraction.
- The tissue was extracted with 18.0 mL of 50% methanol.
- Samples were vortex-mixed at 2500 rpm for 2 minutes.
- Samples were then centrifuged at 4500 rpm for 10 minutes.
- The resulting supernatant was decanted and filtered through a 0.2 µm glass fiber filter into amber HPLC vials.
- Chromatographic analysis was performed using Agilent 1100/1200 LC-UV systems (Agilent, Manchester, United Kingdom) equipped with:
    - quaternary pump
    - vacuum degasser
    - autosampler
    - column oven
    - diode-array detector (242 nm)
- Peaks for DA and epi-DA were quantified using external calibration standards.
- Calibration curves met a minimum correlation coefficient (r^2) of 0.999.

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- Results were reported as mg DA/kg tissue.
- Method performance characteristics[7]:
    - LOD: 0.4 mg/kg
    - LOQ: 1.0 mg/kg

5. Single Laboratory Validation study

A single-laboratory validation study was conducted to measure or evaluate the following parameters for ASP Alert™:

- Comparability[8]
- Probability of Detection[8]
- Ruggedness[8]

For any naturally contaminated shellfish sample, ASP Alert™ results were compared to those of an accepted LC-UV reference method[7]. Matrices tested were mussels and scallops.

5.1 Comparability

5.1.1 Method

- Comparability is demonstrated when a new method intended as an alternative/substitute does not produce a significant difference in results compared with the officially recognized NSSP method for the same matrix and intended use.

- Naturally incurred mussel and scallops samples were first collected from different sites and different seasons. A minimum of 10-11 animals were used for each shellfish Sample ID. These shellfish were shucked, 80-100g of meat was measured and homogenized. Then 2 aliquouts were measured. One 0.5 g aliquot was used to test with ASP Alert™. The second aliquot was tested using the will be tested using the reference method of LC-UV and the correct concentration (mg DA/kg) was obtained.

- 112 mussels and 81 scallop samples were tested. The number of samples tested were based on the availability of these naturally contaminated samples and the ISSC guidelines which state that a minimum of 30 samples need to be tested.

5.1.2 Results

- Results of the comparability study, summarized in Table 1, demonstrate that the ASP Alert™ is an effective screening method for the qualitative determination of DA in mussels and scallops.

- ***Mussels***
    - The assay achieved 100% positive detection for samples at or above the 20 mg/kg regulatory threshold, correctly identifying all 79 LC-UV confirmed positives.
    - There were no false-negative results on samples containing toxin at or above the 20 mg/kg regulatory threshold.
    - The assay achieved 100% negative detection for all samples below 8 mg/kg.

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- Samples containing DA levels between 8 mg/kg and 13.8 mg/kg gave a mix of positive and negative results. All samples above 13.8 mg/kg gave a positive result, except for one sample at 18.9 mg/kg which produced a negative result.
- Consistent with the intended use as a conservative screening tool, the assay is designed to detect DA at levels approaching the regulatory limit (15–20 mg/kg) to ensure a margin of safety.

- ***Scallops***
  - The assay achieved 100% positive detection for samples at or above the 20 mg/kg regulatory threshold, correctly identifying all 40 LC-UV confirmed positives.There were no false-negative results on samples containing toxin at or above the 20 mg/kg regulatory threshold.
  - The assay achieved 100% negative detection for all samples below 9 mg/kg.
  - Samples containing DA levels between 9 mg/kg and 15 mg/kg gave a mix of positive and negative results. All samples above 15 mg/kg gave a positive result, except for one sample at 17.9 mg/kg which produced a negative result.
  - Consistent with the intended use as a conservative screening tool, the assay is designed to detect DA at levels approaching the regulatory limit (15–20 mg/kg) to ensure a margin of safety.

Table 1: Naturally contaminated mussel and scallops tested with ASP Alert™ and the reference method LC-UV

Matrix	Sample ID	DA concentration (mg/kg)	Scanner Qualitative Result
Mussels	Z9	1	-
	Z16	1	-
	Z15	3	-
	Z14	3	-
	40	6	-
	39	6	-
	38	6	-
	37	7	-

36	8	-
35	8	+
34	8	+
33	9	-
32	9	-
31	10	-
30	11	-
29	11	+
I1	11	-
MD16	11.1	-
MD15	11.5	-
MD14	11.9	-
I10	11.9	-
28	12	-
I3	12.1	+
MD13	12.3	-
I9	12.3	-
I2	12.8	+

I8	12.8	+
27	13	+
I7	13.3	-
I6	13.8	+
I5	14.4	+
MD12	14.6	+
26	15	+
I4	15	+
MD11	15.3	+
MD10	16	+
MD9	16.9	+
MD8	17.8	+
MD7	18.9	-
MC16	19	+
MC15	19	+
MD6	20	+
MC14	20	+
MC13	21	+

MD5	21.4	+
MD4	23	+
MD3	25	+
MC12	25	+
MC11	26	+
MD2	27	+
MC10	27	+
Z10	27	+
MC9	29	+
MD1	29	+
MC8	30	+
MB16	32	+
MB16-2-b	32	+
MC7	32	+
MB15	33	+
MB15-b	33	+
MC6	34	+
MB14	34	+

MB14-b	34	+
MB13	36	+
MB13-b	36	+
MC5	36	+
MC4	39	+
MC3	42	+
MB12	42	+
MB12-b	42	+
MB11	44	+
MB11-b	44	+
MC2	45	+
MB10	46	+
MB10-b	46	+
MB9	49	+
MB9-b	49	+
MC1	50	+
MB8	51	+
MB8-b	51	+

MB7	55	+
MB7-b	55	+
MB6	58	+
MB5	62	+
MB4	66	+
MB3	71	+
25	74	+
MA16	77	+
Z23	77	+
MB2	77	+
MA15	79	+
24	79	+
MA14	82	+
MB1	84	+
MA13	86	+
23	86	+
22	93	+
Z25	101	+

	21	101	+
	MA12	101	+
	MA11	106	+
	MA10	111	+
	MA9	117	+
	Z24	120	+
	MA8	124	+
	MA7	131	+
	MA6	139	+
	MA5	148	+
	MA4	159	+
	MA3	171	+
	MA2	185	+
	MA1	202	+
Scallops	Z1	3	-
	Z6	3	-
	SD13	6	-
	Z20	6	-

Z19	6	-
Z12	7	-
SD12	7	-
SD11	8	-
Z34	8	-
SD10	8	-
Z4	8	-
SD9	8	-
SD8	9	-
Z22	9	+
Z21	9	-
SD7	9	-
SD6	10	-
I28	10.9	-
SD5	11	-
I27	11.7	-
SD4	12	+
SD3	12	-

I23	12.4	+
I35	12.5	-
I22	12.6	+
I26	12.7	-
I34	12.9	+
SD2	13	-
I33	13.2	+
I32	13.5	+
I25	13.7	+
I31	13.9	+
I30	14.2	+
I29	14.6	+
Z3	14.6	+
I24	14.9	+
SD1	15	-
SC16	17	+
SC15	17	+
SC14	17.9	-

SC13	19	+
SC12	22	+
I16	22.4	+
SC11	23	+
I15	23.6	+
SC10	24	+
I14	25	+
SC9	25	+
I13	26.5	+
SC8	27	+
SC7	28	+
I12	28.3	+
H12	31.4	+
H19	32.4	+
H10	34.3	+
H3	35.3	+
H18	37.5	+
H2	37.9	+

H11	37.9	+
Z30	40	+
Z29	40	+
H17	42.5	+
H4	49.1	+
H6	50.4	+
H16	54.6	+
H8	58.2	+
H9	63.7	+
Z33	67	+
H15	75.7	+
H20	85.6	+
Z32	95	+
H13	95.1	+
Z35	102	+
Z31	107	+
H5	124.9	+
H14	134	+

	Z5	155	+
	H7	216.7	+
	I11	292.2	+
	Z18	353	+
	H1	424.8	+

5.2 Probability of Detection

5.2.1 Method

- Probability of Detection (POD) is the probability that a qualitative method produces a positive response at a given analyte concentration. POD varies with concentration and provides a single performance description that inherently reflects false-negative and false-positive behavior across the working range.
- A mixture of natural shellfish samples and DA spiked samples were used.
- For POD, a different aliquot from the “Comparability” samples were incorporated into this study. Additional Spiked mussel and scallop samples were also prepared for this analysis. A minimum of 10-11 animals were used for each shellfish Sample ID. These shellfish were shucked, 80-100g of meat was measured and homogenized. Then 2 aliquouts were measured. One aliquot was used to test with ASP Alert™. The second aliquot was tested using the will be tested using the reference method of LC-UV and the correct concentration (mg DA/kg) was obtained.
- For the spiked samples, blank samples were collected for each species and spiked with different volumes of DA-CRM (NRCC, Canada) to yield different DA concentrations. The spiked samples were necessary to comply with the minimum number of samples required for a particular DA concentration range.
- Several mussel samples were then tested and the samples were categorized into 5 distinct concentration ranges. These include:
    - DA level: 0-7 mg DA/kg
    - DA level: 8-13 mg DA/kg
    - DA level: 13.1-19 mg DA/kg
    - DA level: 20-30 mg DA/kg
    - DA level: >30 mg DA/kg
- A minimum of 20 independent samples per category were tested.
- A total of 172 mussel samples and 142 scallop samples were evaluated.

5.2.2 Results

Results for the POD study are shown in Table 2. A dose response curve. A dose-response curve with the combined samples is shown in Figure 1.

- **Mussels:**
  - In mussel, the assay achieved 100% accuracy for samples in the following concentration ranges: 0-7 mg DA/kg, 20-30 mg DA/kg, and for >30 mg DA/kg. All samples in the range of 0-7 mg DA/kg were negative, while all samples in the ranges of 20-30 mg DA/kg and >30 mg DA/kg were positive.
  - In the range of 8-13 mg DA/kg, 16 out of 30 samples were positive and in the range of 13.1-19 mg DA/kg, 21 out of 23 samples were positive.
  - There were no false positive results on un-spiked control samples.

- **Scallops:**
  - In scallops, the assay achieved 100% accuracy for samples in the following concentration ranges: 0-7 mg DA/kg, 20-30 mg DA/kg, and for >30 mg DA/kg. All samples in the range of 0-7 mg DA/kg were negative, while all samples in the ranges of 20-30 mg DA/kg and >30 mg DA/kg were positive.
  - In the range of 8-13 mg DA/kg,7 out of 30 samples were positive and in the range of 13.1-19 mg DA/kg, 19 out of 23 samples were positive.
  - There were no false positive results on un-spiked control samples.

Table 2: Results of POD study of ASP Alert™ with mussels and scallops

Species	DA level (mg/kg)	Number of Samples	Number of Positives	Percentage Positive results
Mussels	0-7	28	0	0.0%
	8-13	30	16	53.3%
	13.1-19	23	21	91.3%
	20-30	34	34	100.0%
	>30	57	57	100.0%
Scallops	0-7	27	0	0.0%

	8-13	31	7	22.6%
	13.1-19	23	19	82.6%
	20-30	32	32	100.0%
	>30	29	29	100.0%

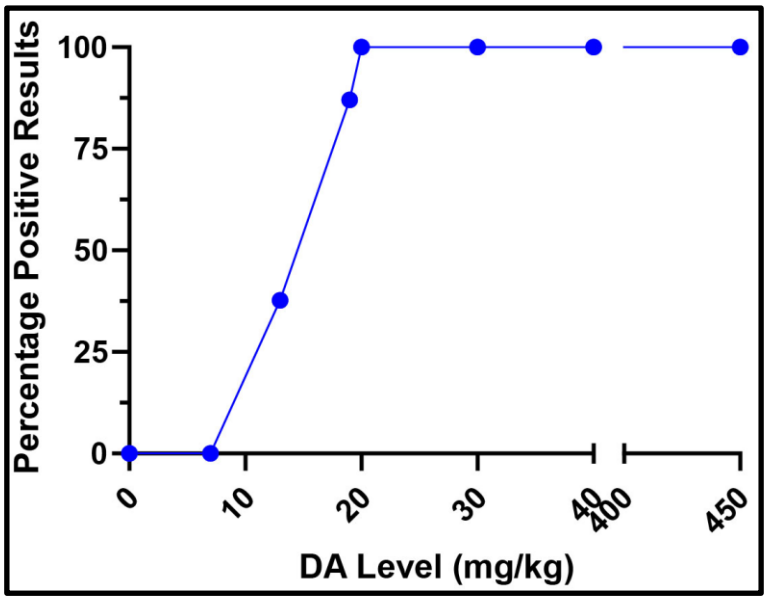


Figure 1: Dose-response curve generated with ASP Alert™ in mussels and scallops

5.3 Ruggedness

5.3.1 Method

- Ruggedness is defined as the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments. For ASP Alert™, the critical parameter being evaluated was the LFA run time of the assay (development time prior to reading).

- Per ISSC guidance, 10–12 analyte-free animals were homogenized to prepare individual mussel and scallop samples respectively. These samples were then spiked with domoic acid (DA) certified reference material at or near the action level, above the ASP Alert™

assay threshold of 15 mg DA/kg, to generate at least 10 samples each for mussels and scallops.

- For each sample, one aliquot was tested using the standard ASP Alert™ procedure. In addition, two aliquots from the same homogenate were processed identically but read at intentionally shortened or extended run times to reflect realistic operator variation. Each aliquot was further sub-aliquoted to yield 3 replicate analyses.
- The different LFA run times tested were:
    - 13 min
    - 15 min (standard procedure)
    - 17 min

5.3.2 Result

Results of the ASP Alert™ ruggedness trials with respect to LFA run time of the assay are summarized in Table 3. No differences were observed in the number of positive results across different LFA run times, indicating consistent assay performance under the tested conditions.

Table 3: Ruggedness trials for ASP Alert™ – effect on LFA run time

Species	DA Concentration (mg DA/kg)	Number of end replicates	Number of Positives at 13 min	Number of Positives at 15 min	Number of Positives at 17 min
Mussels	20.2	3	3	3	3
	23.8	3	3	3	3
	18.7	3	3	3	3
	24.9	3	3	3	3
	22.9	3	3	3	3
	19	3	3	3	3
	19.1	3	3	3	3
	21.8	3	3	3	3
	19.5	3	3	3	3
	17.2	3	3	3	3
Scallops	25	3	3	3	3
	23.6	3	3	3	3
	22.4	3	3	3	3
	19.5	3	3	3	3
	18.7	3	3	3	3
	17.9	3	3	3	3
	17.1	3	3	3	3
	17.9	3	3	3	3

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	21.3	3	3	3	3
	24.3	3	3	3	3

6. Quality Control

Quality control (QC) testing for manufactured ASP Alert™ lots is performed at both the in-process and finished-product stages. For in-process QC, LFA cassettes are run using a blank diluent to confirm correct test and control line placement and acceptable line intensity. For finished-product release testing, domoic acid–containing CRM mussel tissue (CRM ASP Mus-e, NRCC) is extracted and diluted to prepare DA levels of 0, 5, 10, 15, 20, and 30 ppm. Each lot is then evaluated by testing the cassettes with these extracted QC samples to generate a dose–response curve. Lot acceptance criteria require that results at 0 and 5 ppm are negative and results at 15 ppm, 20 ppm and 30 ppm are positive. Results at 10 ppm can be a mix of positive and negative. QC standards are prepared as single-use aliquots and stored frozen to avoid repeated freeze–thaw cycles and to ensure consistent use of freshly prepared DA samples for each QC run.

7. Discussion

The single-laboratory validation (SLV) study confirms that the ASP Alert™ is a robust and effective screening method for the qualitative determination of domoic acid (DA) in mussels and scallops. The study was designed to challenge the assay with naturally contaminated samples collected from diverse geographical sites and seasons, ensuring that performance reflects realistic testing conditions.

The Comparability study demonstrated strong agreement between the ASP Alert™ and the standard LC-UV reference method. For both mussels and scallops, the assay demonstrated 100% agreement for all samples containing DA at or above the regulatory action level of 20 mg/kg. Crucially, there were no false-negative results observed for any sample exceeding the 20 mg/kg limit in either matrix..

ASP Alert™ is engineered with a screening threshold of 15 mg/kg to provide a necessary safety buffer for shellfish growers and public health officials. In the POD study, 100% of samples containing greater than 20 mg/kg of DA were correctly identified as positive. By flagging samples that are approaching the regulatory limit, the ASP Alert™ enables early intervention and prevents borderline products from reaching the market without confirmatory testing. Samples containing levels of DA less than 8 mg/kg) were reliably identified as negative.

The Ruggedness study confirmed that the ASP Alert™ maintains consistent performance despite minor procedural variations that may occur in a routine laboratory or field setting. Testing across multiple manufacturing lots of the ASP Alert™ yielded identical qualitative results, demonstrating manufacturing stability. Additionally, variations in the LFA development time ranging from 13 to 17 minutes did not alter the qualitative outcome for any sample.

Based on the results presented here, it is recommended that the ASP Alert™ be approved by the Interstate Shellfish Sanitation Conference as a screening method for the qualitative determination of domoic acid in mussels and scallops.

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Proposal 27-155

Sensoreal® ASP Alert™ Rapid Test for Domoic Acid in Shellfish

Product ID: ALRT-ASP

Relevance of Domoic Acid Determination

Domoic acid (DA) is a potent marine neurotoxin produced by certain *Pseudo-nitzschia* diatoms during harmful algal blooms (HABs). It is the causative agent of Amnesic Shellfish Poisoning (ASP) in humans. Ingestion of DA-contaminated bivalve mollusks can cause severe gastrointestinal and neurological symptoms, including permanent memory loss, and has been linked to intoxications in marine mammals. Due to the significant health risks, regulatory authorities worldwide (including the US, Canada, and Europe) require routine monitoring of bivalve shellfish, applying a strict maximum regulatory limit of 20 mg/kg (20 ppm) for harvest closures and market restrictions.

Validation Data

Test Sensitivity and Threshold:	ASP Alert™ is engineered with a conservative decision threshold of 15 mg/kg (15 ppm) to provide an added safety margin relative to the regulatory limit.
Comparability with LC-UV:	In single-laboratory validation studies, the assay demonstrated 100% agreement with standard LC-UV reference methods for samples containing DA at or above the regulatory action level (≥20 mg/kg) in both mussels and scallops. No false negatives were observed at or above the regulatory limit.
Ruggedness:	The test exhibits robust stability and maintains consistent qualitative performance despite minor procedural variations and across different manufacturing lots, ensuring reliable operational outcomes in routine laboratory or field settings.
General Limited Warranty:	The ASP Alert™ Rapid Test Kit is provided for research and pre-testing purposes, and is not intended to replace officially validated regulatory testing methods. Sensoreal Inc. makes no warranty, expressed or implied, including any warranty of merchantability or fitness for a particular purpose. **The user assumes responsibility for determining the product's suitability for their specific application, and Sensoreal Inc. shall not be liable for any indirect, incidental, or consequential damages arising from the use of these products.**

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Instructions are effective as of (date): 2026-04-07

Version: 0.1

1. General Description and Principle of the Method

Sensoreal ASP Alert™ is a rapid lateral flow immunoassay (LFA) designed for the qualitative detection of domoic acid (DA) in bivalve mollusks (mussels and scallops). The assay operates on a competitive binding format.

Domoic acid is extracted from homogenized shellfish tissue using a 50% methanol solution. The extract is then diluted and incubated with a lyophilized antibody–gold nanoparticle conjugate. If DA is present in the sample, it binds to the conjugate antibodies, reducing the amount of free antibody–gold complex available. The mixture is then applied to the lateral flow cassette and migrates across a membrane containing immobilized DA-protein at the test line. Consequently, the test line intensity is inversely proportional to the DA concentration (higher DA concentration = fainter test line). A control line will appear in all valid tests to confirm proper assay function. Results are objectively interpreted using the Sensoreal SensoRead™ scanner.

2. Safety Instructions

Treat all samples, extracts, and used test components as potentially contaminated with domoic acid and biological material. Wear appropriate personal protective equipment (PPE) including gloves, safety goggles, and a laboratory coat during testing. The ASP Alert™ protocol utilizes a 50% methanol extraction solvent (1:1 reagent-grade methanol to deionized water, v/v), which is hazardous and flammable. Handle extraction solvents in a well-ventilated area in accordance with institutional chemical safety procedures, and keep away from ignition sources.

3. Storage and Stability

-Lateral Flow Test Strips (Cassettes): Store at the temperature specified on the label. Do not freeze. Keep cassettes in their original, sealed pouches until immediately prior to use to protect them from moisture and humidity.
-Antibody Conjugate Vials (Lyophilized): This is the only kit component that may be frozen. Store frozen or as strictly specified on the product label. Keep vials sealed and do not open until ready to perform the assay.
-Diluent Powder Vials: Store at the temperature specified on the label. Do not freeze. Keep sealed to protect from excess heat and moisture.
-Buffer Solution: Store as directed on the label.
-General Stability: Protect all kit components from excess heat and direct sunlight. Do not utilize kit components past their printed expiration date or if they exhibit any signs of damage or deterioration.

4. Warnings and Precautions

-The ASP Alert™ test is optimized for analyzing freshly harvested bivalves, intact samples refrigerated for under 48 hours, or previously homogenized tissue kept frozen. Prior to beginning the extraction process, it is critical that all chilled or frozen samples are allowed to fully equilibrate to ambient room temperature.
-Unprocessed shellfish can be safely held under refrigeration (2–8°C) provided they will be tested within two days of harvest. If the testing timeline extends beyond this 48-hour window, the shellfish meat must be completely shucked, homogenized (as outlined in Section C), and stored at -20°C to ensure toxin stability prior to extraction.
-Please note that this protocol is exclusively validated for bivalve shellfish matrices. Any application to alternative sample types requires thorough independent validation by the user before routine testing is implemented.
-Follow the specified extraction, dilution, incubation, and read times exactly as detailed in Section D and E. Deviations from the protocol may adversely affect test performance.
- To avoid cross-contamination, use clean, disposable pipette tips and fresh extraction/dilution materials for each individual sample.
-Do not open the lateral flow cassettes or conjugate components until ready to initiate testing.
-Use exclusively the designated Sensoreal SensoRead™ reader and corresponding method settings for the interpretation of results. Visual interpretation is not supported.
-Dispose of sample extracts, methanol-containing waste, and used kit components in strict accordance with institutional, local, and regulatory environmental requirements.
-As with any screening technique, positive results leading to regulatory action or market closure should be confirmed by a recognized alternative quantitative method (e.g., LC-UV).

A. Materials Provided

The ASP Alert™ kit includes the following components:

1. Lateral Flow Test Strips (Cassettes)
2. Conjugate Vials (lyophilized antibody-gold nanoparticle)
3. Diluent Powder Vials ("D")
4. Buffer Solution ("B")

B. Materials and Reagents Required (Not Provided)

1. Sensoreal SensoRead™ Scanner (required for result interpretation)
2. Extraction Solvent: 50% Methanol (Prepared as 1:1 reagent-grade 100% methanol and deionized water)
3. Shellfish shucking tools (knife, etc.)
4. Immersion blender or equivalent laboratory blender for tissue homogenization
5. Analytical weighing balance
6. Centrifuge (compatible with 15 mL conical tubes and capable of 5000 rpm)
7. Electronic timer
8. Adjustable precision pipettes covering volumes of 20–200 µL and 100–1000 µL
9. Disposable pipette tips
10. 15 mL conical tubes (for extraction and collection)
11. The use of test tube racks and a vortex mixer is recommended for operational ease; however, these components are not mandatory for the methodology.

C. Sample Collection and Storage

1. For each sample ID, shuck a representative population of 10 to 12 individual animals to obtain a minimum of 100 g of pooled shellfish tissue.
2. Thoroughly rinse the exterior of the shellfish with deionized or distilled water to remove extraneous debris, such as sand or mud.
3. Transfer the collected tissue to a sieve and allow it to drain to remove excess fluid.
4. Homogenize the tissue using an immersion blender (or equivalent mechanical blender) until a completely uniform, smooth consistency is achieved.

D. Toxin Extraction (Part A)

1. To prepare the 50% methanol extraction solvent, combine 5 mL of deionized (DI) water with 5 mL of 100% methanol in a labeled container and agitate thoroughly to ensure a homogeneous 1:1 (v/v) mixture.
2. Using a clean spatula and weighing balance, weigh exactly 0.5 g of the homogenized shellfish sample and transfer it into a 15 mL conical tube.
3. Add 10 mL of 50% methanol extraction solvent to the conical tube containing the sample.
4. Cap the conical tube tightly and shake vigorously (or utilize a vortex mixer) for 1 minute.
5. Transfer the tube to a centrifuge and spin at 5000 rpm for 15 minutes to separate the particulate matter from the toxin-containing solvent.
6. Following centrifugation, carefully transfer the supernatant (the clear liquid) into a clean 15 mL conical tube.

E. Test Strip Sample Analysis Procedure (Part B)

Ensure the Sensoreal SensoRead™ scanner is turned on and the correct lot QR code has been scanned prior to initiating the test.

1. Sample Dilution: Locate the Diluent powder tube ("D"). Using a clean pipette, add 2 mL of Buffer "B" and 20 µL of the sample supernatant to Tube "D". Mix thoroughly by vortexing or pipetting up and down.
2. Conjugate Incubation: Using a clean pipette tip, transfer 70 µL of the Diluted Sample (from tube “D”) into the Conjugate vial. Pipette the mixture up and down to achieve complete dissolution of the lyophilized pellet, ensuring that no red specks remain adhered to the interior walls of the tube. Once the pellet is fully dissolved and the solution is homogeneous, let the vial sit uninterrupted at room temperature for exactly 10 minutes.
3. Lateral Flow Application: After the 10-minute incubation, transfer 70 µL of the Conjugate mix into the sample port of the test cassette.
4. Development: Leave the cassette on a flat, horizontal surface. Set a timer and allow the assay to develop for exactly 15 minutes.

F. SensoRead™ Scanner Analysis

1. After exactly 15 minutes of development, immediately insert the test cassette into the reader slot of the SensoRead™ device. Follow the on-screen prompts: Select Cassette -> ASP Alert
2. You will be prompted to identify the batch being used. Either select an existing Lot from the on-screen list, or for a new Lot, press “Add New Lot” and scan the batch-specific QR code located on the LFA test pouch or the Certificate of Analysis. This QR code provides the system with integral calibration parameters required for accurate interpretation. *(Note: Each Lot only needs to be scanned once; for subsequent tests with the same Lot, simply select it from the saved list).*
3. Once the Lot is confirmed, enter the unique Sample ID using the on-screen keypad and press “Run Test”. The device will immediately initiate the analysis process to receive the qualitative result (T/C ratio and classification).

G. Interpretation of Results

Assay results are determined by the SensoRead™ scanner through a digital analysis of the Test-to-Control (T/C) line intensity ratio. While the absolute intensity of the control line may fluctuate between runs, a visible control line must be present for the assay to be considered valid; the absence of a control line indicates an invalid result requiring a retest.

The SensoRead™ scanner provides an objective determination of line intensities for consistent interpretation, as well as a digital photographic record of all test strips.

-Positive: The scanner has detected Domoic Acid at or above the 15 mg/kg threshold. The reader will flag this sample, indicating it has reached or exceeded the precautionary limit.
-Negative: The T/C ratio indicates concentrations below the 15 mg/kg threshold, and the sample is considered compliant for harvest.

Note: It is essential to perform the reading at the designated 15-minute mark. Determinations made using strips that have dried for more or less than the required time may be inaccurate, as chromatographic line intensities vary with drying time. To ensure a rigorous safety margin against the 20 mg/kg NSSP regulatory limit, the ASP Alert™ system is intentionally calibrated to a conservative early-warning threshold of 15 mg/kg.

Test Line	**Control Line**	**Interpretation**
Absent	Absent	Invalid Test (Repeat Test)
Faint or Non-Visible	Visible	Positive: ≥ 15 mg/kg
Moderate Intensity	Visible	Negative: ≤ 15 mg/kg
More Intense than Control Line	Visible	Negative: Non-Detect

For supplemental technical data or methodological assistance, please direct all inquiries to Sensoreal Technical Support at sales@sensoreal.com.

SHELLFISH LABORATORY EVALUATION CHECKLIST

ASP Alert™ (Lateral Flow Assay) for Amnesic Shellfish Poisoning (ASP) — Domoic Acid

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
ASP Alert™ — LFA for ASP (Domoic Acid) in Shellfish

LABORATORY:	
ADDRESS:	
TELEPHONE:	**FAX:**
EMAIL:	
DATE OF EVALUATION:	**DATE OF REPORT:**
LAST EVALUATION:	
LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	
SHELLFISH SPECIALIST:	
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: _____ Conformity is noted by a "√"

C- Critical K - Key O - Other N/A - Not Applicable

SHELLFISH LABORATORY EVALUATION CHECKLIST

ASP Alert™ (Lateral Flow Assay) for Amnesic Shellfish Poisoning (ASP) — Domoic Acid

PART I – QUALITY ASSURANCE (QA)		
1.1 Quality Assurance (QA) Plan		
Code	**Ref**	**Item**
K	1,2	**1.1.1 Written QA Plan exists** (check √ those items which apply): ☐ Organization of laboratory ☐ Staff training requirements ☐ SOPs/Work Instructions ☐ Internal QC measures (equipment, calibration, maintenance, acceptance criteria) ☐ Laboratory safety (chemical safety applicable to methanol) ☐ Data integrity/record control ☐ Internal performance assessment ☐ External performance assessment (if applicable)
C	2	**1.1.2 QA Plan is implemented** (evidence of use, current version controlled, staff awareness).
1.2 Educational / Experience / Training Requirements		
Code	**Ref**	**Item**
C	1,2	**1.2.1 Supervisor/technical lead is qualified** per lab policy and applicable program requirements.
K	1.2	**1.2.2 Analysts are qualified and authorized** to perform ASP Alert™ testing; authorization list maintained.
C	3	**1.2.3 Method-specific training documented** (ASP Alert™ IFU/SOP, critical timings, pipetting, scanner operation, invalid criteria, reporting).
K	1	**1.2.4 Competency is assessed and recorded** (initial + periodic).
1.3 Work Area		
Code	**Ref**	**Item**
O	2	**1.3.1 Work area adequate** for workload, sample prep, and storage.
K	2	**1.3.2 Work area clean and well lighted.**
K	2	**1.3.3 Work area has adequate temperature control** to support kit/storage requirements and stable testing conditions.
O	2	**1.3.4 Work surfaces nonporous/easily cleaned**; contamination prevention practices in place.
K	2,3	**1.3.5 Chemical safety controls in place** for 50% methanol (labeling, SDS access, waste handling).
1.4 Laboratory Equipment (General + Method-Relevant)		
Code	**Ref**	**Item**
K	3	**1.4.1 Equipment inventory exists** (includes model/ID, location, calibration/verification needs).
C	2	**1.4.2 Micropipettors used in method are calibrated/verified** for volumes used (e.g., 20-100 µL, 1 mL or appropriate devices). Records maintained.

SHELLFISH LABORATORY EVALUATION CHECKLIST

ASP Alert™ (Lateral Flow Assay) for Amnesic Shellfish Poisoning (ASP) — Domoic Acid

K	2	**1.4.3 Timer(s) available and controlled** for 10-min conjugate incubation and 15-min cassette development.
K	2	**1.4.4 Balance used for 0.5 g tissue is verified/calibrated** at relevant range; records maintained.
C	2	**1.4.5 Centrifuge performance is verified** (speed/time appropriate; maintenance/service records maintained).
C	3	**1.4.6 Sensoreal Sensoread™ Scanner is installed and functional** (method/settings controlled; alignment/cassette insertion per IFU).
K	3	**1.4.7 SensoRead™ scanner performance checks documented** (startup/self-test or verification check per IFU/SOP).
O	1,2	**1.4.8 Refrigeration/temperature monitoring where required** (for kits/reagents as specified). Records maintained.
1.5 Reagent / Kit Storage, Preparation, and Inventory Control		
Code	**Ref**	**Item**
C	3	**1.5.1 ASP Alert™ kits stored per IFU** (temperature range, protection from extremes).
K	3	**1.5.2 Lot # and expiry tracked** for kits, cassettes, conjugate vials, diluent (as applicable).
C	3	**1.5.3 Extraction solvent is prepared correctly (50% methanol)** and labeled (contents, date, preparer).
K	3	**1.5.4 Reagents and samples clearly labeled** to prevent mix-ups; chain-of-custody maintained.
O	3	**1.5.5 Receiving inspection documented** (condition upon receipt, storage initiated promptly).
1.6 Records, Data Integrity, and Document Control		
Code	**Ref**	**Item**
C	3	**1.6.1 Controlled documents in use** (current IFU/SOP, version controlled, accessible to staff).
C	3,4	**1.6.2 Traceability is ensured** (sample ID → extraction → dilution → cassette → scanner result → report).
K	3	**1.6.3 Raw data retention** (scanner output files, run sheets, logs) per policy.

SHELLFISH LABORATORY EVALUATION CHECKLIST

ASP Alert™ (Lateral Flow Assay) for Amnesic Shellfish Poisoning (ASP) — Domoic Acid

K	3	**1.6.4 Deviations/nonconformities documented** with corrective actions (CAPA or equivalent).
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR ASP TOXINS — ASP ALERT™ (LFA)		
2.1 Collection, Receipt, and Transportation of Samples (Laboratory Controls)		
Code	**Ref**	**Item**
C	2,3	**2.1.1 Representative sample is received and logged** (sample identifiers, species, harvest area/source, date/time).
K	2,3	**2.1.2 Sample condition assessed on receipt** (temperature/condition noted; acceptance/rejection criteria defined).
K	2.3	**2.1.3 Time-to-processing controls** defined (or contingency plan if delays occur).
O	2,3	**2.1.4 Storage conditions documented** prior to preparation/extraction (refrigerated/frozen as defined).
2.2 Preparation of Samples for Analysis — Homogenization		
Code	**Ref**	**Item**
C	3	**2.2.1 Shellfish are shucked and homogenized to uniform consistency** suitable for representative subsampling.
K	3	**2.2.2 Homogenization method is standardized** (equipment, time, cleaning between samples to prevent carryover).
K	3	**2.2.3 Cross-contamination controls in place** (clean tools, workspace cleaning, single-use consumables where appropriate).
2.3 Preparation of Samples for Analysis — Extraction and Clarification (ASP Alert™)		
Code	**Ref**	**Item**
C	3	**2.3.1 Tissue mass for extraction is 0.5 g** (within defined tolerance).
C	3	**2.3.2 Extraction solvent is 50% methanol and 10 mL** is added per method (within tolerance).
K	3	**2.3.3 Sample is vigorously shaken or vortexed for 1 minute to** ensure thorough mixing.
C	3	**2.3.4 Centrifugation is performed at 5000 rpm for 15 minutes** (or validated equivalent); supernatant is collected.
K	3	**2.3.5 Supernatant collection avoids pellet disturbance** (minimize particulates that can affect flow).
K	2,3	**2.3.6 Extract handling/storage defined** if not analyzed immediately (conditions/time limits documented).
2.4 Sample Assay — Dilution, Conjugate Incubation, Cassette Development, SensoRead™ scanner		
Code	**Ref**	**Item**
C	3	**2.4.1 Dilution is performed as specified:** 20 µL supernatant into 2.0 mL diluent (within tolerance).

SHELLFISH LABORATORY EVALUATION CHECKLIST

ASP Alert™ (Lateral Flow Assay) for Amnesic Shellfish Poisoning (ASP) — Domoic Acid

C	3	**2.4.2 70 µL of diluted sample is added to the conjugate vial** (within tolerance).
C	3	**2.4.3 Conjugate is fully resuspended** (aspirate/mix until uniform red solution; no visible clumps).
K	3	**2.4.4 Conjugate incubation is 10 minutes** (within defined window).
C	3	**2.4.5 Entire red solution is dispensed into cassette** (complete transfer; no leakage).
C	3	**2.4.6 Cassette development time is 15 minutes** (results read within defined window).
C	3	**2.4.7 QR code is scanned with the SensoRead™ scanner** to load the correct lot calibration information.
C	3	**2.4.8 Cassette is inserted into SensoRead™ scanner, sample ID is entered and result obtained** using controlled settings/method file.
C	3	**2.4.9 Invalid test criteria are defined and followed** (e.g., scanner error/invalid flag; actions documented).
K	2	**2.4.10 Proper disposal practices followed** for methanol-containing waste and biohazard materials.
2.5 Quality Control (QC), Acceptance/Rejection, and Reporting		
Code	**Ref**	**Item**
C	3	**2.5.1 Internal validity check met** (control indicator per IFU/scanner validity). Invalid results are not reported as valid.
K	3	**2.5.2 SensoRead™ scanner check/verification performed** at defined frequency; failures addressed before reporting results.
K	2,3	**2.5.3 Run documentation complete** (operator, date/time, kit lot/expiry, sample ID, deviations).
C	2,4	**2.5.4 Reporting and notification procedures defined** for results requiring program action (as applicable).
K	2	**2.5.5 Data retention and traceability maintained** (scanner outputs + run sheets retained per policy).

REFERENCES

1. NSSP Model Ordinance / NSSP Guide for the Control of Molluscan Shellfish (FDA/ISSC) — current edition.
2. Good Laboratory Practice (GLP) and laboratory quality system/document control requirements.
3. ASP Alert™ Method Documentation: ASP Alert™ IFU/Procedure Card; internal SOPs; SensoRead™ Scanner user manual/method file ID; SLV/ISSC proposal documents (as applicable).
4. Laboratory sample receipt/chain-of-custody procedures and applicable program requirements (state/agency procedures).

LABORATORY:	**DATE OF EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

SHELLFISH LABORATORY EVALUATION CHECKLIST

ASP Alert™ (Lateral Flow Assay) for Amnesic Shellfish Poisoning (ASP) — Domoic Acid

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
ASP DETECTION FOR ASP ALERT™ WITH ASP COMPONENT: (Part I-II)	

SHELLFISH LABORATORY EVALUATION CHECKLIST

ASP Alert™ (Lateral Flow Assay) for Amnesic Shellfish Poisoning (ASP) — Domoic Acid

A. Results	
Total # of Critical (C) Nonconformities	_____
Total # of Key (K) Nonconformities	_____
Total # of Critical (C), Key (K), and Other (O) Nonconformities	_____

B. Criteria for Determining Laboratory Status of ASP Alert™ assay for ASP:

1. **Conforms Status:** The ASP Alert™ for ASP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The ASP Alert™ assay for ASP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. The number of Critical nonconformities is ≥ 1 but < 4;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status**: The ASP Alert™ assay for ASP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is ≥4;
   b. or total # of Key nonconformities is ≥ 6;
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____
LEO Signature:_____ Date:_____

Proposal for Task Force Consideration at the ISSC 2027 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ Administrative
Submitter	Hosseinali Safavieh
Affiliation	Sensoreal Inc.
Address Line 1	6674 Esplanade Avenue (Suite B)
Address Line 2	
City, State, Zip	Montreal, QC H2V 4L5 Canada
Phone	438-728-1243
Fax	Not applicable
Email	roozbeh@sensoreal.com
Proposal Subject	PSP Alert™: Qualitative Detection of Paralytic Shellfish Toxins in Mussels
Specific NSSP Guide Reference	Section IV Guidance Documents, Chapter III. Laboratory, @.02 Methods, .01 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	Request for approving PSP Alert™ as a qualitative screening tool for the detection of paralytic shellfish toxins in mussels.
Public Health Significance	Paralytic shellfish toxins (PSTs) are a group of potent, heat-stable marine biotoxins produced primarily by certain dinoflagellates, including species of *Alexandrium*, *Gymnodinium*, and *Pyrodinium*. PSTs, which include saxitoxin (STX) and several structurally related analogues, can accumulate in filter-feeding bivalve molluscs such as mussels, clams, oysters, and scallops. Consumption of contaminated shellfish can cause Paralytic Shellfish Poisoning (PSP), characterized by symptoms including tingling or numbness of the lips and extremities, headache, dizziness, muscle weakness, loss of coordination, and, in severe cases, respiratory paralysis and death. The toxic effects of PSTs are primarily associated with their ability to block voltage-gated sodium channels, thereby interfering with normal nerve and muscle function. PST-producing harmful algal blooms occur in many shellfish-growing regions worldwide, including coastal areas of North America, and can result in rapid increases in toxin concentrations in shellfish. Such events may lead to harvesting restrictions or closures and can present significant risks to public health and the shellfish industry. Rapid and reliable detection of PSTs in shellfish is therefore important for supporting routine monitoring, identifying potentially unsafe harvesting areas, and enabling timely management decisions.
Cost Information	Refer to “ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP” document attached for Sensoreal Inc.

ISSC Method Application and Single Lab Validation Checklist For Acceptance of a Method for Use in the NSSP

The purpose of single laboratory validation in the National Shellfish Sanitation Program (NSSP) is to ensure that the analytical method under consideration for adoption by the NSSP is fit for its intended use in the Program. A Checklist has been developed which explores and articulates the need for the method in the NSSP; provides an itemized list of method documentation requirements; and, sets forth the performance characteristics to be tested as part of the overall process of single laboratory validation. For ease in application, the performance characteristics listed under validation criteria on the Checklist have been defined and accompany the Checklist as part of the process of single laboratory validation. Further a generic protocol has been developed that provides the basic framework for integrating the requirements for the single laboratory validation of all analytical methods intended for adoption by the NSSP. Methods submitted to the Interstate Shellfish Sanitation Conference (ISSC) Laboratory Methods Review (LMR) Committee for acceptance will require, at a minimum, six (6) months for review from the date of submission.

Name of the New Method	PSP Alert™
Name of the Method Developer	Sensoreal Inc.
Developer Contact Information	Hosseinali Safavieh 6674 Esplanade Avenue (Suite B), Montreal QC H2V 4L5

A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		There is a need for a simple, rapid detection tool for reliably detecting paralytic shellfish toxins (PSTs) such as saxitoxin (STX) in shellfish, that can be used in laboratory settings.
2. What is the intended purpose of the method?		Qualitative screening of PSTs in mussels.
3. Is there an acknowledged need for this method in the NSSP?		There is a need for simple assays that deliver rapid and accurate results for limited-use screening in marine biotoxin testing.
4. What type of method? i.e. chemical, molecular, culture, etc.		Lateral flow immunoassays
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		PSP Alert™
Method Scope		Qualitative screening and detection of PSTs such as STX, neosaxitoxin (NeoSTX), etc in mussels only.
References		Study Report, kit insert/IFU and SDS and Quality Manual (QM) included in submission
Principle		Competitive lateral flow immunoassay for detection of PSTs using aqueous-based extraction.
Any Proprietary Aspects		Yes, commercial test kit
Equipment Required		Centrifuge, shucking tools, conical tubes, blender, timer, pipettes, Sensoreal Sensoread™ scanner.
Reagents Required		PSP Alert™ test devices and provided reagents inside the kit.

Sample Collection, Preservation and Storage Requirements		Shellfish should be collected according to standard industry practices and stored at 2–8°C prior to testing.
Safety Requirements		Used test devices, concical tubes containing slurry or shellfish extracts, and pipettes should be handled as though contaminated with PSTs and disposed of accordingly. Gloves and lab coats should be worn during testing.
Clear and Easy to Follow Step-by-Step Procedure		Step-by-step procedure card included in kit insert and submission
Quality Control Steps Specific for this Method		Each test device includes an internal control line to confirm proper function. A mussel certified reference material (CRM-PSP-Mus) from the National Research Council Canada can be used as positive control.
C. Validation Criteria		
1. Accuracy / Trueness (Probability of Detection)		100% accurate detection of PSTs was achieved for the following conditions: ● 100% of mussel samples with overall PST levels at or above the regulatory limit of 80 µg STX eq/100g (0% false negatives). ● 100% of mussel samples with overall PST levels below 49 µg STX eq/100g were all accurately detected as negative.
2. Measurement Uncertainty		Not applicable
3. Precision Characteristics (repeatability and reproducibility)		Not applicable.
4. Recovery		Not applicable
5. Specificity		Not applicable
6. Working and Linear Ranges		Not applicable
7. Limit of Detection		The positive threshold of the PSP Alert™ has been set at 65 µg STX eq/100g.
8. Limit of Quantitation / Sensitivity		Not applicable.
9. Ruggedness		Several parameters were evaluated. ● LFA development time and initial sample volume support tolerances of ±2 min (13-17 min) and ± 4 µL (16-24 µL), respectively. ● Assay performance remained consistent across 12–34°C and 46–77% Relative Humidity (RH). ● Sample–conjugate volume between 68–75 µL produced the expected result in all replicates. ● Lot consistency: Results were consistent across two LFA lots and two conjugate lots.
10. Matrix Effects		Matrix effects cannot be properly evaluated as only one species was evaluated.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		The assay demonstrated comparability to the HPLC-PCOX reference method when testing naturally incurred mussel samples, with 0% false negatives being observed.
D. Other Information		
1. Cost of the Method		Approximately $32/test
2. Special Technical Skills Required to Perform the Method		None. Training on assay operation is provided by Sensoreal Inc..

3. Special Equipment Required and Associated Cost		Centrifuge. Sensoreal Sensoread™ scanner (approximately $4000)
4. Abbreviations and Acronyms Defined		All relative abbreviations and acronyms have been defined in the study report.
5. Details of Turn Around Times (time involved to complete the method)		The test can be operated in under 60 minutes, including sample preparation.
6. Provide Brief Overview of the Quality Systems Used in the Lab		

Submitters Signature 	Date: August 21, 2026
Submission of Validation Data and Draft Method to Committee	Date:
Reviewing Members	Date:
Accepted	Date:
Recommendations for Further Work	Date:
Comments:	

DEFINITIONS

1. **Accuracy/Trueness** - Closeness of agreement between a test result and the accepted reference value.
2. **Analyte/measurand** - The specific organism or chemical substance sought or determined in a sample.
3. **Blank** - Sample material containing no detectable level of the analyte or measurand of interest that is subjected to the analytical process and monitors contamination during analysis.
4. **Comparability** – The acceptability of a new or modified method as a substitute for an established method in the NSSP. Comparability must be demonstrated for each substrate or tissue type by season and geographic area if applicable.
5. **Fit for purpose** – The analytical method is appropriate to the purpose for which the results are likely to be used.
6. **HORRAT value** – HORRAT values give a measure of the acceptability of the precision characteristics of a method.[4]
7. **Limit of Detection** – the minimum concentration at which the analyte or measurand can be identified. Limit of detection is matrix and analyte/measurand dependent.[4]
8. **Limit of Quantitation/Sensitivity** – the minimum concentration of the analyte or measurand that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.
9. **Linear Range** – the range within the working range where the results are proportional to the concentration of the analyte or measurand present in the sample.
10. **Measurement Uncertainty** – A single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.
11. **Matrix** – The component or substrate of a test sample.
12. **Method Validation** – The process of verifying that a method is fit for purpose.[1]
13. **Precision** – the closeness of agreement between independent test results obtained under stipulated conditions.[1, 2] There are two components of precision:
    a. **Repeatability** – the measure of agreement of replicate tests carried out on the same sample in the same laboratory by the same analyst within short intervals of time.
    b. **Reproducibility** – the measure of agreement between tests carried out in different laboratories. In single laboratory validation studies reproducibility is the closeness of agreement between results obtained with the same method on replicate analytical portions with different analysts or with the same analyst on different days.
14. **Quality System** - The laboratory's quality system is the process by which the laboratory conducts its activities so as to provide data of known and documented quality with which to demonstrate regulatory compliance and for other decision–making purposes. This system includes a process by which appropriate analytical methods are selected, their capability is evaluated, and their performance is documented. The quality system shall be documented in the laboratory's quality manual.
15. **Recovery –** The fraction or percentage of an analyte or measurand recovered following sample analysis.
16. **Ruggedness** – the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.[4]
17. **Specificity** – the ability of a method to measure only what it is intended to measure.[1]
18. **Working Range –** the range of analyte or measurand concentration over which the method is applied.

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Date: August 21, 2026

Laboratory Methods Review & Quality Assurance Committee
Interstate Shellfish Sanitation Conference
209-2 Dawson Road
Columbia, SC 29223-1740

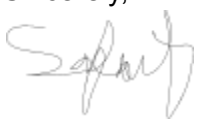
Please find enclosed the single laboratory validation (SLV) study report and supporting documentation for PSP Alert™, Sensoreal Inc.'s rapid qualitative assay for the detection of paralytic shellfish toxins (PSTs), including saxitoxin (STX), in shellfish. PSTs are responsible for paralytic shellfish poisoning and represent an important public-health concern in areas affected by harmful algal blooms.

We respectfully request the Committee's review of this submission and consideration of PSP Alert™ for acceptance as an ISSC-approved screening method for the determination of STX and other relevant PSTs in mussels. The assay offers important advantages in rapid time-to-result, procedural simplicity, and ease of implementation in laboratory settings. Its acceptance by the ISSC could provide meaningful benefits to shellfish growers, testing laboratories, regulators, and public-health authorities by supporting timely screening and informed management decisions.

The SLV study was designed and conducted in accordance with feedback provided by the ISSC Laboratory Methods Review Committee following its review of the PSP Alert™ pre-proposal. The study therefore included evaluation of assay performance using naturally contaminated mussel samples through comparability and probability of detection (POD) studies, assessment of cross-reactivity and potential interference from other marine biotoxins, and an extensive ruggedness evaluation. The resulting validation demonstrated reliable detection at the regulatory action level, a conservative safety margin below that level, strong sensitivity, acceptable specificity, broad recognition of relevant PST congeners, and robust performance across reasonable procedural and environmental variations.

We would be pleased to answer any questions or provide any additional information that may assist the Committee in its review. Thank you for your time and consideration of this submission.

Sincerely,



Hosseinali Safavieh
CEO, Sensoreal Inc.

Enclosures:	SLV Study Report	ISSC Method Application Checklist
	PSP Alert™ IFU & Procedure card	PSP Alert Safety Data Sheet
	Safety Data Sheet	Sensoreal PSP Alert Laboratory Checklist
	PSP Alert-ISSC Proposal form	

Single Laboratory Validation Study of the Sensoreal PSP Alert Lateral Flow Assay for the Qualitative Detection of Saxitoxin and other Paralytic Toxins in Mussels

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1. Introduction

Paralytic shellfish toxins (PSTs), including saxitoxin (STX) and related analogues, are a major food safety concern in shellfish harvesting and aquaculture[1]. These toxins can accumulate in shellfish during harmful algal bloom (HAB) events and pose serious health risks if contaminated shellfish enter the food supply as consumption of these shellfish result in paralytic shellfish poisoning (PSP)[2,3]. Existing laboratory-based methods for PST detection, such as HPLC with post-column oxidation (HPLC-PCOX)[4,5], provide high analytical reliability but require specialized equipment, trained personnel, and centralized laboratory access.

This creates significant practical limitations for timely decision-making, particularly for shellfish harvesters, small producers, and remote communities[6]. Delays in sample transport and testing can reduce responsiveness during toxin events and increase operational and public health risks.

To address this gap, Sensoreal Inc. developed PSP Alert, a rapid, portable, lateral flow assay (LFA)-based screening tool intended to support faster, more accessible PST monitoring. PSP Alert is designed to classify shellfish samples into qualitative results and additionally practical concentration categories and to provide a user-friendly alternative or complement to centralized laboratory testing.

2. Principle of the Method

Sensoreal PSP Alert™ is a rapid lateral flow immunoassay (LFA) designed for the qualitative detection of saxitoxin (STX) and related PSTs in bivalve mollusks. The assay uses a competitive binding format. PSTs are first extracted from homogenized shellfish tissue using a water-based extraction solution. The extract is then diluted and incubated with a lyophilized antibody–gold nanoparticle conjugate. We respectfully request your review of this submission and consideration of PSP Alert for acceptance as an ISSC-approved method for the determination of PSTs in mussels only.

When STX and other PSP toxins are present in the sample, they bind to the conjugate antibodies, reducing the amount of free antibody–gold complex available to bind at the test line. The mixture is then added to the lateral flow cassette, where it migrates across a membrane

containing immobilized STX-protein at the test line. As a result, test-line intensity is inversely proportional to the total PSP toxin concentration (reported as µg STX eq/100g): higher PSP levels produce a fainter test line. A control line appears in all valid tests to confirm proper assay performance. Because the antibody was raised against STX, its affinity varies across individual PSP congeners, with the strongest affinity observed for STX.

Results are read using the Sensoreal SensoRead™ scanner, which provides a numerical test/control (T/C) ratio and a qualitative classification (High, Medium, or Low). In this validation study, High results were considered positive, while Low and Medium results were considered negative unless otherwise specified. The assay is intended for users with basic laboratory skills (e.g., shellfish handling, homogenization, and simple pipetting) and is designed as a screening tool to support harvest-area closure decisions and help prevent contaminated shellfish from reaching the market. Although the technology can be applied to bivalve mollusks more broadly, the current validation and approval effort is focused specifically on mussels only.

3. Intended Use

PSP Alert™ is intended for the qualitative screening of shellfish for STX and other PSTs in mussels only, with a positive result indicating samples at or above the toxic/regulatory threshold of 80 µg STX eq/100g. The test kit is designed for use by quality control personnel, laboratory staff, and other trained users familiar with handling shellfish samples and performing basic sample preparation and pipetting procedures.

4. PSP Alert Method

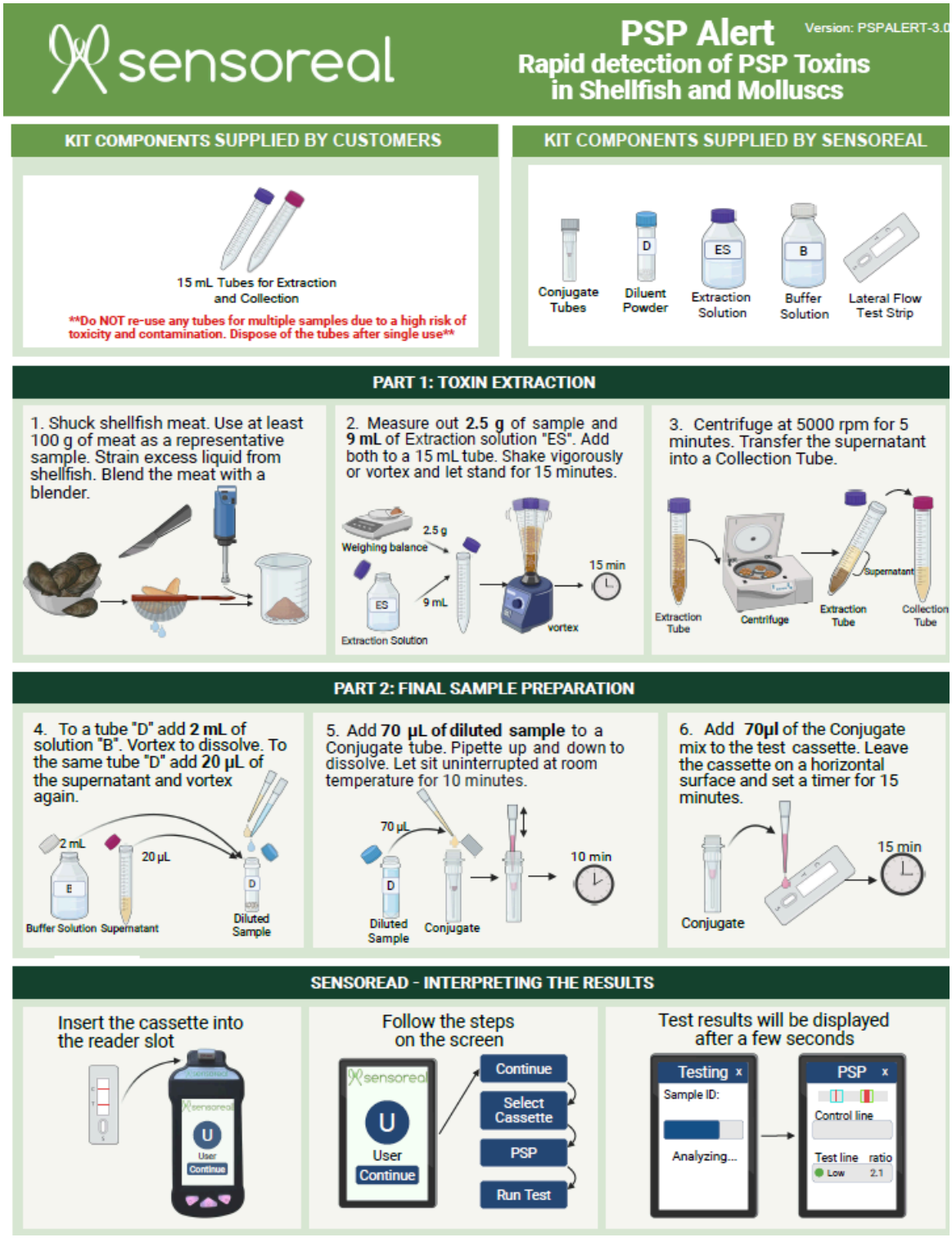


Figure 1: Procedure card illustrating the PSP Alert method.

4.1 Materials Provided

The PSP Alert™ kit includes the following:

- LFA cassettes,
- lyophilized antibody conjugate vials,
- proprietary buffer solution for extraction and lyophilized reagent for dilution,
- polypropylene sample tubes,
- 1X phosphate-buffered saline (PBS) (required for making diluent solution),
- Sensoreal SensoRead™ scanner (required for interpreting results).

4.2 Additional materials required, but not provided

Equipment and consumables not provided with the kit but necessary for operation include:

- shellfish shucking tools
- blenders for shellfish tissue homogenization
- an analytical balance
- electronic timer
- Centrifuge
- adjustable laboratory pipettes covering volumes of 20–200 µL, and 100–1000 µL.
- Additional accessories such as test tube racks, disposable pipette tips, and a vortex mixer are also required.

4.3 Precautions

- Do not open cassette or conjugate components until ready to use. Keep all test components in their original packaging/containers until the time of testing.
- Do not use kit contents beyond the expiration date.
- Treat all samples, extracts, and used test components as potentially contaminated with domoic acid and biological material. Wear appropriate PPE (e.g., gloves, lab coat, eye protection) during testing.
- To avoid cross-contamination, use clean pipette tips/tubes and fresh extraction/dilution materials for each sample
- Follow the specified extraction, dilution, incubation, and read times exactly; deviations may affect test performance.
- Use only the designated Sensoreal reader/method settings for interpretation, as applicable.
- Dispose of sample extracts, methanol-containing waste, and used kit components in accordance with institutional, local, and regulatory requirements.
- Safety documentation (SDS) for relevant reagents/solutions should be available to users upon request / maintained by the laboratory.

4.4 Storage Requirements

- Store PSP Alert™ kit components according to the product IFU labeling (controlled storage conditions as specified by Sensoreal).
- Do not freeze kit components, except the antibody conjugate.
- Keep components in their original packaging until use.
- Protect kit components from excess heat, moisture, and direct sunlight.
- Do not use components that are expired or show signs of damage/deterioration.

4.5 Sensoread reader set-up

- Turn on the reader and scan the QR code to select the correct test for the lot number.

4.6 PSP Alert Method Protocol

- For LFA testing, homogenized whole flesh was prepared using at least 10 individual shellfish per sampling site. This is equivalent to 80-100g of sample meat.
- A 2.5 g aliquot of homogenized tissue was weighed into an empty tube.
- 9 mL of provided extraction solution was added. The sample was vigorously shaken and left to stand for 15 minutes.
- The mixture was centrifuged at 5000 rpm for 5 minutes.
- 2 mL of 1X PBS was added to the vial containing the dry diluent powder to prepare the diluent solution. A 20 µL aliquot of the extract supernatant was then transferred into the freshly prepared diluent solution.
- 70 µL of the diluted extract was added to a conjugate vial.
- The conjugate vial contents were mixed thoroughly and incubated for 10 minutes.
- The incubated mixture was then applied to the absorbent pad of the test strip.
- The test strip was allowed to develop for 15 minutes.
- After exactly 15 minutes of development, the test cassette was immediately inserted into the reader slot of the SensoRead™ device. Cassette → PSP Alert™ was selected using the on-screen prompts.
- The Lot used for testing was then identified. An existing Lot was selected from the on-screen list, or, for a new Lot, “Add New Lot” was selected and the batch-specific QR code located on the LFA test pouch or Certificate of Analysis was scanned. The QR code provided the lot-specific calibration parameters required for result interpretation. Each Lot was scanned only once and was subsequently selected from the saved list for additional tests using the same Lot.
- Once the Lot was confirmed, the unique Sample ID was entered using the on-screen keypad and “Run Test” was selected. The test was analyzed by the device, and the resulting T/C ratio and qualitative classification were recorded.
    - Note: To minimize the risk of false negatives and provide a safety buffer for shellfish growers, the PSP Alert threshold has been set at 65 µg STX eq/100g, rather than the regulatory limit of 80 µg STX eq/100g. As a result, samples above 65 µg STX eq/100g are classified as positive, enabling earlier detection before reaching the regulatory action threshold.

4.7 HPLC-PCOX method analysis

- The reference or ISSC-approved method utilized for this study was HPLC-PCOX, as detailed by Riet and colleagues[4].

4.7.1 Sample collection and preparation

- The outside of the shellstock was thoroughly cleaned with fresh water.
- Meat was removed from a representative sample of 10–15 animals by opening the shells and separating the tissue.
- The tissue was collected on a 2 mm sieve, allowed to drain for 5 minutes, and then homogenized using a blender.

4.7.2 Acid extraction of toxins

- 5.0 g of homogenized shellfish tissue was transferred into a 50 mL polypropylene centrifuge tube.
- 5.0 mL of 0.1 M HCl was added, and the tube was capped and vortexed thoroughly.
- The pH was checked and adjusted to 2.0–4.0 (preferably ~3.0) using 5 M HCl or 5 M NaOH, if needed.
- The capped tube was placed in a boiling water bath for 5 minutes and then cooled to room temperature.
- The pH was rechecked and readjusted to 2.0–4.0 if necessary.
- The supernatant was separated by gravity or by centrifugation at 3000 × g for 10 minutes.
- The supernatant was decanted into a vial.

4.7.3 Deproteination and cleanup

- 500 µL of the supernatant was transferred into a microcentrifuge tube.
- 25 µL of 30% (v/v) trichloroacetic acid (TCA) was added for deproteination.
- The mixture was vortexed thoroughly and centrifuged at 16,000 × g for 5 minutes.
- 35 µL of 1.0 M NaOH was added to raise the pH.
- The mixture was centrifuged again at 16,000 × g for 5 minutes.
- The supernatant was filtered through a 0.2 µm nylon syringe filter into an autosampler vial for LC analysis.

4.7.4 LC-PCOX system setup and equilibration

- The liquid chromatography (LC) and post-column oxidation (PCOX) system was set up with the required analytical column, mobile phases, and post-column reagents for the toxin group being analyzed.
- The LC gradient conditions were programmed, and all reagent lines were primed with fresh reagents.
- The system was equilibrated for at least 20 minutes at 30–40°C using:
    - 100% solvent A at 0.8 mL/min
    - post-column oxidant at 0.4 mL/min
    - post-column acid at 0.4 mL/min
- Stable temperature conditions were maintained because temperature fluctuations can affect retention times.

4.7.5 System suitability check

- Mixed working standards were injected to confirm chromatographic separation and overall system performance.
- Typical injection volumes for system suitability were:
    - GTX/STX toxins: 10 µL
    - C toxins: 5 µL
- Gradient conditions or column temperature were adjusted, when needed, to improve peak resolution.
- The required signal-to-noise performance was verified to ensure method sensitivity targets (LOD/LOQ) were met. Injection volumes were increased within the acceptable range, when necessary, to improve detectability.

4.7.6 Sample and calibration analysis

- Calibration solutions, sample extracts, blanks, and spiked samples were injected into the LC system.
- The injection volume was selected based on the system suitability evaluation and within the allowed range for each toxin group.
- Chromatographic peaks were integrated for each analyte.

4.7.7 Quantification and calculation

- Calibration curves were generated by plotting peak area vs. concentration (µM) for the calibration solutions.
- Linear regression from the calibration curves was used to calculate toxin concentrations in the sample extracts.
- Results were expressed as µmoles STX·diHCll equivalents/L, using the appropriate relative toxicities for individual toxins.

5. Single Laboratory Validation study

A single-laboratory validation study was conducted to measure or evaluate the following parameters for PSP Alert:

- Comparability[7]
- Probability of Detection[7]
- Ruggedness[7]
- Interference or Selectivity study[8]
- Cross-reactivity[7]

For any naturally contaminated shellfish sample, PSP Alert results were compared to those of an accepted HPLC-PCOX reference method[4]. Matrices tested were mussels only.

5.1 Comparability

Method

- Comparability is demonstrated when a new method intended as an alternative/substitute does not produce a significant difference in results compared with the officially recognized NSSP method for the same matrix and intended use.

- Naturally incurred mussel samples were first collected from different sites and different seasons. A minimum of 10-11 animals were used for each shellfish Sample ID. These shellfish were shucked, 80-100g of meat was measured and homogenized. Then 3 aliquots were measured. One aliquot was used to test with PSP Alert. The second aliquot was tested using the will be tested using the reference method of HPLC-PCOX and the correct concentration (µg STX eq/100g) was obtained. The final aliquot was used in the following Probability of Detection Study.

- For this portion of the study, 30 naturally contaminated mussel samples were tested. False negative and false positive rates were recorded[10].

Results

- Results of the comparability study, summarized in Table 1, demonstrate that the PSP Alert™ is an effective screening method for the qualitative determination of PSP toxins in mussels.

- The accuracy for the assay for mussel samples containing PSP toxins at or above the regulatory limit of 80 µg STX eq/100g was 100%. All 10 samples tested with HPLC-PCOX concentrations of ≥ 80 µg STX eq/100g produced positive results with PSP Alert with no false negative results.

- All mussel samples containing PSP toxin levels below 53 µg STX eq/100g (14 unique samples tested) yielded negative results.

- Consistent with the intended use as a conservative screening tool, the assay is designed to detect PSTs at levels approaching the regulatory limit to ensure a margin of safety. As the threshold of the assay was set at 65 µgSTX eq/100g, mussel samples with a concentration between 53-62 µg STX eq/100g (n =4) yielded a mix of negative and positive results. Consequently, all samples above 65 µg STX eq/100g (n = 10) were reliably detected as positive.

- Based on the precautionary limit of 65 µg STX eq/100g for shellfish meat, no false negatives were observed. One sample (3.3%) yielded a false positive result.

- The PST breakdown or composition of each unique sample ID is provided in Appendix 1.

Table 1. Naturally contaminated mussel samples tested with PSP Alert and reference method HPLC-PCOX.

Matrix	Sample ID	PSP level (ugSTX/100g)	Reader Qualitative Result
Mussels	BUR-FI-2025-MT-00109	<25	-
	BUR-FI-2025-MT-00143	<25	-
	BUR-FI-2025-MT-00759	26	-
	BUR-FI-2024-MT-02753	29	-
	BUR-FI-2025-MT-00540	32	-
	BUR-FI-2025-MT-01500	34	-
	BUR-FI-2024-MT-01389	36	-
	BUR-FI-2025-MT-00041	39	-
	BUR-FI-2025-MT-03508	40	-
	BUR-FI-2024-MT-02016	42	-
	BUR-FI-2024-MT-02714	44	-
	BUR-FI-2024-MT-03726	45	-
	BUR-FI-2024-MT-01103	46	-
	BUR-FI-2025-MT-01495	47	-
	BUR-FI-2024-MT-01299	53	+
	BUR-FI-2024-MT-03008	59	-
	BUR-FI-2024-MT-02004	60	-

	BUR-FI-2024-MT-01778	62	-
	BUR-FI-2025-MT-03210	66	+
	BUR-FI-2024-MT-01835	67	+
	BUR-FI-2024-MT-02657	94	+
	BUR-FI-2024-MT-02770	88	+
	BUR-FI-2025-MT-01490	91	+
	BUR-FI-2024-MT-02065	110	+
	BUR-FI-2024-MT-02736	130	+
	BUR-FI-2024-MT-02036	140	+
	BUR-FI-2024-MT-02807	150	+
	BUR-FI-2024-MT-01786	170	+
	BUR-FI-2024-MT-02037	200	+
	BUR-FI-2024-MT-02724	210	+

5.2 Probability of Detection

Method

- Probability of Detection (POD) is the probability that a qualitative method produces a positive response at a given analyte concentration. POD varies with concentration and provides a single performance description that inherently reflects false-negative and false-positive behavior across the working range.

- Naturally contaminated mussel samples were used.

- For the POD study, an aliquot of the 30 natural samples used in the previous Comparability study were used, along with 70 additional natural samples were from different regions and seasons, totalling to a 100 unique samples. A minimum of 10-11 animals were used for each shellfish Sample ID. These shellfish were shucked, 80-100g of meat was measured and homogenized. Then 2 aliquouts were measured. One aliquot was used to test with PSP Alert. The second aliquot was tested using the reference method of HPLC-PCOX and the correct concentration (ugSTX eq/100g) was obtained.

- Several mussel samples were then tested and the samples were categorized into 5 distinct concentration ranges, each containing 20 independent samples. These include:
    - PST level: < 25 µg STX eq/100g
    - PST level: 25-39 µg STX eq/100g
    - PST level: 40-59 µg STX eq/100g
    - PST level: 60-79 µg STX eq/100g
    - PST level: 80-210 µg STX eq/100g

- False-positive and false-negative results were recorded, and the sensitivity and specificity of PSP Alert™ were subsequently calculated[10].

Result

- In the POD study, PSP Alert was evaluated using mussel samples spanning five PSP toxin concentration ranges expressed as µgSTXeq/100g (<25, 25–39, 40–59, 60–79, and 80-210). Results of the POD study are summarized in Table 2.

- PSP Alert yielded 100% negative results for all samples in the two lowest toxin ranges (<25 and 25–39 µg STX eq/100g), and the proportion of positive results increased as PSP concentration rose in the mid-range groups:
    - 40–59 µg STX eq/100g: 5/20 (25%)
    - 60–79 µg STX eq/100g: 17/20 (85%).

- Consistent with the comparability study, PSP Alert achieved 100% correct classification for mussel samples at or above the regulatory limit of 80 µg STX eq/100g.

- With the assay's conservative safety buffer, all mussel samples containing PST concentrations above 65 µg STX eq/100 g were classified as positive (n = 31), supporting the use of PSP Alert™ as a protective screening tool for growers and other end users. Using the precautionary PSP Alert™ threshold of 65 µg STX eq/100 g, no false-negative results were observed, while 9 samples (9%) yielded false positive results. Based on these classification outcomes, PSP Alert™ demonstrated a sensitivity of 1.00 and a specificity of 0.87.

- A dose–response curve for the combined sample set, illustrating the increase in the percentage of positive results with increasing PST concentration, is presented in Figure 2. The individual PST profile or toxin composition, and corresponding test results for each unique sample ID are provided in Appendix 1.

Table 2. Results of the POD study of PSP Alert with mussels.

Species	PST level (µg STX eq/100g)	Number of samples	Number of Positive results	Percentage Positive results
Mussels	Less than 25	20	0	0%
	25-39	20	0	0%
	40-59	20	5	25%
	60-79	20	17	85%
	80-210	20	20	100%

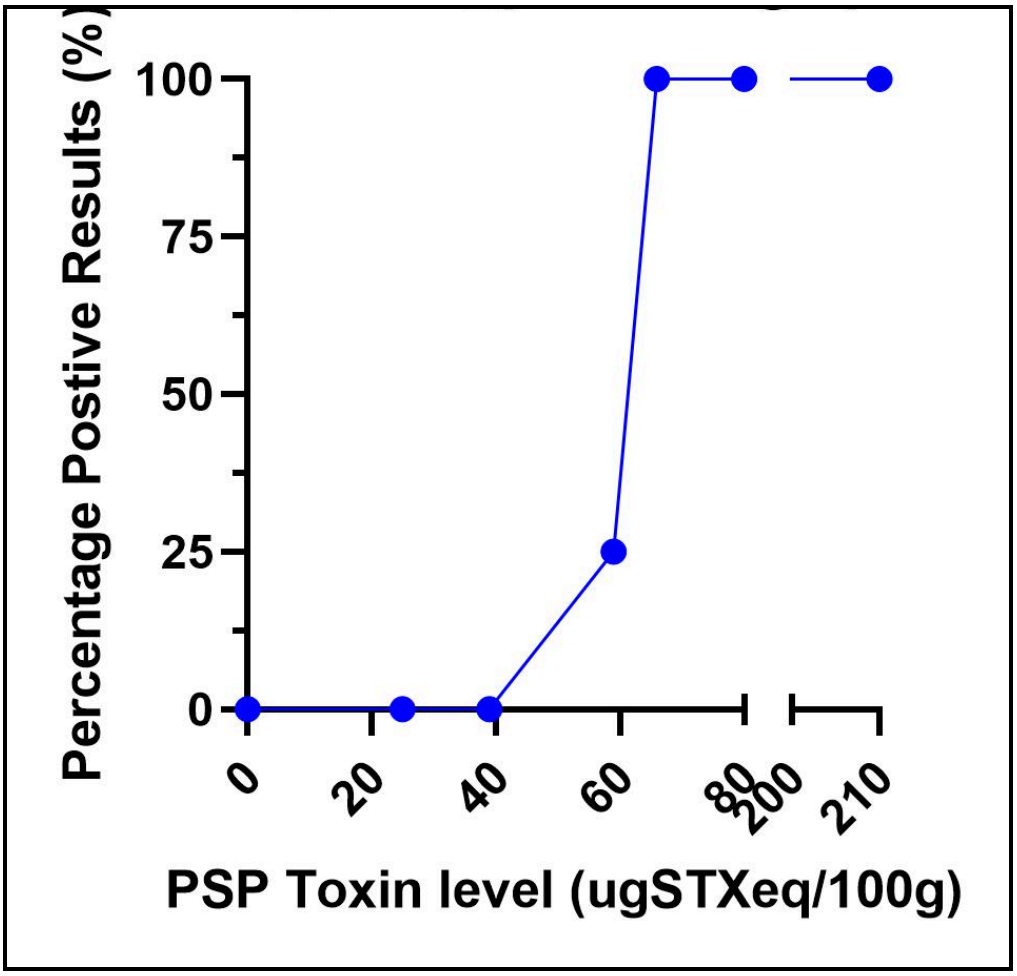


Figure 2: Dose-response curve generated with PSP Alert on mussels samples.

5.3 Ruggedness

Method

- Ruggedness is defined as the ability of a particular method to withstand relatively minor changes in analytical technique, reagents, or environmental factors likely to arise in different test environments.

- For PSP Alert™, several critical parameters were systematically evaluated to assess the assay's resilience to such variations and to characterize its overall ruggedness. These parameters included:
    - LFA development time before result interpretation;
    - Volume of sample used in the initial dilution step;
    - Environment or laboratory room temperature during assay performance;
    - Volume of sample-conjugate solution for the LFA run; and
    - Relative humidity during assay performance.

5.3.1 LFA development time

Method

- Per ISSC guidance, 10–12 analyte-free animals were homogenized to prepare individual mussel samples respectively. These samples were then spiked with STX certified reference material (CRM) slightly above the action level (81 µgSTX eq/100g), above the PSP Alert™ assay threshold of 65 µgSTX eq/100g to generate multiple samples

- The STX CRM was purchased from the National Research Council Canada (NRCC; CRM STX-g; Lot # 20201215).

- For each sample, one aliquot was tested using the standard PSP Alert™ procedure. An additional aliquot from the same homogenate was processed identically but read at intentionally shortened or extended run times to reflect realistic operator variation. Both were further sub-aliquoted to yield 3 replicate analyses for the specific LFA run time.

- The different LFA run times are:
    - 15 mins - standard LFA run time
    - 13 mins
    - 17 mins

Results

- Results of the PSP Alert™ ruggedness trials for the LFA development time are summarized in Table 3. No differences were observed in the number of positive results across the incubation times (13 mins -17 mins) evaluated for any spike level (Table 3), indicating consistent assay performance within ± 2 minutes of the standard time.

Table 3: Ruggedness trials for PSP Alert – effect of LFA run time.

Species	PST concentration (µg STX eq/100g)	Sample ID#	Number of Replicates	Number of Positives at 13 min	Number of Positives at 15 min (standard time)	Number of Positives at 17 min
Mussels	81	1	3	3	3	3
	81	2	3	3	3	3
	81	3	3	3	3	3
	81	4	3	3	3	3
	81	5	3	3	3	3
	81	6	3	3	3	3
	81	7	3	3	3	3
	81	8	3	3	3	3
	81	9	3	3	3	3
	81	10	3	3	3	3

5.3.2 Volume of sample used in the initial dilution step

Method

- A total of 10 mussel samples were used in this part of the study. One (1) sample was a naturally contaminated mussel sample containing 88 µg STX eq/100 g. The toxin breakdown is provided in Appendix 1. The remaining nine (9) were PST-free mussel homogenates spiked with STX CRM to approximately 84 µg STX eq/100 g. Each homogenate consisted of at least 10–12 animals.
- For each sample, one aliquot was tested using the standard PSP Alert™ procedure. Additional aliquots from the same homogenate were processed identically, except that the volume of sample used during the dilution step was varied. Each aliquot was further sub-aliquoted to provide three replicate analyses for each volume condition.
- The sample volumes (used in the dilution step) tested were:
    - 20 µL - standard volume
    - 16 µL
    - 18 µL
    - 22 µL
    - 24 µL

Results

- Results of the PSP Alert™ ruggedness evaluation assessing variations in the sample volume used during the dilution step are summarized in Table 4. No differences were observed in the number of positive results across the sample volumes evaluated, indicating consistent assay performance under the tested conditions.

Table 4: Ruggedness trials for PSP Alert – changes in sample volume used for dilution.

Species	PST concentration (µg STX eq/100g)	Sample ID#	Number of Replicates	Number of Positives for 16 µL	Number of Positives for 18 µL	Number of Positives for 20 µL (SOP time)	Number of Positives for 22 µL	Number of Positives for 24 µL	Conjugate/LFA Lot No.
Mussels (naturally contaminated)	88	BUR-FI-2025-MT-02770	3	3	3	3	3	3	Conjugate Lot# 2460766_40STXAb;
Mussel	84	M2	3	3	3	3	3	3	LFA

s (spiked)									Lot# 2461088
Mussels (spiked)	84	M3	3	3	3	3	3	3	
Mussels (spiked)	84	M4	3	3	3	3	3	3	
Mussels (spiked)	84	M5	3	3	3	3	3	3	
Mussels (spiked)	84	M6	3	3	3	3	3	3	Conjugate Lot# 2460739_40StxAb; LFA Lot# 2460893
Mussels (spiked)	84	M7	3	3	3	3	3	3	
Mussels (spiked)	84	M8	3	3	3	3	3	3	
Mussels (spiked)	84	M9	3	3	3	3	3	3	
Mussels (spiked)	84	M10	3	3	3	3	3	3	

5.3.3 Laboratory Room/Ambient temperature

Method

- A total of 10 mussel samples were used in this part of the study. One (1) sample was a naturally contaminated mussel sample containing 88 µg STX eq/100 g. The toxin breakdown is provided in Appendix 1. The remaining nine (9) were PST-free mussel

homogenates spiked with STX CRM to approximately 84 µg STX eq/100 g. Each homogenate consisted of at least 10–12 animals.

- For each sample, one aliquot was tested using the standard PSP Alert™ procedure under standard laboratory conditions. Additional aliquots from the same homogenate were processed identically, except that the ambient laboratory temperature was varied. Each aliquot was further sub-aliquoted to provide three replicate analyses for each temperature condition.
- Relative humidity (RH) was maintained between 46% - 48%, while the ambient temperature was varied. The following temperature conditions were evaluated:
    - 26°C -standard lab room temperature (RTP)
    - 12°C
    - 34°C

Results

- Results of the PSP Alert™ ruggedness evaluation assessing variations in ambient temperature are summarized in Table 5. No differences were observed in the number of positive results across 12°C- 34°C, indicating consistent assay performance throughout the tested range.

Table 5: Ruggedness trials for PSP Alert – changes in ambient temperatures.

Species	PST concentration (µg STX eq/100g)	Sample ID#	Number of Replicates	Number of Positives at 12°C	Number of Positives at 26°C (standard RTP)	Number of Positives at 34°C	Conjugate/LFA Lot No.
Mussels (naturally contaminated)	88	BUR-FI-2025-MT-02770	3	3	3	3	Conjugate Lot# 2460766_40STXAb; LFA Lot# 2461088
Mussels (spiked)	84	M2	3	3	3	3	
Mussels (spiked)	84	M3	3	3	3	3	
Mussels (spiked)	84	M4	3	3	3	3	
Mussels (spiked)	84	M5	3	3	3	3	

Mussels (spiked)	84	M6	3	3	3	3	Conjugate Lot# 2460739_40StxAb; LFA Lot# 2460893
Mussels (spiked)	84	M7	3	3	3	3	
Mussels (spiked)	84	M8	3	3	3	3	
Mussels (spiked)	84	M9	3	3	3	3	
Mussels (spiked)	84	M10	3	3	3	3	

5.3.4 Volume of sample-conjugate solution for LFA run

Method

- A total of 10 mussel samples were used in this part of the study. One (1) sample was a naturally contaminated mussel sample containing 88 µg STX eq/100 g. The toxin breakdown is provided in Appendix 1. The remaining nine (9) were PST-free mussel homogenates spiked with STX CRM to approximately 84 µg STX eq/100 g. Each homogenate consisted of at least 10–12 animals.
- For each sample, one aliquot was tested using the standard PSP Alert™ procedure. A second aliquot from the same homogenate was processed identically, except that the volume of the final sample–conjugate solution applied to the LFA was modified. Each aliquot was then further sub-aliquoted to provide three replicate measurements for each test condition.
- The different volumes of the sample–conjugate solution evaluated were:
    - 70 µL - standard method
    - 65 µL
    - 68 µL
    - 72 µL
    - 75 µL

Results

- Results of the PSP Alert™ ruggedness evaluation assessing variations in the final volume of the sample–conjugate solution applied to the LFA are summarized in Table 6.
- All 120 tests performed using sample–conjugate volumes between 68-75 µL produced the expected positive results. For the 65 µL sample–conjugate volume group, 19 of 30 tests were correctly identified as positive, while the remaining 11 produced negative

results. These findings indicated that a reduction to 65 µL was not acceptable for reliable testing, whereas sample–conjugate volumes between 68-75 µL were well tolerated.

Table 6: Ruggedness trials for PSP Alert – changes in sample-conjugate solution volumes.

Species	PST concentration (µg STX eq/100g)	Sample ID#	Positive results for 65 µL	Positive results for 68 µL	Positive results for 70 µL (standard volume)	Positive results for 72 µL	Positive results for 75 µL	Conjugate/LFA Lot No.
Mussels (naturally contaminated)	88	BUR-FI-2025-MT-02770	2	3	3	3	3	Conjugate Lot# 2460766_40STX Ab; LFA Lot# 2461088
Mussels (spiked)	84	M2	2	3	3	3	3	
Mussels (spiked)	84	M3	3	3	3	3	3	
Mussels (spiked)	84	M4	0	3	3	3	3	
Mussels (spiked)	84	M5	3	3	3	3	3	
Mussels (spiked)	84	M6	3	3	3	3	3	Conjugate Lot# 2460739_40StxAb; LFA Lot# 2460893
Mussels (spiked)	84	M7	2	3	3	3	3	
Mussels (spiked)	84	M8	1	3	3	3	3	
Mussels (spiked)	84	M9	3	3	3	3	3	
Mussels (spiked)	84	M10	1	3	3	3	3	

5.3.5 Relative humidity

Method

- A total of 10 mussel samples were used in this part of the study. One (1) sample was a naturally contaminated mussel sample containing 88 µg STX eq/100 g. The toxin breakdown is provided in Appendix 1. The remaining nine (9) were PST-free mussel

homogenates spiked with STX CRM to approximately 84 µg STX eq/100 g. Each homogenate consisted of at least 10–12 animals.

- For each sample, one aliquot was tested using the standard PSP Alert™ procedure. Additional aliquots from the same homogenate were processed identically, except that the ambient relative humidity (RH) of the laboratory was varied. Each aliquot was further sub-aliquoted to provide three replicate analyses for each RH condition.
- Ambient temperature was maintained within a narrow range of 24.7°C - 25.4°C. The following RH conditions were evaluated:
    - 46-48% RH, 24.7ºC- standard lab RH
    - 60-61% RH, 24.9ºC
    - 76-77% RH, 25.4ºC

Results

- Results of the PSP Alert™ ruggedness evaluation assessing variations in ambient RH are summarized in Table 7. No differences were observed in the number of positive results between 46-77% RH, indicating consistent assay performance.

Table 7: Ruggedness trials for PSP Alert– changes in ambient relative humidity.

Species	PST concentration (µg STX eq/100g)	Sample ID#	Positive results for 46-48% RH	Positive results for 60-61% RH	Positive results for 76-77% RH	Conjugate/ LFA Lot No.
Mussels (naturally contaminated)	BUR-FI-2025-MT-02770	BUR-FI-2025-MT-02770	3	3	3	Conjugate Lot# 2460766_40STXAb; LFA Lot# 2461088
Mussels (spiked)	84	M2	3	3	3	
Mussels (spiked)	84	M3	3	3	3	
Mussels (spiked)	84	M4	3	3	3	
Mussels (spiked)	84	M5	3	3	3	
Mussels (spiked)	84	M6	3	3	3	Conjugate Lot# 2460739_40StxAb;
Mussels (spiked)	84	M7	3	3	3	

Mussels (spiked)	84	M8	3	3	3	LFA Lot# 2460893
Mussels (spiked)	84	M9	3	3	3	
Mussels (spiked)	84	M10	3	3	3	

5.4 Selectivity or Interference study with potentially interfering toxins

Method

- LFA tests for PST detection should remain highly selective in the presence of other marine biotoxins, such as domoic acid (DA) and tetrodotoxin (TTX), which may co-occur during harmful algal blooms.
- Aliquots from three naturally contaminated mussel samples containing total PST concentrations of <25, 45, and 88 µg STX eq/100g were divided into two groups, Group A and Group B. PST congener profiles of these samples are provided in Appendix 1.
- For Group A, each mussel sample was fortified with DA CRM (CRM-DA-h; Lot# 20210922; NRCC) to a final concentration 21 mg DA/kg shellfish tissue, slightly above the regulatory limit of 20 mg DA/kg[9]. Each fortified sample was sub-aliquoted to produce three replicates.
- For Group B, each mussel sample was fortified with TTX CRM (CRM-TTX; Lot# 20170328; NRCC) to a final concentration of 48 µg TTX/kg shellfish tissue, slightly above the 44 µg TTX/kg reference level established by the European Food Safety Authority (EFSA)[11]. Each fortified sample was sub-aliquoted to produce three replicates.

Results

- The results of the selectivity or interference study are summarized in Table 8. All samples produced the expected qualitative result, indicating no observable interference from either DA or TTX under the conditions evaluated.

Table 8. Results of selectivity study with potentially interfering toxins with naturally contaminated mussel samples.

Group	Interfering toxin and level	Sample ID	PST level (µg STX eq/100g)	Number of tests	Number of positive results
A	Domoic acid-21 mg/kg	BUR-FI-2024-MT-00040	< 25	3	0
		BUR-FI-2024-MT-03012	45	3	0

		BUR-FI-2024-MT-02770	88	3	3
B	Tetrodotoxin - 48 µg/kg	BUR-FI-2024-MT-00040	< 25	3	0
		BUR-FI-2024-MT-03012	45	3	0
		BUR-FI-2024-MT-02770	88	3	3

5.5 Cross-reactivity

Method

For LFA-based methods, cross-reactivity describes the relative response of the assay to individual analytes within the targeted toxin class, such as different PST congeners.

- Mussel tissue confirmed to be free of PSTs was used to prepare the sample homogenates. Each homogenate was prepared from a minimum of 10–12 mussels to provide a representative matrix.

- For each PST congener evaluated, multiple aliquots of PST-free mussel homogenate were fortified with the respective CRMs obtained from NRCC. Concentrations ranging from 0 to 200 ng/mL were prepared for each congener.The maximum molar concentration varied per PST congener based on molecular weight, ranging from 420 to 670 nM.

- Each fortified sample was further sub-aliquoted to provide three replicate analyses at each concentration.

- Following analysis using PSP Alert™, the test line-to-control line (T/C) ratio obtained for each concentration was normalized relative to the corresponding blank sample (0 nM) for that congener. Dose–response curves were generated by plotting the normalized assay response against the molar concentration of each PST congener.

- The following PST congeners were evaluated:
    - Saxitoxin (STX): CRM-STX-g; Lot# 20201215
    - Neosaxitoxin (NeoSTX): CRM-NEO-d; Lot# 20170411
    - Gonyautoxins 1 and 4 (GTX1&4): CRM-GTX1&4-e; Lot# 20211027
    - Gonyautoxins 2 and 3 (GTX2&3): CRM-GTX2&3-d; Lot# 20170419
    - Gonyautoxin 5 (GTX5): CRM-GTX5-d; Lot# 20191024
    - C1 and C2 toxins (C1&2): CRM-C1&2-c; Lot# 20201208
    - Decarbamoyl gonyautoxins 2 and 3 (dcGTX2&3): CRM-dcGTX2&3-d; Lot# 20220630

- IC_{50} values were obtained from each dose–response curve[12]. Cross-reactivity was determined relative to STX using the following equation:
    - Cross-reactivity (%) = [IC_{50} (STX) / IC_{50} (PST congener)] × 100

Result

- The results of the PSP Alert™ cross-reactivity study are summarized in Table 9, and the corresponding dose–response curves are presented in Figure 3.
- The cross-reactivity response relative to STX (100% by definition) for GTX2&3, NeoSTX, GTX1&4, GTX5, C1/C2 and dcGTX2&3 was 118.1%, 86.4%, 78.1%, 15.4%, 12.3% and 7.6% respectively.

Table 9. Cross-reactivity data for PSP Alert with selected PST congeners.

Toxin	IC_{50} concentration (nM)	Cross-reactivity (%)
STX	2.07	100.0
GTX2&3	1.75	118.1
NeoSTX	2.39	86.4
GTX1&4	2.65	78.1
GTX5	13.42	15.4
C1/C2	16.74	12.3
dcGTX2&3	27.27	7.6

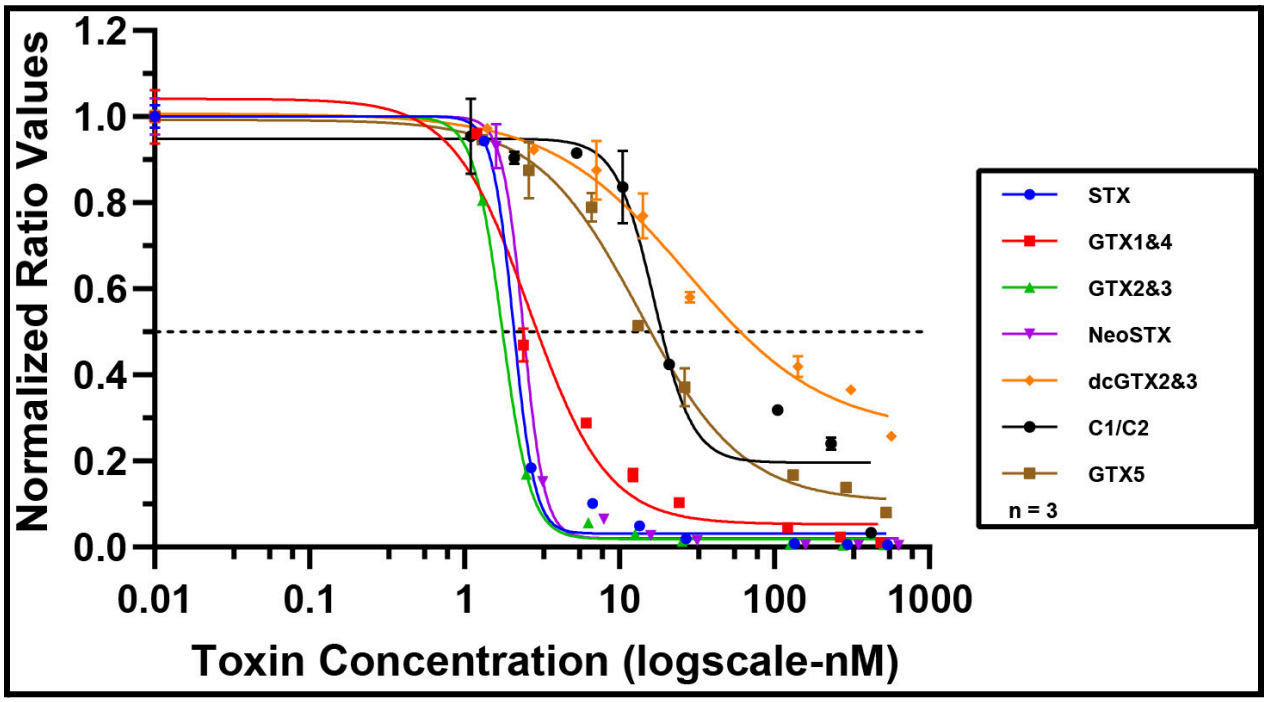


Figure 3. Cross-reactivity dose–response curves of PSP Alert™ for selected paralytic shellfish toxin (PST) congeners.

6. Quality Control

- Quality control (QC) testing for manufactured PSP Alert lots is performed at both the in-process and finished-product stages. For in-process QC, LFA cassettes are run using a blank diluent to confirm correct test and control line placement and acceptable line intensity.

- For finished-product release testing, PST–containing CRM mussel tissue (CRM PSP Mus, NRCC) is extracted and diluted to prepare equivalent toxin levels of 0, 20, 40, 70, 90, and 180 µg STX eq/100g. Each lot is then evaluated by testing the cassettes with these extracted QC samples (minimum of three replicates per level) to generate a dose–response curve. Lot acceptance criteria require that results between 0-40 µg STX eq/100g are negative and results at or above 70 µg STX eq/100g are all positive. QC standards are prepared as single-use aliquots and stored frozen to avoid repeated freeze–thaw cycles and to ensure consistent use of freshly prepared PSP toxin samples for each QC run.

7. Discussion

The present SLV study evaluated PSP Alert™ as a qualitative screening method for PST detection in mussels. Performance was assessed through comparability, POD, ruggedness, interference, and cross-reactivity studies using naturally contaminated and spiked samples. The results characterized assay classification near the regulatory action level, as well as its operating tolerances and selectivity.

Comparability and Probability of Detection

Comparability testing was performed using 30 naturally contaminated mussel samples, with PST concentrations determined using the HPLC-PCOX reference method. PSP Alert™ used a conservative assay threshold of 65 µg STX eq/100 g to provide a margin below the regulatory action level. All 12 samples at or above this threshold were correctly classified as positive, with no false-negative results and one false-positive result. Samples between approximately 50 and 65 µg STX eq/100 g showed mixed classifications, consistent with the transition region of a qualitative assay.

The POD study included 100 naturally contaminated samples, including separate aliquots of samples from the comparability study, and was used to further assess concentration-dependent assay performance. The proportion of positive results increased with PST concentration, from 0% in both the <25 and 25–39 µg STX eq/100 g groups, to 25% in the 40–59 µg STX eq/100 g group, 85% in the 60–79 µg STX eq/100 g group, and 100% in the 80–210 µg STX eq/100 g group. All 31 samples at or above the 65 µg STX eq/100 g assay threshold were classified as positive. Across the complete dataset, no false-negative results were observed, while 9% of samples produced false-positive results. This corresponded to a sensitivity of 1.00 and a specificity of 0.87.

Ruggedness

Ruggedness testing was performed to assess whether reasonable variations in assay testing parameters or environmental conditions affected qualitative classification. Parameters included

LFA development time, sample volume used for dilution, ambient temperature, sample–conjugate volume applied to the LFA, and relative humidity.

LFA development times of 13–17 minutes produced the expected positive results, indicating that a ±2-minute variation from the standard 15-minute run time was tolerated under the conditions tested. Similarly, varying the initial sample volume from 16–24 µL (±4 µL variation), compared with the standard 20 µL, did not affect qualitative classification. Environmental variation also had no observed effect within the tested ranges. Consistent results were obtained between 12–34°C at approximately 46–48% RH and between 46–77% RH when temperature was maintained near 25°C.

The sample–conjugate volume was identified as the most sensitive parameter. All tests using sample-conjugate volume between 68–75 µL produced the expected positive results, whereas the 65 µL condition produced 11 negative results among 30 replicates. These findings indicated that deviations from approximately −3 to +5 µL around the standard 70 µL volume were tolerated, while larger reductions from 65 µL could affect test development.

Interference or Selectivity and Cross-reactivity

Interference or selectivity testing was performed to assess other marine biotoxins such as DA and TTX could affect PSP Alert™ classification. Naturally contaminated mussel samples representing low, intermediate, and toxic PST concentrations were fortified with DA at 21 mg/kg or TTX at approximately 48 µg/kg. The addition of either toxin did not change the expected qualitative result. Samples containing total PST levels of <25 and 45 µg STX eq/100 g remained negative, while the sample containing 88 µg STX eq/100 g remained positive in all replicates. These results indicated no observable interference from DA or TTX under the conditions tested.

Cross-reactivity testing was performed separately to evaluate the response of PSP Alert™ to individual PST congeners. Dose-dependent responses were observed for all congeners tested. Relative to STX at 100%, cross-reactivity was highest for GTX2&3, NeoSTX, and GTX1&4 at 118.1%, 86.4%, and 78.1%, respectively, while lower but measurable responses were observed for GTX5, C1/C2, and dcGTX2&3 at 15.4%, 12.3%, and 7.6%, respectively. These differences were consistent with expected variation in antibody affinity among structurally different PST analogues.

The results therefore indicated that PSP Alert™ maintained the expected PST classification in the presence of DA and TTX while also responding to a range of PST congeners relevant to naturally contaminated shellfish.

Suitability for ISSC Adoption

PSP Alert™ provided rapid qualitative detection of PSTs with a simplified workflow compared with instrumental methods. In the SLV study, all naturally contaminated mussel samples at or above 65 µg STX eq/100 g were detected as positive, with no false-negative results. The 100-sample POD study showed a concentration-dependent response, with 9% false positives, a sensitivity of 1.00, and a specificity of 0.87 based on the precautionary threshold level of 65 µg STX eq/100 g. The assay also correctly detected the PST levels in mussel samples spiked with either DA or TTX, indicating no potential interference being caused by the presence of the toxins

Ruggedness testing also showed consistent qualitative results across LFA development times of 13–17 minutes (±2 µL deviation) , sample volumes of 16–24 µL (±4 µL deviation), ambient temperatures between 12–34°C, and relative humidity of approximately 46–77%. Sample–conjugate volumes of 68–75 µL were also well tolerated, while 65 µL produced inconsistent results and was identified as the most critical procedural variation.

Based on the data presented, PSP Alert™ performed consistently under the conditions evaluated and may be suitable for consideration by ISSC as a rapid qualitative screening method for PSTs in mussels.

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Appendix 1.

Table S1: PST profile breakdown of all the naturally-contaminated mussel samples used in SLV study for PSP Alert.

Sample ID	PST concentration (µg STX eq/100g)	GTX4	GTX1	dcGTX3	dcGTX2	GTX5	GTX3	GTX2	NEO	dcSTX	STX	C1	C2
BUR-FI-2025-MT-00109	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00
BUR-FI-2025-MT-00143	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00
BUR-FI-2025-MT-00956	<25	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00
BUR-FI-2024-MT-01398	<25	0.00	0.04	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.06	0.00	0.00
BUR-FI-2024-MT-01563	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00
BUR-FI-2024-MT-01653	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00
BUR-FI-2024-MT-01716	<25	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.05	0.00	0.00
BUR-FI-2024-MT-01720	<25	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUR-FI-2024-MT-01759	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUR-FI-2024-MT-01799	<25	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.04	0.00	0.01
BUR-FI-2024-MT-01805	<25	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.05	0.00	0.00
BUR-FI-2024-MT-01880	<25	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.00	0.05	0.00	0.00
BUR-FI-2024-MT-01881	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.06	0.00	0.00
BUR-FI-2024-MT-01900	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.00	0.00

BUR-FI-2024-MT-01914	<25	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.15	0.00	0.05	0.00	0.01
BUR-FI-2024-MT-01923	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUR-FI-2024-MT-01985	<25	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.10	0.00	0.07	0.00	0.01
BUR-FI-2024-MT-02800	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUR-FI-2024-MT-02801	<25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
BUR-FI-2024-MT-02804	<25	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.08	0.00	0.04	0.00	0.01
BUR-FI-2024-MT-03003	25	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.14	0.00	0.07	0.00	0.01
BUR-FI-2024-MT-03004	25	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.13	0.00	0.08	0.00	0.01
BUR-FI-2025-MT-00759	26	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.14	0.00	0.07	0.00	0.00
BUR-FI-2024-MT-03514	26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.12	0.00	0.01
BUR-FI-2025-MT-01496	26	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.16	0.00	0.09	0.00	0.01
BUR-FI-2025-MT-00763	27	0.00	0.16	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.11	0.00	0.00
BUR-FI-2024-MT-02808	28	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.18	0.00	0.06	0.00	0.01
BUR-FI-2024-MT-02753	29	0.00	0.06	0.00	0.00	0.00	0.01	0.01	0.13	0.00	0.05	0.00	0.02
BUR-FI-2024-MT-02748	29	0.00	0.11	0.00	0.00	0.00	0.02	0.02	0.00	0.00	0.13	0.00	0.01
BUR-FI-2024-MT-02761	29	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.17	0.00	0.08	0.00	0.04

BUR-FI-2025-MT-00352	29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.23	0.00	0.05	0.00	0.01
BUR-FI-2024-MT-02763	31	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.16	0.00	0.07	0.00	0.06
BUR-FI-2025-MT-00382	31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.24	0.00	0.06	0.00	0.01
BUR-FI-2025-MT-01497	31	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.20	0.00	0.09	0.00	0.01
BUR-FI-2025-MT-00540	32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.26	0.00	0.05	0.00	0.01
BUR-FI-2025-MT-00798	34	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.30	0.00	0.00	0.00	0.03
BUR-FI-2025-MT-01498	34	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.19	0.00	0.13	0.00	0.01
BUR-FI-2025-MT-01500	34	0.00	0.00	0.00	0.00	0.00	0.06	0.01	0.15	0.00	0.11	0.00	0.00
BUR-FI-2024-MT-01389	36	0.00	0.05	0.00	0.00	0.00	0.01	0.00	0.23	0.00	0.06	0.00	0.01
BUR-FI-2025-MT-00041	39	0.00	0.00	0.00	0.00	0.02	0.03	0.03	0.08	0.00	0.21	0.00	0.01
BUR-FI-2024-MT-02889	40	0.00	0.00	0.00	0.00	0.00	0.03	0.01	0.19	0.00	0.14	0.00	0.03
BUR-FI-2025-MT-03508	49	0.08	0.09	0.00	0.00	0.00	0.03	0.01	0.17	0.00	0.07	0.00	0.02
BUR-FI-2024-MT-02016	42	0.00	0.00	0.00	0.00	0.02	0.02	0.01	0.22	0.00	0.06	0.00	0.10
BUR-FI-2024-MT-02714	44	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.25	0.00	0.12	0.01	0.05
BUR-FI-2025-MT-00040	44	0.00	0.00	0.00	0.00	0.01	0.03	0.02	0.00	0.00	0.32	0.00	0.05
BUR-FI-2024-MT-03726	45	0.04	0.08	0.00	0.00	0.01	0.02	0.02	0.15	0.00	0.13	0.00	0.01

BUR-FI-2024-MT-03012	45	0.03	0.00	0.00	0.00	0.00	0.03	0.01	0.28	0.00	0.08	0.00	0.02
BUR-FI-2024-MT-01103	46	0.00	0.05	0.00	0.00	0.00	0.02	0.01	0.24	0.00	0.12	0.00	0.02
BUR-FI-2025-MT-00520	47	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.37	0.00	0.10	0.00	0.01
BUR-FI-2025-MT-00570	47	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.39	0.00	0.07	0.00	0.01
BUR-FI-2025-MT-01495	47	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.31	0.00	0.14	0.00	0.01
BUR-FI-2025-MT-01494	49	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.43	0.00	0.00	0.00	0.04
BUR-FI-2024-MT-01299	53	0.04	0.00	0.00	0.00	0.00	0.05	0.01	0.30	0.00	0.07	0.00	0.06
BUR-FI-2024-MT-01975	55	0.07	0.11	0.00	0.00	0.00	0.06	0.03	0.18	0.00	0.06	0.00	0.04
BUR-FI-2025-MT-01354	55	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.37	0.00	0.13	0.00	0.03
BUR-FI-2024-MT-02700	56	0.03	0.05	0.00	0.00	0.00	0.06	0.01	0.22	0.00	0.13	0.00	0.04
BUR-FI-2024-MT-03005	58	0.04	0.06	0.00	0.00	0.00	0.03	0.02	0.25	0.00	0.16	0.00	0.02
BUR-FI-2024-MT-01337	59	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.14	0.00	0.41	0.00	0.01
BUR-FI-2024-MT-02510	59	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.49	0.00	0.07	0.00	0.02
BUR-FI-2024-MT-03008	59	0.05	0.00	0.00	0.00	0.00	0.04	0.02	0.27	0.00	0.18	0.00	0.03
BUR-FI-2024-MT-02004	60	0.00	0.00	0.00	0.00	0.00	0.02	0.01	0.38	0.00	0.17	0.00	0.03
BUR-FI-2025-MT-03295	60	0.00	0.05	0.00	0.01	0.00	0.02	0.03	0.23	0.00	0.22	0.01	0.03

BUR-FI-2024-MT-01643	77	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.42	0.00	0.34	0.00	0.00
BUR-FI-2024-MT-01479	61.00	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.48	0.00	0.10	0.00	0.02
BUR-FI-2024-MT-02735	61	0.06	0.06	0.00	0.00	0.01	0.04	0.01	0.27	0.00	0.10	0.00	0.06
BUR-FI-2025-MT-03461	61	0.00	0.09	0.00	0.00	0.02	0.03	0.03	0.27	0.00	0.15	0.00	0.01
BUR-FI-2024-MT-01778	62	0.00	0.00	0.00	0.00	0.00	0.02	0.02	0.27	0.00	0.29	0.00	0.02
BUR-FI-2024-MT-02888	62	0.11	0.15	0.00	0.00	0.00	0.11	0.03	0.11	0.00	0.09	0.00	0.02
BUR-FI-2025-MT-01460	65	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.50	0.00	0.09	0.00	0.04
BUR-FI-2024-MT-01869	65	0.00	0.08	0.00	0.00	0.02	0.02	0.01	0.40	0.00	0.08	0.00	0.05
BUR-FI-2024-MT-01515	66	0.09	0.07	0.00	0.00	0.00	0.03	0.01	0.34	0.00	0.11	0.00	0.01
BUR-FI-2025-MT-03210	66	0.00	0.00	0.00	0.00	0.01	0.05	0.01	0.33	0.00	0.17	0.00	0.08
BUR-FI-2024-MT-01835	67	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.12	0.00	0.53	0.00	0.01
BUR-FI-2025-MT-00744	67	0.00	0.04	0.00	0.00	0.00	0.03	0.01	0.24	0.00	0.21	0.00	0.13
BUR-FI-2025-MT-03497	68	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.39	0.00	0.17	0.00	0.09
BUR-FI-2024-MT-01464	70	0.00	0.00	0.00	0.00	0.00	0.01	0.01	0.37	0.00	0.30	0.00	0.01
BUR-FI-2024-MT-02606	72	0.00	0.10	0.01	0.01	0.02	0.01	0.02	0.37	0.00	0.13	0.01	0.06
BUR-FI-2025-MT-03237	72	0.00	0.04	0.00	0.01	0.01	0.03	0.04	0.20	0.00	0.33	0.01	0.04

BUR-FI-2025-MT-03426	77	0.04	0.05	0.00	0.01	0.00	0.03	0.03	0.20	0.00	0.34	0.01	0.06
BUR-FI-2025-MT-03016	79	0.04	0.07	0.00	0.01	0.01	0.05	0.03	0.30	0.00	0.16	0.01	0.10
BUR-FI-2024-MT-02657	94	0.00	0.07	0.02	0.02	0.01	0.04	0.03	0.48	0.00	0.16	0.01	0.10
BUR-FI-2024-MT-01392	82	0.00	0.00	0.00	0.00	0.01	0.01	0.00	0.61	0.00	0.17	0.00	0.02
BUR-FI-2024-MT-02770	88	0.20	0.20	0.00	0.00	0.01	0.08	0.03	0.24	0.00	0.09	0.00	0.03
BUR-FI-2024-MT-02850	89	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.72	0.00	0.12	0.00	0.04
BUR-FI-2025-MT-01490	91	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.76	0.00	0.12	0.00	0.02
BUR-FI-2024-MT-00719	93	0.06	0.05	0.00	0.00	0.00	0.05	0.00	0.69	0.00	0.08	0.00	0.00
BUR-FI-2024-MT-02065	110	0.19	0.09	0.00	0.00	0.02	0.16	0.02	0.36	0.00	0.21	0.00	0.05
BUR-FI-2024-MT-03157	110	0.00	0.07	0.00	0.00	0.01	0.01	0.01	0.52	0.00	0.40	0.01	0.02
BUR-FI-2024-MT-01887	110	0.13	0.09	0.00	0.00	0.01	0.18	0.04	0.31	0.00	0.26	0.00	0.09
BUR-FI-2024-MT-01105	120	0.00	0.00	0.00	0.00	0.00	0.01	0.00	0.63	0.00	0.49	0.00	0.02
BUR-FI-2024-MT-01359	130	0.05	0.04	0.00	0.00	0.00	0.05	0.01	0.95	0.00	0.04	0.00	0.17
BUR-FI-2024-MT-01942	130	0.06	0.06	0.00	0.00	0.00	0.05	0.01	0.68	0.00	0.34	0.00	0.07
BUR-FI-2024-MT-02736	130	0.05	0.06	0.01	0.00	0.00	0.05	0.00	0.61	0.00	0.19	0.00	0.30
BUR-FI-2024-MT-02036	140	0.05	0.12	0.00	0.00	0.02	0.06	0.03	0.71	0.00	0.26	0.00	0.11

BUR-FI-2024-MT-02807	150	0.08	0.16	0.00	0.01	0.03	0.07	0.06	0.80	0.00	0.23	0.00	0.05
BUR-FI-2024-MT-01968	150	0.06	0.11	0.01	0.00	0.01	0.07	0.01	0.81	0.00	0.12	0.01	0.30
BUR-FI-2024-MT-02746	150	0.31	0.32	0.00	0.00	0.03	0.19	0.06	0.35	0.00	0.25	0.00	0.04
BUR-FI-2024-MT-01786	170	0.07	0.00	0.01	0.00	0.02	0.07	0.01	1.03	0.00	0.18	0.00	0.34
BUR-FI-2024-MT-02037	200	0.09	0.06	0.01	0.00	0.00	0.06	0.01	1.13	0.00	0.10	0.00	0.53
BUR-FI-2024-MT-02724	210	0.55	0.62	0.00	0.00	0.04	0.18	0.08	0.45	0.00	0.13	0.00	0.04

Table S2: Results of the POD study on naturally contaminated mussel samples with PSP Alert.

Group	Sample ID	HPLC- PCOX concentration (µg STX eq/100g)	Qualitative Result
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-00109	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-00143	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-00956	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01398	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01563	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01653	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01716	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01720	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01759	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01799	<25	-

PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01805	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01880	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01881	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01900	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01914	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01923	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-01985	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-02800	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-02801	<25	-
PST Level < 25 µg STX eq/100g	BUR-FI-2025-MT-02804	<25	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-03003	25	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-03004	25	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-00759	26	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-03514	26	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-01496	26	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-00763	27	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-02808	28	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-02753	29	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-02748	29	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-02761	29	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-00352	29	-

PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-02763	31	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-00382	31	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-01497	31	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-00540	32	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-00798	34	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-01498	34	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-01500	34	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-01389	36	-
PST Level: 25-39 µg STX eq/100g	BUR-FI-2025-MT-00041	39	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-02889	40	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-03508	40	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-02016	42	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-02714	44	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-00040	44	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-03726	45	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-03012	45	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-01103	46	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-00520	47	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-00570	47	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-01495	47	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-01494	49	+

PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-01299	53	+
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-01975	55	+
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-01354	55	+
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-02700	56	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-03005	58	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-01337	59	-
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-02510	59	+
PST Level: 40-59 µg STX eq/100g	BUR-FI-2025-MT-03008	59	-
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-02004	60	-
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-03295	60	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-01479	61	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-02735	61	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-03461	61	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-01778	62	-
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-02888	62	-
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-01460	65	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-01869	65	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-01515	66	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-03210	66	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-01835	67	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-00744	67	+

PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-03497	68	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-01464	70	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-02606	72	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2024-MT-01643	77	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-03237	77	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-03426	77	+
PST Level: 60-79 µg STX eq/100g	BUR-FI-2025-MT-03016	79	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01392	82	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02770	88	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02850	89	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01490	91	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-00719	93	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02657	94	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02065	110	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-03157	110	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01887	110	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01105	120	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01359	130	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01942	130	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02736	130	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02036	140	+

PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02807	150	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01968	150	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02746	150	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-01786	170	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02037	200	+
PST Level ≥ 80 µg STX eq/100g	BUR-FI-2025-MT-02724	210	+

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
5001 CAMPUS DRIVE
COLLEGE PARK, MD 20740-3835
TEL. 240-402-4960/9258/7629, 301-796-0788
CFSANDSSLEOS@FDA.HHS.GOV

SHELLFISH LABORATORY EVALUATION CHECKLIST
PSP Alert — LFA for PSP toxins in Shellfish

LABORATORY:	
ADDRESS:	
TELEPHONE:	**FAX:**
EMAIL:	
DATE OF EVALUATION:	**DATE OF REPORT:**
LAST EVALUATION:	
LABORATORY REPRESENTED BY:	**TITLE:**
LABORATORY EVALUATION OFFICER:	
SHELLFISH SPECIALIST:	
OTHER OFFICIALS PRESENT:	**TITLE:**

Items which do not conform are noted by: _____ Conformity is noted by a "√"

C- Critical K - Key O - Other N/A - Not Applicable

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

PART I – QUALITY ASSURANCE (QA)		
1.1 Quality Assurance (QA) Plan		
Code	**Ref**	**Item**
K	1,2	**1.1.1 Written QA Plan exists** (check √ those items which apply): ☐ Organization of laboratory ☐ Staff training requirements ☐ SOPs/Work Instructions ☐ Internal QC measures (equipment, calibration, maintenance, acceptance criteria) ☐ Laboratory safety ☐ Data integrity/record control ☐ Internal performance assessment ☐ External performance assessment (if applicable)
C	2	**1.1.2 QA Plan is implemented** (evidence of use, current version controlled, staff awareness).
1.2 Educational / Experience / Training Requirements		
Code	**Ref**	**Item**
C	1	**1.2.1 Supervisor/technical lead is qualified** per lab policy and applicable program requirements.
K	1,2	**1.2.2 Analysts are qualified and authorized** to perform PSP Alert testing; authorization list maintained.
C	2,3	**1.2.3 Method-specific training documented** (P Alert IFU/SOP, critical timings, pipetting, reader operation, invalid criteria, reporting).
K	1,2	**1.2.4 Competency is assessed and recorded** (initial + periodic).
1.3 Work Area		
Code	**Ref**	**Item**
O	2	**1.3.1 Work area adequate** for workload, sample prep, and storage.
K	2	**1.3.2 Work area clean and well lighted.**
K	2	**1.3.3 Work area has adequate temperature control** to support kit/storage requirements and stable testing conditions.
O	2	**1.3.4 Work surfaces nonporous/easily cleaned**; contamination prevention practices in place.
1.4 Laboratory Equipment (General + Method-Relevant)		
Code	**Ref**	**Item**
K	2	**1.4.1 Equipment inventory exists** (includes model/ID, location, calibration/verification needs).
C	1,2	**1.4.2 Micropipettors used in method are calibrated/verified** for volumes used (e.g., 10 µL, 70 µL, 1 mL or appropriate devices). Records maintained.
K	2	**1.4.3 Timer(s) available and controlled** for 10-min conjugate incubation and 15-min cassette development.
K	2	**1.4.4 Balance used for 2.5 g tissue is verified/calibrated** at relevant range; records maintained.
C	2,3	**1.4.5 Centrifuge performance is verified** (speed/time appropriate; maintenance/service records maintained).

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

C	3	**1.4.6 PSP Alert reader is installed and functional** (method/settings controlled; alignment/cassette insertion per IFU).
K	3	**1.4.7 Reader performance checks documented** (startup/self-test or verification check per IFU/SOP).
O	2	**1.4.8 Refrigeration/temperature monitoring where required** (for kits/reagents as specified). Records maintained.
1.5 Reagent / Kit Storage, Preparation, and Inventory Control		
Code	**Ref**	**Item**
C	3	**1.5.1 PSP Alert kits stored per IFU** (temperature range, protection from extremes).
K	3	**1.5.2 Lot # and expiry tracked** for kits, cassettes, conjugate vials, diluent (as applicable).
K	3	**1.5.4 Reagents and samples clearly labeled** to prevent mix-ups; chain-of-custody maintained.
O	3	**1.5.5 Receiving inspection documented** (condition upon receipt, storage initiated promptly).
1.6 Records, Data Integrity, and Document Control		
Code	**Ref**	**Item**
C	3,4	**1.6.1 Controlled documents in use** (current IFU/SOP, version controlled, accessible to staff).
C	3,4	**1.6.2 Traceability is ensured** (sample ID → extraction → dilution → cassette → reader result → report).
K	3	**1.6.3 Raw data retention** (reader output files, run sheets, logs) per policy.
K	3,4	**1.6.4 Deviations/nonconformities documented** with corrective actions (CAPA or equivalent).
PART II – ANALYSIS OF SHELLFISH SAMPLES FOR PSP TOXINS — PSP ALERT (LFA)		
2.1 Collection, Receipt, and Transportation of Samples (Laboratory Controls)		
Code	**Ref**	**Item**

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

C	2,3	**2.1.1 Representative sample is received and logged** (sample identifiers, species, harvest area/source, date/time).
K	2	**2.1.2 Sample condition assessed on receipt** (temperature/condition noted; acceptance/rejection criteria defined).
K	2	**2.1.3 Time-to-processing controls** defined (or contingency plan if delays occur).
O	2,4	**2.1.4 Storage conditions documented** prior to preparation/extraction (refrigerated/frozen as defined).
2.2 Preparation of Samples for Analysis — Homogenization		
Code	**Ref**	**Item**
C	3	**2.2.1 Shellfish are shucked and homogenized to uniform consistency** suitable for representative subsampling.
K	3	**2.2.2 Homogenization method is standardized** (equipment, time, cleaning between samples to prevent carryover).
K	2,3	**2.2.3 Cross-contamination controls in place** (clean tools, workspace cleaning, single-use consumables where appropriate).
2.3 Preparation of Samples for Analysis — Extraction and Clarification (PSP Alert)		
Code	**Ref**	**Item**
C	3	**2.3.1 Tissue mass for extraction is 2.5 g** (within defined tolerance).
C	3	**2.3.2 Extraction solvent is provided and is 9 mL** is added per method (within tolerance).
K	3	**2.3.3 Sample is thoroughly mixed and left to stand for 15 minutes** ensure thorough mixing and extraction of toxins from tissue.
C	3	**2.3.4 Centrifugation is performed at 5000 rpm for 5 minutes** (or validated equivalent); supernatant is collected.
K	3	**2.3.5 Supernatant collection avoids pellet disturbance** (minimize particulates that can affect flow).
K	2,3	**2.3.6 Extract handling/storage defined** if not analyzed immediately (conditions/time limits documented).
2.4 Sample Assay — Dilution, Conjugate Incubation, Cassette Development, Reader		
Code	**Ref**	**Item**
C	3	**2.4.1 Dilution is performed as specified:** 10 µL supernatant into 1.0 mL diluent (within tolerance).
C	3	**2.4.2 70 µL of diluted sample is added to the conjugate vial** (within tolerance).
C	3	**2.4.3 Conjugate is fully resuspended** (aspirate/mix until uniform red solution; no visible clumps).
K	3	**2.4.4 Conjugate incubation is 10 minutes** (within defined window).
C	3	**2.4.5 Entire red solution is dispensed into cassette** (complete transfer; no leakage).

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

C	3	**2.4.6 Cassette development time is 15 minutes** (results read within defined window).
C	3	**2.4.7 Cassette is inserted into reader and result obtained** using controlled settings/method file.
C	3	**2.4.8 Invalid test criteria are defined and followed** (e.g., reader error/invalid flag; actions documented).
K	2	**2.4.9 Proper disposal practices followed** for biohazard materials.
2.5 Quality Control (QC), Acceptance/Rejection, and Reporting		
Code	**Ref**	**Item**
C	3	**2.5.1 Internal validity check met** (control indicator per IFU/reader validity). Invalid results are not reported as valid.
K	3	**2.5.2 Reader check/verification performed** at defined frequency; failures addressed before reporting results.
K	2,3	**2.5.3 Run documentation complete** (operator, date/time, kit lot/expiry, sample ID, deviations).
C	2,4	**2.5.4 Reporting and notification procedures defined** for results requiring program action (as applicable).
K	2	**2.5.5 Data retention and traceability maintained** (reader outputs + run sheets retained per policy).

REFERENCES

1. NSSP Model Ordinance / NSSP Guide for the Control of Molluscan Shellfish (FDA/ISSC) — current edition.
2. Good Laboratory Practice (GLP) and laboratory quality system/document control requirements.
3. PSP Alert Method Documentation: PSP Alert IFU/Procedure Card; internal SOPs; reader user manual/method file ID; SLV/ISSC proposal documents (as applicable).
4. Laboratory sample receipt/chain-of-custody procedures and applicable program requirements (state/agency procedures).

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

LABORATORY:			DATE OF EVALUATION:
SHELLFISH LABORATORY EVALUATION CHECKLIST SUMMARY OF NONCONFORMITIES			
Page	Item	Observation	Documentation Required

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
PSP ALERT LFA ASSAY FOR PSP COMPONENT: (Part I-II)	
A. Results Total # of Critical (C) Nonconformities Total # of Key (K) Nonconformities Total # of Critical (C), Key (K), and Other (O) Nonconformities	 _____ _____ _____

B. Criteria for Determining Laboratory Status of PSP Alert assay for PSP:

1. **Conforms Status:** The PSP Alert for PSP component of this Laboratory is in conformity with NSSP requirements if all of the following apply.
   a. No Critical nonconformities;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

2. **Provisionally Conforms Status:** The PSP Alert assay for PSP component of this laboratory is determined to be provisionally conforming to NSSP requirements if all of the following apply.
   a. The number of Critical nonconformities is ≥ 1 but < 4;
   b. and <6 Key nonconformities;
   c. and <12 Total Nonconformities.

3. **Does Not Conform Status**: The PSP Alert assay for PSP component of this laboratory is not in conformity with NSSP requirements when any of the following apply.
   a. The total # of Critical nonconformities is ≥4;
   b. or total # of Key nonconformities is ≥ 6;
   c. or the total # of Critical, Key and Others is ≥ 12.

C. Laboratory Status (*circle appropriate*)

Does Not Conform **Provisionally Conforms** **Conforms**

SHELLFISH LABORATORY EVALUATION CHECKLIST

PSP Alert (Lateral Flow Assay) for Paralytic Shellfish Poisoning (PSP)

Acknowledgment by Laboratory Director/Supervisor:

All corrective Action will be implemented and verifying substantiating documentation received by the Laboratory Evaluation Officer on or before _____.

Laboratory Signature:_____ Date:_____

LEO Signature:_____ Date:_____

Proposal 27-156

Sensoreal® PSP Alert™ Rapid Test for detection of Paralytical Shellfish Toxins in Shellfish

Product ID: ALRT-PSP

Relevance of Saxitoxin and Paralytic Shellfish Toxin (PST) Determination

Paralytic shellfish toxins (PSTs), including saxitoxin (STX) and its analogues (e.g., neosaxitoxin and gonyautoxins), are potent marine neurotoxins produced by certain dinoflagellates during harmful algal blooms (HABs). These toxins are the causative agents of Paralytic Shellfish Poisoning (PSP) in humans. Consumption of PST-contaminated bivalve mollusks can lead to a range of symptoms, from tingling and numbness to severe neurological effects, including respiratory paralysis, which can be life-threatening. PST exposure has also been associated with intoxication events in marine wildlife. Due to these significant health risks, regulatory authorities worldwide (including the United States, Canada, and Europe) require routine monitoring of bivalve shellfish. A strict maximum regulatory limit of 80 µg STX equivalents per 100 g of shellfish tissue (0.8 ppm) is enforced for harvest closures and market access control.

Validation Data

Test Sensitivity and Threshold:	PSP Alert™ is engineered with a conservative decision threshold of 65 µg STX equivalent per 100g of shellfish tissue (0.65 ppm) to provide an added safety margin relative to the regulatory limit.
Selectivity:	The assay detects Saxitoxin and other PSTs to varying degrees.
Comparability with HPLC-PCOX:	In single-laboratory validation studies, the assay demonstrated 100% agreement with standard HPLC-PCOX reference methods for samples containing PSTs at or above the regulatory action level (≥ 80 µg STX eq/100g) in mussels. No false negatives were observed at or above the regulatory limit.
Cross-reactivity	Saxitoxin – 100% GTX 2&3 – 118.1% GTX5- 15.4% Decarbamoyl GTX 2&3 – 7.6% Neosaxiton – 86.4% GTX 1&4 – 78.1% C1/C2 toxins – 12.3%
General Limited Warranty:	The PSP Alert™ Rapid Test Kit is intended for research and pre-testing purposes only and is not a replacement for officially validated regulatory methods. **Users are responsible for determining product suitability. Sensoreal Inc. provides no expressed or implied warranties and is not liable for indirect, incidental, or consequential damages.**

:

To order or to learn more please contact:

Sensoreal Inc.
6674 Avenue de l'Esplanade, Suit B
Montreal QC H2V4L5
Website: sensoreal.com

Ordering and Technical Support:
sales@sensoreal.com

Instructions are effective as of (date): 2026-08-21

Version: US.1.0

1. General Description and Principle of the Method

Sensoreal PSP Alert™ is a rapid lateral flow immunoassay (LFA) designed for the qualitative detection of paralytic shellfish toxins (PSTs), mainly saxitoxin (STX) in bivalve mollusks (mussels). The assay operates on a competitive binding format. Consumption of PST-contaminated shellfish result in symptoms of paralytic shellfish poisoning (PSP).

PSTs like STX are extracted from homogenized shellfish tissue using our proprietary aqueous extraction solution. The extract is then diluted and incubated with a lyophilized antibody–gold nanoparticle conjugate. If STX is present in the sample, it binds to the conjugate antibodies, reducing the amount of free antibody–gold complex available. The mixture is then applied to the lateral flow cassette and migrates across a membrane containing immobilized STX-protein at the test line. Consequently, the test line intensity is inversely proportional to the STX concentration (higher STX concentration = fainter test line). A control line will appear in all valid tests to confirm proper assay function. Results are objectively interpreted using the Sensoreal SensoRead™ scanner.

2. Safety Instructions

Treat all samples, extracts, and used test components as potentially contaminated with STX, other PSTs, and biological material. Wear appropriate PPE, including gloves, safety goggles, and a laboratory coat, during testing. The PSP Alert™ protocol uses an environmentally friendly aqueous extraction solution that can be safely discarded down the drain. Dispose of all samples and used test components in appropriate waste containers.
.

3. Storage and Stability

-Lateral Flow Test Strips (Cassettes): Store at the temperature specified on the label. Do not freeze. Keep cassettes in their original, sealed pouches until immediately prior to use to protect them from moisture and humidity.
-Antibody Conjugate Vials (Lyophilized): This is the only kit component that may be frozen. Store frozen or as strictly specified on the product label. Keep vials sealed and do not open until ready to perform the assay.
-Diluent Powder Vials: Store at the temperature specified on the label. Do not freeze. Keep sealed to protect from excess heat and moisture.
-Buffer Solution: Store as directed on the label.
-General Stability: Protect all kit components from excess heat and direct sunlight. Do not utilize kit components past their printed expiration date or if they exhibit any signs of damage or deterioration.

4. Warnings and Precautions

-The PSP Alert™ test is optimized for analyzing freshly harvested bivalves, intact samples refrigerated for under 48 hours, or previously homogenized tissue kept frozen. Prior to beginning the extraction process, it is critical that all chilled or frozen samples are allowed to fully equilibrate to ambient room temperature.
-Unprocessed shellfish can be safely held under refrigeration (2–8°C) provided they will be tested within two days of harvest. If the testing timeline extends beyond this 48-hour window, the shellfish meat must be completely shucked, homogenized (as outlined in Section C), and stored at -20°C to ensure toxin stability prior to extraction.
-Please note that this protocol is exclusively validated for bivalve shellfish matrices. Any application to alternative sample types requires thorough independent validation by the user before routine testing is implemented.
-Follow the specified extraction, dilution, incubation, and read times exactly as detailed in Section D and E. Deviations from the protocol may adversely affect test performance.
- To avoid cross-contamination, use clean, disposable pipette tips and fresh extraction/dilution materials for each individual sample.
-Do not open the lateral flow cassettes or conjugate components until ready to initiate testing.
-Use exclusively the designated Sensoreal SensoRead™ reader and corresponding method settings for the interpretation of results. Visual interpretation is not supported.
-Dispose of sample extracts, and used kit components in strict accordance with institutional, local, and regulatory environmental requirements.
-As with any screening technique, positive results leading to regulatory action or market closure should be confirmed by a recognized alternative quantitative method (e.g., HPLC-PCOX).

A. Materials Provided

The PSP Alert™ kit includes the following components:

1. Lateral Flow Test Strips (Cassettes)
2. Extraction solution (bottle – “E”)
3. Conjugate Vials (lyophilized antibody-gold nanoparticle)
4. Diluent Powder Vials ("D")
5. Buffer Solution ("B")

B. Materials and Reagents Required (Not Provided)

1. Sensoreal’s SensoRead™ Scanner (required for result interpretation)
2. Immersion blender or equivalent laboratory blender for tissue homogenization
3. Analytical weighing balance (if required)
4. Centrifuge (compatible with 15 mL conical tubes and capable of 5000 rpm)
5. Electronic timer
6. Adjustable precision pipettes covering volumes of 20–200 µL and 100–1000 µL
7. Disposable pipette tips
8. 15 mL conical tubes (for extraction and collection)
9. The use of test tube racks and a vortex mixer is recommended for operational ease; however, these components are not mandatory for the methodology.

C. Sample Collection and Storage

1. For each sample ID, shuck a representative population of 10 to 12 individual animals to obtain a minimum of 100 g of pooled shellfish tissue.
2. Thoroughly rinse the exterior of the shellfish with deionized or distilled water to remove extraneous debris, such as sand or mud.
3. Transfer the collected tissue to a sieve and allow it to drain to remove excess fluid.
4. Homogenize the tissue using an immersion blender (or equivalent mechanical blender) until a completely uniform, smooth consistency is achieved.

D. Toxin Extraction (Part A)

1. Using a clean spatula and weighing balance, weigh exactly 2.0 g of the homogenized shellfish sample and transfer it into a 15 mL conical tube.
2. Add 9 mL of extraction solution to the conical tube containing the sample.
3. Cap the conical tube tightly and shake vigorously and incubate for 15 minutes.
4. Transfer the tube to a centrifuge and spin at 5000 rpm for 15 minutes to separate the particulate matter from the toxin-containing solvent. Following centrifugation, carefully transfer the supernatant (the clear liquid) into a clean 15 mL conical tube.

E. Test Strip Sample Analysis Procedure (Part B)

Ensure the Sensoreal SensoRead™ scanner is turned on and the correct lot QR code has been scanned prior to initiating the test.

1. Sample Dilution: Locate the Diluent powder tube ("D"). Using a pipette fitted with a new tip, add 2 mL of Buffer "B" and 20 µL of the sample supernatant to Tube "D". Mix thoroughly by vortexing or pipetting up and down.
2. Using a pipette fitted with a new tip, transfer 70 µL of the Diluted Sample (from tube “D”) into the Conjugate vial. Pipette the mixture up and down to achieve complete dissolution of the lyophilized pellet, ensuring that no red specks remain adhered to the interior walls of the tube. Once the pellet is fully dissolved and the solution is homogeneous, let the vial sit uninterrupted at room temperature for exactly 10 minutes.
3. After the 10-minute incubation, transfer 70 µL of the Conjugate mix into the sample port of the test cassette.
4. Leave the cassette on a flat, horizontal surface. Set a timer and allow the assay to develop for exactly 15 minutes.

F. SensoRead™ Scanner Analysis

1. After exactly 15 minutes of development, immediately insert the test cassette into the reader slot of the SensoRead™ device. Follow the on-screen prompts: Select Cassette -> PSP Alert
2. You will be prompted to identify the batch being used. Either select an existing Lot from the on-screen list, or for a new Lot, press “Add New Lot” and scan the batch-specific QR code located on the LFA test pouch or the Certificate of Analysis. This QR code provides the system with integral calibration parameters required for accurate interpretation. *(Note: Each Lot only needs to be scanned once; for subsequent tests with the same Lot, simply select it from the saved list).*
3. Once the Lot is confirmed, enter the unique Sample ID using the on-screen keypad and press “Run Test”. The device will immediately initiate the analysis process to receive the qualitative result (T/C ratio and classification).

G. Interpretation of Results

Assay results are determined by the SensoRead™ scanner through a digital analysis of the Test-to-Control (T/C) line intensity ratio. While the absolute intensity of the control line may fluctuate between runs, a visible control line must be present for the assay to be considered valid; the absence of a control line indicates an invalid result requiring a retest.

The SensoRead™ scanner provides an objective determination of line intensities for consistent interpretation, as well as a digital photographic record of all test strips.

-**Positive**: The scanner has detected STX and/or other PSTs at or above the 65 µg STX eq/100g threshold. The reader will flag this sample, indicating it has reached or exceeded the precautionary limit.
-**Negative:** The T/C ratio indicates concentrations below the 65 µg STX eq/100g threshold, and the sample is considered compliant.

Test Line	Control Line	Interpretation
Absent	Absent	Invalid Test (Repeat Test)
Faint or Non-Visible	Visible	Positive: ≥ 65 µg STX eq/100g
Moderate Intensity	Visible	Negative: < 65 µg STX eq/100g
More Intense than Control Line	Visible	Negative: Non-Detect

Note: It is essential to perform the reading at the designated 15-minute mark. Determinations made using strips that have dried for more or less than the required time may be inaccurate, as chromatographic line intensities vary with drying time. To ensure a rigorous safety margin against the 80 µg STX eq/100g NSSP regulatory limit, the PSP Alert™ system is intentionally calibrated to a conservative early-warning threshold of the 65 µg STX eq/100g

END OF PAGE

Proposal 27-156

sensoreal

PSP Alert

Version: PSPALERT-3.0

Rapid detection of PSP Toxins in Shellfish and Molluscs

KIT COMPONENTS SUPPLIED BY CUSTOMERS



15 mL Tubes for Extraction and Collection

****Do NOT re-use any tubes for multiple samples due to a high risk of toxicity and contamination. Dispose of the tubes after single use****

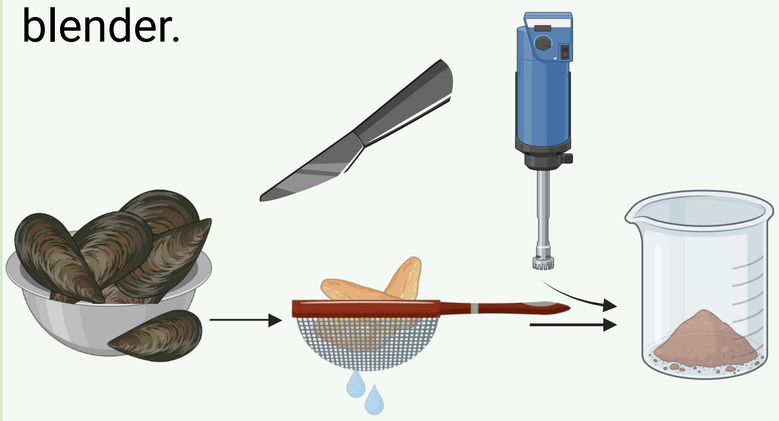
KIT COMPONENTS SUPPLIED BY SENSOREAL



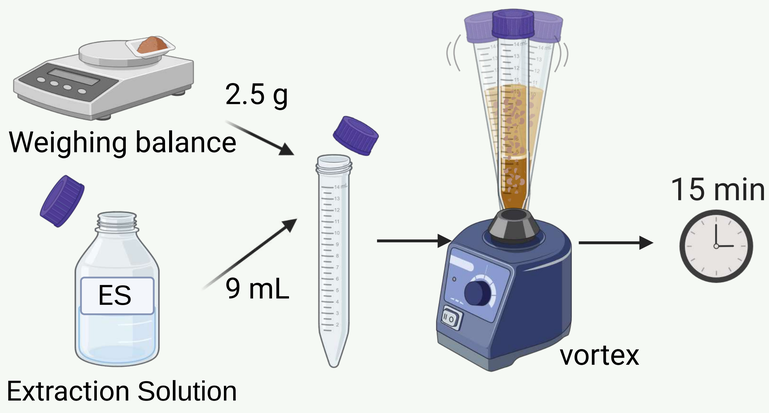
Conjugate Tubes | Diluent Powder | Extraction Solution | Buffer Solution | Lateral Flow Test Strip

PART 1: TOXIN EXTRACTION

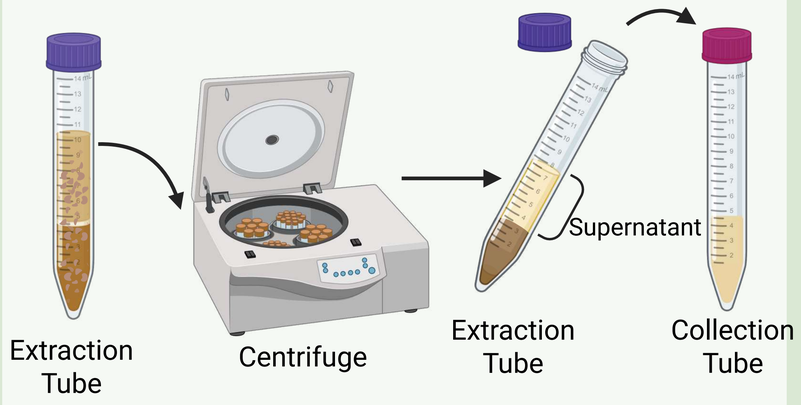
1. Shuck shellfish meat. Use at least 100 g of meat as a representative sample. Strain excess liquid from shellfish. Blend the meat with a blender.



2. Measure out **2.5 g** of sample and **9 mL** of Extraction solution "ES". Add both to a 15 mL tube. Shake vigorously or vortex and let stand for 15 minutes.

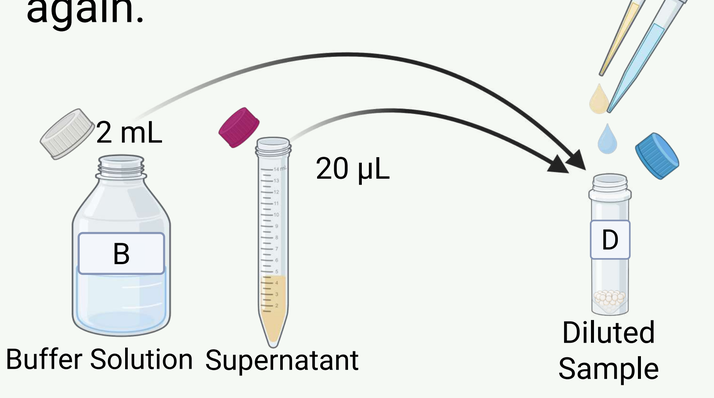


3. Centrifuge at 5000 rpm for 5 minutes. Transfer the supernatant into a Collection Tube.

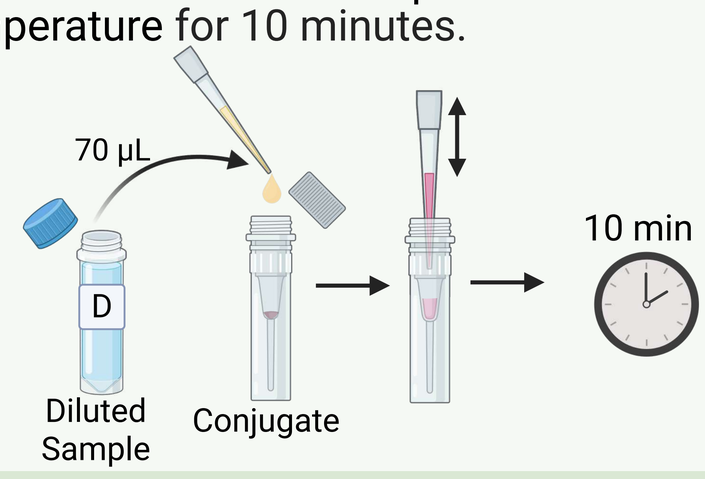


PART 2: FINAL SAMPLE PREPARATION

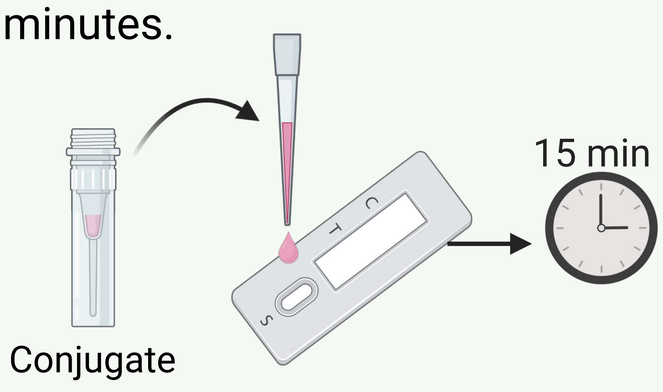
4. To a tube "D" add **2 mL** of solution "B". Vortex to dissolve. To the same tube "D" add **20 µL** of the supernatant and vortex again.



5. Add **70 µL of diluted sample** to a Conjugate tube. Pipette up and down to dissolve. Let sit uninterrupted at room temperature for 10 minutes.

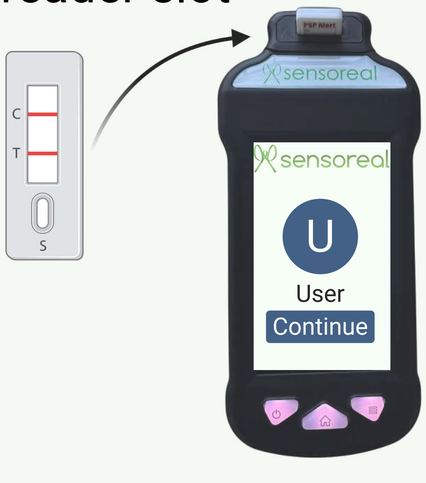


6. Add **70µl** of the Conjugate mix to the test cassette. Leave the cassette on a horizontal surface and set a timer for 15 minutes.

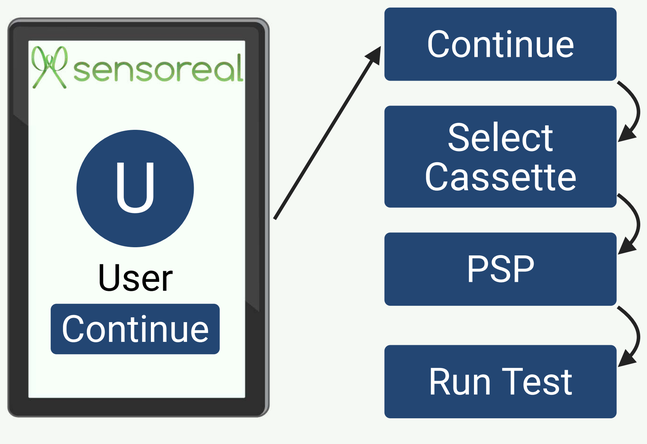


SENSOREAD - INTERPRETING THE RESULTS

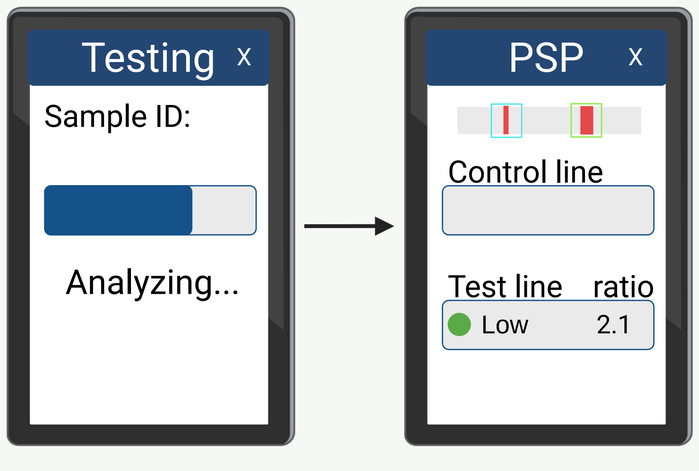
Insert the cassette into the reader slot



Follow the steps on the screen



Test results will be displayed after a few seconds



Safety Data Sheet

Section 1: Product and Company Identification

Product Identifiers

Product Name:	PSP Alert™ Test Kit
Catalog Number:	ALRT-PSP
Relevant Identified Uses	For research, laboratory, and pre-screening use only. Not for diagnostic, therapeutic, drug, household, or other unintended uses.
Ingredients Information	This product contains antibody of animal origin. Surfactants, such as Triton X100, and Bovine Serum Albumin (BSA) may be present in some reagents but are supplied at non-hazardous levels. See individual product insert for information and refer to guidance below.

Company Identifiers

Company:	Sensoreal Inc.
Address:	6674 Esplanade Avenue, Suite B, Montréal, Quebec, H2V 4L5, Canada
E-mail:	sales@sensoreal.com
Internet:	www.sensoreal.com

Section 2: Hazard Identification

Classification of the Substance or Mixture

Classification according to Regulation (EC) No 1272/2008 [CLP]

Physical hazards	Not Hazardous
Health Hazards	Not Hazardous as supplied
Environmental Hazards	Not Hazardous
Additional information	Not applicable

Label elements

Labelling according to Regulation (EC) No 1272/2008 [CLP]

Hazard pictograms	None
Signal word	None
Hazard Statements	Not Applicable

EU Specific Hazard Statements	Not Applicable

Precautionary Statements

Prevention:	Use standard laboratory precautions. Avoid ingestion, inhalation, and contact with skin or eyes.
Responses:	Wash exposed skin with soap and water. Rinse eyes with water if contact occurs. Seek medical attention if symptoms persist.
Storage:	Store as directed on the product insert or component labels.
Disposal	Dispose of contents and containers according to local, regional, and national regulations.
Other Hazards	This mixture does not contain substances known to be PBT or vPvB at reportable levels.

Section 3: Composition / Information on Ingredients

Substance: This substance is a mixture.

Mixture:

Component	CAS No.	EC No.	Classification	Concentration
Triton X-100	9036-19-5	618-344-0	H302; H318; H411	< 0.05%
Sodium Chloride	7647-14-5	231-598-3	Not hazardous at supplied concentration for skin, but may be harmful if swallowed and cause eye irritation	Proprietary*
Sodium phosphate dibasic	7558-79-4	231-448-7	Not hazardous at supplied concentration	0.1%
Dihydrogen potassium phosphate	7778-77-0	231-913-4	Not hazardous at supplied concentration	<0.05%
Potassium chloride	7447-40-7	231-211-8	Not hazardous at supplied concentration	<0.05%
Bovine Serum Albumin	7647-14-5	231-598-3	Not hazardous at this concentration.	<5%

***Note: Proprietary reagent identities and concentrations are withheld as confidential business information where permitted. Components are supplied in small quantities for laboratory use.**

Section 4: First Aid Measures

General advice:	Consult a doctor and show this safety data sheet.
Eye contact:	Flush eyes with plenty of water for at least 10 minutes, occasionally

	lifting the upper and lower eyelids. Get medical attention if symptoms occur.
Skin contact:	Immediately wash skin with copious amounts of soap and water for at least 15 minutes. Remove contaminated clothing and wash before reuse. Get medical attention if discomfort persists.
If inhaled:	Remove to fresh air and monitor breathing. Give oxygen if breathing becomes difficult, give artificial respiration if breathing stops. Get medical attention if symptoms appear.
If ingested:	Rinse mouth thoroughly with water. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. Get medical attention if symptoms appear.
Notes to physician:	No specific antidote. Medical staff must contact Poison Control Center. To the best of our knowledge, the chemical, physical and toxicological properties have not been thoroughly investigated.

Section 5: Fire-Fighting Measures

Extinguishing media:	Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide.
Special exposure hazards:	Nature of decomposition products is not known.
Precautions for fire-fighters:	Firefighters should wear appropriate protective equipment and self-contained breathing apparatus with a full face-piece operated in positive pressure mode.
Further information:	Not available

Section 6: Accidental Release Measures

Personal precautions:	Immediately contact emergency personnel. Use suitable protective equipment. Evacuate personnel to safe areas. Ensure adequate ventilation. Avoid breathing vapors, mist, dust or gas. Dispose cloth in accordance with local disposal guidelines.
Environmental precautions:	Used test products/cassettes can be disposed of in proper garbage and avoid disposing liquid waste can be in waterways, drains and soil.
Cleaning up:	Absorb liquid spills with suitable inert material. Collect into a suitable container for disposal according to local, regional, or national regulations. Avoid creating aerosols or dusty conditions. Dispose of contaminated materials according to institutional laboratory waste procedures.

Section 7: Handling And Storage

Handling:	Do not ingest. Avoid contact with skin and eyes. Wash thoroughly after handling. Use according to product instructions and standard laboratory practices.

Storage:	Store as directed on the individual product insert or component labels.

Section 8: Exposure Controls/ Personal Protection

Personal Protective:	Safety glasses; Lab coat; Rubber or plastic gloves.
Engineering Controls:	Use in a laboratory setting with adequate ventilation. Ensure laboratory is equipped with a safety shower and eye wash station.

Section 9: Physical And Chemical Properties

Physical State:	Liquid and lyophilized powder
Odor:	Little to none
Solubility in water	All chemical reagents are miscible in water.
Additional information:	Not available.

Section 10: Stability and Reactivity

Chemical stability:	Stable under recommended transport and storage conditions.
Reactivity:	Stable under normal handling conditions.
Hazardous reactions:	Will not occur under normal storage, handling and use conditions.

Section 11: Toxicological Information

Inhalation:	There may be irritation of the throat or respiratory tract if aerosols, mist, or dried material are inhaled.
Ingestion:	There may be irritation of the mouth or throat. Ingestion of reagents should be avoided.
Skin:	There may be mild irritation at the site of contact.
Eyes:	There may be mild irritation and redness.
Additional Information:	Triton X-100, in concentrated form, may be harmful if swallowed, may cause skin irritation, and may cause serious eye irritation. In this kit, Triton X-100 is present only at trace, non-hazardous levels, approximately 10 µL per test and <0.05% overall.

Section 12: Ecological Information

Toxicity:	Concentrated Triton X-100 may be hazardous to aquatic life. Avoid dispersal of spilled material, runoff, and contact with soil, waterways, drains, and sewers.

Section 13: Disposal Considerations

Waste product:	Transfer unused reagents, used test devices, tubes, and contaminated materials to a suitable container and arrange for disposal in accordance with national, regional, or local legislation.
Contaminated Packaging :	Dispose of according to institutional, local, regional, or national waste disposal procedures.

Section 14: Transport Information

IATA / ADR /DOT-US / IMDG
Not regulated in the meaning of transport regulations.

UN Number	Not applicable for complete kit
UN proper shipping name	Not applicable for complete kit
Transport hazard class(es)	Not applicable for complete kit
Packing group	Not applicable for complete kit
Environmental hazards	Not applicable for complete kit
Special precautions for user	Not applicable for complete kit

Transport in bulk according to Annex II of MARPOL 73/78 and the IBC Code
Not applicable for complete kit

Note for Triton X100:	Triton X-100 may be associated with UN 3082, Environmentally Hazardous Substance, Liquid, N.O.S., Class 9, Packing Group III. In PSP Alert™, Triton X-100 is present only at trace levels, approximately 10 µL per test and <0.05% overall. Based on the supplied quantity and concentration and under IATA Special Provision A197, the complete kit is not expected to be regulated as dangerous goods for transport and the Triton X100 does not exceed the small inner packaging volume limit.

Section 15: Regulatory Information

Safety, health and environmental regulations/legislation specific for the substance or mixture

Substances of Very High Concern
None.

Substances listed under Annex I of Regulation (EC) No 689/2008
None.

Restricted substances under Annex V of Regulation (EC) No 689/2008
None

Substances under Regulation (EC) No 850/2004 of the European Parliament and of the Council of 29 April 2004 on persistent organic pollutants and amending Directive 79/117/EEC
None

German Water hazard classes (Wassergefährdungsklassen)
Not classified

Other International Inventories
No information available.

Chemical Safety Assessment
No chemical safety assessment has been carried out for the complete kit.

Section 16: Other Information

The information contained in this SDS relates only to the material(s) designated and does not relate to use(s) in combination with any other material, process(es) and/or chemical reaction(s).

Note to Users:

For research and in vitro use only. Not for therapeutic, household, or clinical diagnostic use. Sensoreal Inc. provides this information in good faith and based on present knowledge. This SDS is provided without warranty of any kind. Sensoreal Inc. assumes no liability for the accuracy or completeness of the information contained herein. Final determination of suitability, handling, storage, transport, and disposal is the sole responsibility of the user.

All materials may present unknown hazards and should be used with caution. Although certain hazards are described herein, Sensoreal Inc. cannot guarantee that these are the only hazards that exist. The recipient is responsible for ensuring that, where applicable, existing laws and guidelines are observed.

End of Safety Data Sheet