ISSC Task Force I
2019 Proposal Inventory

Proposal Number	Submitter / Proposal Subject
11-103	Spinney Creek Shellfish, Inc. (Tom Howell) Alternative Male-Specific Coliphage Meat Standard for Restricted Classification of Growing Areas Impacted by wastewater treatment plant outfall.
13-107	East Coast Shellfish Growers' Association (Bob Rheault) Sources of Seed for Aquaculture
13-111	Abraxis, LLC (Dave Deardorff) DSP PPIA Kit for Determination of Okadaic Acid Toxins Group (OA, DTX1, DTX2) in Molluscan Shellfish
13-114	Resource Access International (Darcie Couture) Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination
13-116	Florida Deparment of Agriculture and Consumer Affairs (Kim Norgren) Shellfish Quarantine Guidance Document
15-109	Maine Department of Marine Resources & Alaska State Environmental Health Laboratory PSP HPLC-PCOX Species Expansion
15-112	ISSC Executive Board (Developer Jessica Jones) Direct Plating Method for trh
15-114	ISSC Executive Board (Developer Kevin Calci) MSC Enumeration in Wastewater by Direct Double-Agar Overlay
17-100	Massachusetts Division of Marine Fisheries (Mike Hickey) Marina Definition
17-103	US Food & Drug Administration LC MS MS for Monitoring DSP Toxins
17-106	PAC RIM (Michael Jamros) RBA PSP Geoduck
17-108	Beacon Analytical Systems, Inc. Detection of ASP biotoxins in *Mytilus edulis* (Blue Mussel) shellfish by ELISA for Domoic Acid
17-110	US Food & Drug Administration Vibrio Probe Checklist
17-116	US Food & Drug Administration Aquaculture in Federal Waters
17-121	US Food & Drug Administration Disposal of Human Sewage and Bodily Fluids
19-100	US Food & Drug Administration (FDA) Determining Emergency Conditions
19-101	Massachusetts Division of Marine Fisheries (Michael Hickey, Jeff Kennedy, Diane Regan) Conditionally Conforming Laboratory Status
19-102	US Food & Drug Administration (FDA) Updating epidemiological investigation reference
19-103	Taylor Shellfish Farms (Bill Dewey) Alternative for allowing harvest for raw consumption from a growing area closed due

Proposal Number	Submitter / Proposal Subject
	to *V.p.*
19-104	Centers for Disease Control and Prevention (CDC) *Vibrio vulnificus* risk evaluation
19-105	Washington State Department of Health (Scott Berbells) Laboratory approval for sample analysis with no Model Ordinance defined method or action level
19-106	ISSC Executive Office Delete Notification Requirement to Pollution Control Agencies
19-107	US Food & Drug Administration (FDA) Determining shoreline survey area
19-108	ECSGA (Robert Rheault) Aquaculture Seed Shellstock
19-109	Florida Department of Agriculture and Consumer Services (Jill Fleiger) Offshore State Water classification requirements
19-110	US Food & Drug Administration (FDA) Point source approved standard station locations
19-111	Washington State Department of Health (Scott Berbells) Allowing the use of the SRS method in areas impacted by point sources
19-112	US Food & Drug Administration (FDA) Nonpoint source approved standard station locations
19-113	US Food & Drug Administration (FDA) Authorizing unclassified areas and multiple classifications for single area
19-114	US Food & Drug Administration (FDA) Emergency Conditions re-opening studies
19-115	Maryland Department of Environment (Kathy Brohawn) Emergency Conditions/closed status to reflect Chapter II use of harvest area
19-116	Massachusetts Division of Marine Fisheries (J. Michael Hickey) Adding a time frame to the limited or temporary period an area can be remain under a closed status prior to being reclassified
19-117	Massachusetts Division of Marine Fisheries (J. Michael Hickey) Shellfish cleansing studies
19-118	US Food & Drug Administration (FDA) Conditional areas not based on predicting microbiological indicator levels
19-119	Washington State Department of Health (Scott Berbells) Reduced marine water sampling in conditionally approved areas impacted by point sources
19-120	Surfside Foods (Tom Dameron) Classification of Federal Waters
19-121	ISSC Executive Office *Karenia brevis*
19-122	US Food & Drug Administration (FDA) Use of “growing area” rather than “harvest area” in Patrol requirements language
19-123	State of Alaska Department of Environmental Conservation (Kim Stryker) Marine Biotoxin Control - Public Health Reasons
19-124	State of Alaska Department of Environmental Conservation (Kim Stryker) Marine Biotoxin Control - Guidance Document

Proposal Number	Submitter / Proposal Subject
19-125	ISSC Executive Office *Karenia brevis* Guidance
19-126	US Food & Drug Administration (FDA) MPN-Real-Time PCR for Enumeration of Vibrio vulnificus in Oysters
19-127	Florida Fish and Wildlife Conservation Commission (Leanne J. Flewelling) Modification of the MARBIONC Brevetoxin ELISA Standard Operating Procedures
19-128	Washington State Dept of Health (Gina Olson) Laboratory Method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* Enumeration and Detection Through MPN and Real-Time PCR
19-129	Northeast Laboratory Evaluation Officers and Managers (NELEOM) (Leonora Porter) Micropipettor Verification
19-130	Northeast Laboratory Evaluation Officers and Managers (NELEOM) (Leonora Porter) Microbiology Laboratory Evaluation Checklist- Standards Thermometer
19-131	Northeast Laboratory Evaluation Officers and Managers (NELEOM) (Leonora Porter) NSSP Microbiology Laboratory Evaluation Checklist – Reagent Water Quality
19-132	Northeast Laboratory Evaluation Officers and Managers (NELEOM) (Leonora Porter) NSSP Microbiology Laboratory Evaluation Checklist – Working Thermometers
19-133	Northeast Laboratory Evaluation Officers and Managers (NELEOM) (Leonora Porter) Microbiology & PCR Laboratory Evaluation Checklists - Working Thermometers
19-134	Massachusetts Division of Marine Fisheries (J. Michael Hickey, Jeff Kennedy, Diane Regan) Membrane Filtration Technique for Seawater using mEndo Agar LES Checklist
19-135	Northeast Laboratory Evaluation Officers and Managers (NELEOM) (Leonora Porter) Microbiology Laboratory Evaluation Checklist - Sterilization
19-136	US Food & Drug Administration (FDA) NSSP DSP Laboratory Evaluation Checklist
19-137	US Food & Drug Administration (FDA) Checklist for the Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES
19-138	US Food & Drug Administration (FDA) NSSP Microbiology Laboratory Evaluation Checklist
19-139	US Food & Drug Administration (FDA) NSSP Microbiology Laboratory Evaluation Checklist
19-140	US Food & Drug Administration (FDA) NSSP Microbiology Laboratory Evaluation Checklist
19-141	US Food & Drug Administration (FDA) NSSP Receptor Binding Assay for Paralytic Shellfish Poisoning (PSP) Laboratory Evaluation Checklist
19-142	WA DOH Public Health Laboratories (Shelley Lankford) Add the use of a mechanical shaker to the water microbiology methods checklist in the sample preparation requirements section and include a reference
19-143	Florida Fish and Wildlife Conservation Commission (Leanne Flewelling) MARBIONC Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA Method Laboratory Evaluation Checklist
19-144	Spinney Creek Shellfish, Inc. (Tom Howell) Guidance for Assessing the Viral Impact from Waste Water Treatment Plant Outfall on

Proposal Number	Submitter / Proposal Subject
	Adjacent Growing Areas using the Male-specific Coliphage Method on Effluent Samples
19-145	US Food & Drug Administration (FDA) Guidance on cleansing studies
19-146	Northeast Laboratory Evaluation Officers and Managers (NELEOM) (Leonora Porter) Micropipettor Verification
19-147	US Food & Drug Administration (FDA) Relay contaminant reduction studies
19-148	ISSC Executive Office Correct language of MO to reflect current checklists
19-149	ISSC Executive Office Biotoxin Guidance

Proposal No. **11-103**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Thomas L. Howell
Affiliation	Spinney Creek Shellfish, Inc.
Address Line 1	PO Box 310
Address Line 2	
City, State, Zip	Eliot, ME 03903
Phone	207-439-2719
Fax	207-439-7643
Email	tlhowell@spineycreek.com
Proposal Subject	Alternative Male-specific Coliphage Meat Standard for Restricted Classification of Growing Areas Impacted by wastewater treatment plant outfall.
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Area @ .02 Bacteriological Standards
Text of Proposal/ Requested Action	G. Standard for the Restricted Classification of Growing Areas Affected by Point Sources and Used as a Shellstock Source for Shellstock Depuration. (4) Exception. If the Male-specific Coliphage indicator is used for supplemental process verification using an end-point meat standard of < 50PFU/100gm and existing fecal coliform testing requirements in Chapter XV .03 J. are used, then FC water quality monitoring is not required for the restricted classification of growing areas affected by point sources such as wastewater treatment plant outfall.
Public Health Significance	Under shellfish relay, water quality requirements are not needed for the restricted classification when a contaminant reduction study is conducted and a minimum time period of two weeks is used. For depuration, the restricted classification requires water quality monitoring and standards. The reason for these upper FC limits is that FC meat indicator does not adequately reflect the viral risk and/or viral depuration kinetics. Male-specific coliphage is a viral indicator organism to be used in growing areas impacted by point source sewage contamination. MSC demonstrates significant advantages over FC alone for both the assessment of viral contamination and assessment of viral depuration kinetics. Upper FC limits were put into the NSSP to prevent shellfish with higher levels of viruses from being depurated. Several studies clearly show that conventional depuration using FC for process validation is not adequate to protect public health with respect to virus contamination in growing areas with significant wastewater treatment plant and sewage impact. Studies have also shown that viral levels in shellfish impacted by sewage and partially treated sewage detected using MSC and molecular techniques are much lower in the summer months than the winter months. Additionally, the viral depuration rate is higher in the summer with process waters >18°C. Recent studies have also shown that MSC is an appropriate viral indicator to assess viral depuration. Therefore, seasonal viral depuration using male-specific coliphage as well as FC for process verification is a superior approach to taking water samples using FC in a growing area adjacent to wastewater treatment plant outfall. Combining the bacterial indicator of FC and the viral indicator MSC for mitigation strategies that use meat scores is far more direct and effective than water quality sampling in this context.

Cost Information	The Male-specific Coliphage (MSC) method is an inexpensive double-agar pour plate method that can be run in any state-certified microbiological laboratory. A refrigerated centrifuge capable of 9,000G is required which costs $10K to $12K (USD). Significant cost savings and a higher level of public health protection may be realized using strategies such as seasonal coliphage depuration process validated using MSC and seasonal coliphage relay using MSC in contaminant reduction studies than requiring water quality limits using FC.
Action by 2011 Task Force I	Recommend referral of Proposal 11-103 to the appropriate committee as determined by the Conference Chairman.
Action by 2011 General Assembly	Adopted recommendation of 2011 Task Force I on Proposal 11-103.
Action by FDA February 26, 2012	Concurred with Conference action on Proposal 11-103.
Action by 2013 Growing Area Classification Committee	Recommend referral of Proposal 11-103 to the appropriate committee as determined by the Conference Chairman. It was additionally recommended that a workgroup be formed to look at current MSC data and the science behind its potential use and applicability for use in the NSSP. The workgroup will organize a summit of outside experts, academia, and scientists to present current information and science on MSC. The group will meet at least quarterly and respond back to the Growing Area Classification Committee on its findings and recommendations. Recommended that the ISSC pursue funding to facilitate scheduling a summit to bring together experts to present the current science in the use of MSC.
Action by 2013 Task Force I	Recommended adoption of Growing Area Classification Committee action on Proposal 11-103.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 11-103.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 11-103.
Action by 2015 Growing Area Classification Committee	Recommended referral of Proposal 11-103 to appropriate committee as determined by the Conference Chair.
Action by 2015 Task Force I	Recommended adoption of Growing Area Classification Committee recommendation on Proposal 11-103.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 11-103.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 11-103.
Action by 2017 Growing Area Committee	Recommended adoption of Proposal 11-103 as amended. Add a new section as follows: Chapter XV. Depuration .03 Other Model Ordinance requirements K. Supplemental Requirements for Depuration using MSC Viral Controls for Shellstock Harvested from Conditionally Restricted Growing Areas Impacted by Wastewater System Discharge (WWSD).

	If the conditionally restricted growing area from which the shellstock is being depurated is impacted by wastewater treatment system discharge (generally that section of the conditionally restriced growing area located within the 300:1 to 1000:1 dilution lines), then supplemental requirements for depuration using MSC viral controls may be required. Depuration using MSC viral controls may be seasonally limited and may be species and depuration facility specific. Contaminant reduction studies as described in (1) below are recommended unless the SSCA and the Depuration Facility Operator have significant experience with the depuration process using MSC viral controls. (1) Male-specific coliphage may be used in addition to fecal coliform for species-specific, growing area-specific, and depuration system-specific contaminant reduction studies. These contaminant reduction studies should demonstrate that; (a) Predictable periods of time exist when male-specific coliphage levels are less than 1,000 PFU/100gm in shellfish meats, (b) Male-specific coliphage and fecal coliform can be consistently reduced below end-point requirements, and (c) Critical limits of season, process water temperature and salinity, and system design and operation limitations can be assessed and determined (d) Species-specific operating protocols may be developed from the contaminant reduction studies for each conditionally restricted growing area that includes; (i) Calendar dates when depuration shall be permitted, (ii) Water temperature and salinity limitations, (iii) Minimum processing time, (iv) Sampling requirements and release criteria, and (v) Operating Protocol. (2) All requirements of Chapter XV shall be followed, (3) A single 0-day MSC shellfish meat sample is required. (4) The MSC end-point requirement for depuration is 50 PFU/100gm. If the single 0-day sample exceeds 50 PFU/100gm, then triplicate samples are required prior to release of product. (5) The geometric mean of the triplicate samples used for product release must not exceed 50PFU/100gm and no single sample over 100 PFU/100gm. (6) Extended depuration may be permitted to achieve end-point requirements. (7) Evaluation of male-specific coliphage samples shall be performed in an NSSP conforming laboratory,
Action of 2017 Task Force I	Recommended adoption of Growing Area Classification Committee recommendation on Proposal 11-103.

Action by FDA February 7, 2018	Did not concur with Conference action on proposal 11-103
Action by ISSC Executive Board	Referred Proposal 11-103 to an appropriate committee as determined by the Conference Chair.

Proposal No. **13-107**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Robert Rheault
Affiliation	East Coast Shellfish Growers Association
Address Line 1	1623 Whitesville Road
Address Line 2	
City, State, Zip	Toms River, NJ 08755
Phone	401-783-3360
Fax	
Email	bob@ecsga.org
Proposal Subject	Sources of Seed for Aquaculture
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter VI. Shellfish Aquaculture
Text of Proposal/ Requested Action	.03 Seed Shellstock Seed may come from any growing area, or from any growing area in any classification, provided that: A. The source of the seed is sanctioned by the Authority B. Seed from growing areas ~~or growing areas~~ in the restricted or prohibited classification have acceptable levels of poisonous or deleterious substances; and C. Seed from growing areas ~~or growing areas~~ in the prohibited classification are cultured for a minimum of ~~six (6) months~~ one month while average daily water temperatures are above 50 degrees F.
Public Health Significance	Shellfish seed collected or cultured in certain growing areas that are in the prohibited classification have been shown through repeated sampling to be free of deleterious substances (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 viral and bacterial contaminants provided water temperatures are high enough to maintain active metabolic activity (above 60 degrees F or 15 degrees C) (Richards 1988). Once the Authority is satisfied that adequate sampling has demonstrated that the seed have "acceptable levels of deleterious substances", then a 30 day period of culture in open waters should be adequate to allow purging of bacterial and viral contaminants to ensure that public health is protected. The Authority retains the right to deny seed collection and culture in any area, or to require additional testing for deleterious substances, or to require longer periods to purge contaminants as necessary. The original intent of this section was to provide for purging of viral and bacterial contamination prior to harvest for consumption on the assumption that deleterious substances were at acceptable levels prior to moving the seed to grow out areas The six-month requirement was implemented as a short-hand way to ensure that seed were grown for at least one month when water temperatures exceeded 60 degrees F. It makes little sense to require relay times in excess of one month for seed that are

	typically more than six months from harvest size when shellstock relay times as short as two weeks are common. References Cited: Richards, G. (1988), Microbial Purification of Shellfish: A Review of Depuration and Relaying, J. Food Protection 51(3)218-251. 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	This change should facilitate record keeping and documentation efforts required to ensure that seed from prohibited waters do not get harvested until bacterial and viral contamination has been purged.
Action by 2013 Task Force I	Recommended referral of Proposal 13-107 to an appropriate committee as determined by the Conference Chairman.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-107.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-107.
Action by 2015 Aquaculture Facility Inspection Committee	Recommended the following: (1) Referral of Proposal 13-107 back to Committee as appointed by the Conference Chair. (2) The charge of the Committee be expanded to include updating and revising the Aquaculture Chapter of the Model Ordinance to reflect current practices and methods and submit proposals for the next Annual Meeting.
Action by 2015 Task Force I	Recommended adoption of Aquaculture Facility Inspection Committee recommendations on Proposal 13-107.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 13-107.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-107.
Action by 2017 Aquaculture Facilities Inspection Committee	Recommended adoption of Proposal 13-107 as substituted. Section I. Definitions Replace definition 9. in Section I of the Model Ordinance as follows: 9. Aquaculture means cultivating shellfish in controlled conditions for human consumption. Cultivation includes propagation and growing of shellfish. These activities may occur in natural or man-made water bodies. These activities include seed production, cultivation in natural water bodies when shellfish are held off the bottom such as the use of racks, bags, or cages, and when shellfish are held in man-made water bodies such as the use of tanks, ponds, or raceways. These activities do not include depuration, wet storage or the broadcasting of spat or seed shellfish being left to mature the same as wild shellfish. Modify definition 93. in Section I of the Model Ordinance as follows: (93) Prohibited means a classification used to identify a growing area where the harvest of shellstock for any purpose, except depletion or gathering or nursery culture of seed for aquaculture, is not permitted.

Section IV. Chapter IV. Shellstock Growing Areas
Change @03 E. (2)(a) to read:
(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 the depletion of the areas classified as prohibited; and

Replace Chapter VI. Aquaculture in its entirety as follows:

Chapter VI. Aquaculture
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.]
@ .01 General.

A. Activities which have been determined to pose a significant public health concern and need regulation outlined in this Chapter include, but are not limited to:
(1) Seed production in waters classified as Prohibited or Unclassified;
(2) Aquaculture that attracts 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.

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.
B. Restricted or Conditionally Restricted would be exempt from these requirements but subject to relay requirements in Chapter V 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;
(3) The open status of the conditionally restricted classification; or
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;
(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;
(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 mammals 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) 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) A program of sanitation, maintenance, and supervision to prevent contamination of the shellfish products;
(7) A description of the water source, including the details of any water treatment process or method;
(8) 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) If applicable, collection of data concerning the quality of food production (algae or other) used in the artificial harvest system; and
(10) 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.

(1) 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 shall meet 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 of Section D. (1) shall be relayed or depurated consistent with Chapter IV 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 of and species of all organisms to be cultivated, cultured, and harvested;

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.

Move Chapter VI Section .07 to a new Chapter:

Chapter XVII Shellfish Gardening

@ .01 Shellfish Gardening.

If a State recognizes shellfish gardening the Authority:

A. Shall permit or register shellfish gardening activities.

B. Shall establish permit or registration conditions and determine classification of waters where shellfish gardening can take place prior to its implementation.

C. Shall provide information to the shellfish gardener on the risk of consuming shellfish from private docks, piers, and shellfish floats attached to piers or docks and from waters not classified and open to harvest for direct

	consumption. D. May require that the shellfish gardener maintain records on the disposition of the shellfish product and provide these records to the Authority. @ . 02 Requirements for the Shellfish Gardener. A. Shellfish gardening shall be practiced only in strict compliance with the provisions of the permit issued by the Authority for the oyster/shellfish gardening activity. B. Shellfish gardeners shall document that they understand the risks associated with consumption for shellfish grown from docks or private piers. C. If required by the Authority, shellfish gardeners shall keep accurate records on the fate or final destination of all shellfish grown at their shellfish garden site and provide these records to the Authority upon request.
Action by 2017 Task Force I	Recommended adoption of Aquaculture Committee recommendation on Proposal 13-107 as amended. Section I. Definitions Replace definition 9. in Section I of the Model Ordinance as follows: 9. Aquaculture means cultivating shellfish in controlled conditions for human consumption. Cultivation includes propagation and growing of shellfish. These activities may occur in natural or man-made water bodies. These activities include seed collection, production, cultivation in natural water bodies when shellfish are held off the bottom such as the use of racks, bags, or cages, and when shellfish are held in man-made water bodies such as the use of tanks, ponds, or raceways. These activities do not include depuration or~~,~~ wet storage~~. or the broadcasting of spat or seed shellfish being left to mature the same as wild shellfish.~~ Modify definition 93. in Section I of the Model Ordinance as follows: (93) Prohibited means a classification used to identify a growing area where the harvest of shellstock for any purpose, except depletion or gathering or nursery culture of seed for aquaculture, is not permitted. Section IV. Chapter IV. Shellstock Growing Areas Change @03 E. (2)(a) to read: (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 the depletion of the areas classified as prohibited; and Replace Chapter VI. Aquaculture in its entirety as follows: Change @03 E. (2)(a) to read: (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 the depletion of the areas classified as prohibited; and

Chapter VI. Aquaculture
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.]

@ .01 General.

A. Aquaculture ~~A~~activities which may~~have been determined to~~ pose a significant public health concern and are regulated~~need regulation outlined~~ in this Chapter include, but are not limited to:
   (1) Seed production in waters classified as Prohibited or Unclassified;
   (2) Aquaculture structures that attracts 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.

Requirements for the Harvester/Dealer

.1 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.

B. Restricted or Conditionally Restricted would be exempt from these requirements but subject to relay requirements in Chapter V for seed that exceeds the maximum seed size established by the Authority.

.2 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; (3) The open status of the conditionally restricted classification; or 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; (2) Water source, its treatment method, if necessary, and its quality in land based systems. .3 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; (6) Corrective actions for addressing seed exceeding the maximum seed size as defined by the Authority. .4 Aquaculture that attracts birds or mammals. A. Operational Plan. Each aquaculture site that the Authority determines may attract sufficient birds and/or mammals 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) Maintenance of the required records

.5 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) A program of sanitation, maintenance, and supervision to prevent contamination of the shellfish products;
(7) A description of the water source, including the details of any water treatment process or method;
(8) 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) If applicable, collection of data concerning the quality of food production (algae or other) used in the artificial harvest system; and
(10) 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.
(1) 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 shall meet 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 of Section D. (1) shall be relayed or depurated consistent with Chapter IV prior to direct marketing.

.6 Polyculture Systems.

A polyculture system shall:

A. Meet all requirements in Section .05 Land Based Systems;
B. Provide information concerning all sources of and species of all organisms to be cultivated, cultured, and harvested;
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.

Move Chapter VI Section .07 to a new Chapter:

Chapter XVII Shellfish Gardening

@ .01 Shellfish Gardening.

If a State recognizes shellfish gardening the Authority:
A. Shall permit or register shellfish gardening activities.
B. Shall establish permit or registration conditions and determine classification of waters where shellfish gardening can take place prior to its implementation.
C. Shall provide information to the shellfish gardener on the risk of consuming shellfish from private docks, piers, and shellfish floats attached to piers or docks and from waters not classified and open to harvest for direct consumption.
D. May require that the shellfish gardener maintain records on the disposition of the shellfish product and provide these records to the Authority.

@ . 02 Requirements for the Shellfish Gardener.

A. Shellfish gardening shall be practiced only in strict compliance with the provisions of the permit issued by the Authority for the oyster/shellfish gardening activity.
B. Shellfish gardeners shall document that they understand the risks associated with consumption for shellfish grown from docks or private piers.
C. If required by the Authority, shellfish gardeners shall keep accurate records on the fate or final destination of all shellfish grown at their shellfish

	garden site and provide these records to the Authority upon request. Recommends a committee be appointed by the Conference Chair to review and revise existing guidance documents related to the Aquaculture Chapter.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-107.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 13-107.

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

LAB #

SAMPLE COLLECTION FORM

DATE OF COLLECTION	Anal BY	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
8/4/98	NLS	8/13/98	8/17/98	PCE

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

DATE OF SHIP	FROM LOT OF	COLLECTED AT	REASON
		Billington Cove Aquafarm	01
		ADDRESS	SOURCE: 10

SHIPPER/PACKER & ADDRESS

FOLLOW UP SAMPLE #1

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: [signature]	SIGNED TO: [signature]	DATE 8/4/98	TIME 1:45	HOW STORED Ice Chest	
AGENT PRINT NAME: 164 John Miller	PRINT NAME: MCALLISTER 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. MFDM %	6. ADD H_2O %	7. TOT PROT %	8. TOT FAT %
9. SORBATES ppm	10. TBA ug malig	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/Ot
25. pH	26. BRIX %	27. INSECT	28. ACCIENT	29. BENZOT'S ppm	30. ASCORB'S ppm	31. INDOLE ug/100g	32. VITAMIN D IU/Ot
33. PEG 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 %
49. FRUCTOSE %	50. SUCROSE %	51. LACTOSE %	52. MALTOSE %	53. YEAST	54. MOLD	55. PSF	56. 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. EPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC			

COLLECTOR'S NOTES:

Moonstone Oyster nursery stock. appt. 36 mm

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

Data Collected by Dr. Dale Leavitt, Roger William University

Hard Clam Seed from Warwick Cove Marina

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 Sn/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 Sn/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 No. **13-111**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	David C. Deardorff
Affiliation	Abraxis LLC
Address Line 1	54 Steamwhistle Drive
Address Line 2	
City, State, Zip	Warminster, PA 18974
Phone	215-357-3911
Fax	215-357-5232
Email	ddeardorff@abraxiskits.com
Proposal Subject	DSP PPIA Kit for Determination of Okadaic Acid Toxins Group (OA, DTX1, DTX2) in Molluscan Shellfish
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests Marine Biotoxin Testing
Text of Proposal/ Requested Action	The DSP PPIA kit be approved as a Marine Biotoxin Laboratory Test Method.
Public Health Significance	Okadaic acid (OA) and its analogues, DTX1, DTX2, together with their ester forms are known as the group of OA-toxins. These toxins, lipophilic and heat stable, are produced by dinoflagellates and can be found in various species of shellfish, mainly in filter feeding bivalve molluscs. The OA-toxins group causes Diarrheic Shellfish Poisoning (DSP), which is characterized by symptoms such as diarrhea, nausea, vomiting and abdominal pain. These symptoms may occur in humans shortly after consumption of contaminated bivalve molluscs such as mussels, clams, scallops or oysters. Inhibition of serine/threonine phosphoprotein phosphatases is assumed to be responsible for these toxic effects. Recently in the Pacific Northwest harvest areas, outbreaks of DSP have occurred.
Cost Information	Refer to Para D.1. of the Checklist
Action by 2013 Laboratory Methods Review and Quality Assurance Committee	Recommended referral of Proposal 13-111 to an appropriate committee as determined by the Conference Chairman and directed the Executive Office send a letter to the submitter requesting additional information as provided by the Laboratory Methods Review and Quality Assurance Committee.
Action by 2013 Task Force I	Recommended adoption of Laboratory Methods Review and Quality Assurance Committee recommendation on Proposal 13-111.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-111.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-111.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 13-111 to an appropriate committee as determined by the Conference Chair until additional data are received.
Action by 2015 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 13-111.
Action by 2015 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-111.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-111.
Action by FDA	Concurred with Conference action on Proposal 13-111.

January 11, 2016	
Action by 2017 Laboratory Committee	Recommended referral of Proposal 13-111 to an appropriate committee as determined by the Conference Chair.
Action by 2017 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 13-111.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-111.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 13-111.

OKATEST

ZE/OA48C
ZE/OA96C

Test for detection of Okadaic Acid-toxins group

Test para la detección de las toxinas del grupo del Ácido Okadaico

ZEULAB, S.L.
C/ Bari, 25 dpdo. • 50197 Zaragoza (SPAIN)
Tel.: +34 976 731 533 • Fax: +34 976 524 078
info@zeulab.com • www.zeulab.com

SCOPE

This protocol specifies a method for the quantitative determination of Okadaic Acid (OA) and other carboxylic toxins of the OA group including DTX1, DTX2 and DTX3 by a colorimetric phosphatase inhibition assay. This method is applicable to shellfish species such as mussels, clams, cockle, scallops, etc.

PRINCIPLE

Test based on the phophatase activity inhibition by OA-toxins group, responsible for diarrheic shell-fish poisoning (DSP).
Phosphatase enzyme PP2A is able to hydrolyse a specific substrate, yielding a product that can be detected at 405 nm. Samples containing toxins from the okadaic acid group will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample. The concentration of toxin in the sample can be calculated using a standard curve.

KIT CONTENTS

	48 Tests Kit	96 Tests Kit
Microtiter plate strips (8 wells per strip)	6	12
Vials of Phosphatase *(Phosphatase)*	2	4
Set of Okadaic Acid Standards (*Okadaic acid 0.5, 0.8, 1.2, 1.8 and 2.8 nM)*	1	1
Chromogenic Substrate *(Chromogenic Substrate)*	1	1
Phosphatase Dilution Buffer *(Phosphatase Dilution Buffer)*	1	1
Stock Buffer Solution *(Stock Buffer Solution)*	1	1
Stop Solution *(Stop Solution)*	1	1
Adhesive film	1	2
Kit instructions	1	1

ADDITIONAL MATERIAL AND REAGENTS NEEDED

- Micropipettes
- Blender (Ultraturax) or mortar and pestle
- Heater at 30ºC ± 2 ºC (i.e. FX Incubator, Ref ZE/FX, from ZEULAB)
- Microplate reader (wavelength at 405 nm)
- Water bath for 76 ± 2 ºC
- Methanol (analytical grade)
- NaOH 2.5 N made by titration, (NaOH of analytical grade)
- HCl 2.5 N made by titration, (HCl of analytical grade)
- Deionised water (grade 2, ISO3696)
- Graded 50 mL centrifuge tubes with screw caps
- Tube shaker
- Centrifuge

SOLUTIONS

1.- Okadaic Acid Standards: to make sure these solutions are homogeneous, it is very important to mix well using a vortex, before applying to the plate.

2.- Chromogenic Substrate solution: The solution contains stabilization resin. Make sure this resin is not added to the microwells. To assure that, it is recommended to transfer the volume needed into a transparent labware (i.e.: test tube or eppendorf) and take the solution from that container to add into the wells. *Note:* Do not use this solution if the absorbance of 90 µL of this solution at 405 nm is over 0.6.

3.- Phophatase solution: Add 2.0 mL of phosphatase dilution buffer *(Phosphatase Dilution Buffer)* to one of the phosphatase vials *(Phosphatase)* and dissolve by mixing gently for 1 hour ± 5 minutes at room temperature (22 ± 2 ºC) to ensure that the enzyme is fully hydrated. Do not use the tube shaker at any moment. This solution must be stored under refrigeration if not in use immediately after preparation. Do not use the phosphatase solution for following days. Each enzyme vial contains enough volume for 24 wells. If more than one vial is used in the assay, dissolve each vial as described above, make a pool with the content of the vials and mix gently, by inversion, before use.
*Attention: this reagent is blue and becomes brownish when dissolved. If brownish colour is noticed before hydratation, discard this reagent as it could be damaged.

4.- Buffer solution x1: dilute the *Stock Buffer Solution* included in the kit by mixing 1 volume with 9 volumes of deionised water. Use buffer solution x1 only freshly made, and store under refrigeration if not in use immediately.

5.- 2.5 N NaOH: weigh 100 g of NaOH and add 500 mL of water and dissolve. Transfer to a volumetric flask and add deionised water up to a final volume of 1000 mL.

6.- 2.5 N HCl: add 205 mL of HCl (37 %) to 400 mL of deionised water already contained in a volumetric flask. Make the volume up to 1000 mL with deionised water.

SAMPLES EXTRACTION

The method described below includes a hydrolysis step to detect all toxins forms of okadaic acid (okadaic acid and dinophisistoxins).

1.- Clean the shell thoroughly using water
2.- Open the shellfish by cutting the adductor muscles.
3.- Wash inside the shell thoroughly to remove any dirt.
4.- Remove the tissue inside the shell by cutting all the muscles attached to the shell.
5.- Place the shellfish tissue in a filter paper for few minutes to remove water in excess.

It is recommended to use graded 50 mL centrifuge tubes with screw caps during the following steps of hydrolysis in order to prevent loses due to labware changes.

6.- Mash the shellfish tissue to obtain a representative sample and weigh 5 g. Add 25 mL of Methanol and homogenise the mixture for 2 minutes using a tube shaker.
7.- Centrifuge at 2000 g for 10 min at 4 ºC. The supernatant (*methanolic extract*) is poured into a centrifuge tube.
8.- Take 640 µL of *methanolic extract* and pour into another centrifuge tube.
9.- Add 100 µL of 2.5 N NaOH.
10.- Seal and heat at 76 ± 2 ºC for 40 minutes.

11.- Add 80 µL of 2.5 N HCl (the sample does not need to be cooled down previously).
12.- Add up to 20 mL of Buffer solution x1.

TEST PROCEDURE

Warning:

The volume of some reagents used in this assay is small and special attention must be paid when added to the wells:

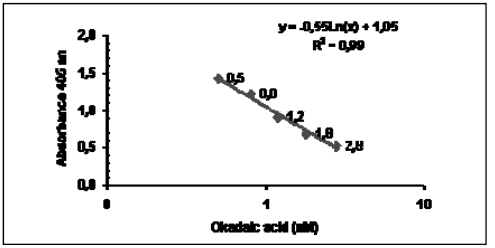
- Make sure the pipettes are calibrated before running the assay.
- Use pipettes according to the volumes to be dispensed. Use pipettes with a maximum pipette volume of 100 or 200 µL.
- Be sure that the incubator's temperature is stabilized before use.

It is recommended to run samples and standards in duplicate.

1.- Add 50 µL of samples or standards.
2.- Add 70 µL of the Phosphatase Solution to each well. Mix well by gentle tapping on the side of the plate.
3.- Cover the plate with the adhesive film provided and incubate for 20 ± 0.5 minutes at 30 ± 2 ºC.
4.- Remove the adhesive film and add 90 µL of Chromogenic Substrate to each well. Mix well by gently tapping on the side of the plate.
5.- Cover the plate with the adhesive film and incubate 30 ± 0.5 minutes at 30 ± 2 ºC.
6.- Remove the adhesive film and add 70 µL of Stop Solution to each well.
7.- Read absorbance of samples and standards at 405 nm.

GRAPHIC REPRESENTATION AND CALCULATIONS OF RESULTS

1.- Obtain a standard curve by plotting the absorbance values in a linear *y axis* and the concentration of okadaic acid in a logarithmic *x axis* and use a logarithmic fitting as shown in the graphic next page. R^2 has to be greater than or equal to 0.96.



2.- The OA concentration contained in the sample (Cs) is calculated by interpolation into the calibration curve or using the following equation:

$$x = \text{EXP}\,(y - b/a)$$

Where *x* is the OA concentration in the sample (Cs) and *y* the absorbance of the sample.

Note: An Excel worksheet to calculate results is available upon request.

3.- Calculate the diarrheic shellfish toxins concentration in tissue (Ct) as follows:

$$\text{Ct}\ (\mu g/kg) = \frac{(\text{Cs (nM)} \times \text{FD} \times \text{MW (g/mol)} \times \text{Ve (L)})}{\text{Mt (g)}}$$

Ct: toxins concentration in tissue, expressed as equivalents of OA; Cs: toxins concentration in sample; FD: Methanolic extract dilution factor (i.e. 640 μL/20 mL → x 31.25); MW: Okadaic acid molecular weight = 805; Ve: Methanolic extract volume (0.025L); Mt: Tissue weight (5g).

Example: for OA concentration of 1.5 nM: 1.5 nM x 31.25 x 805 g/mol x 0.025L / 5g = 189 μg OA eq/kg.

NOTE: For samples with OA concentration falling outside the working range (< 0.5 nM or > 2.8 nM), results will be reported as < 0.5 nM (or < 63 μg/Kg) or > 2.8 nM (or > 352 μg/kg), respectively. When sample absorbance is below the value obtained for 2.8 nM the methanolic extract could be diluted up to 1:4 and samples re-tested

STABILITY AND STORAGE

The kit contents must be stored at 4 - 12 ºC and protected from light. This kit has a shelf life of 8 months when stored under optimal conditions. See the expiry date on the kit package.

SAFETY

Safety clothing should be worn and skin contact with the reagents avoided. Do not ingest.
A SAFETY DATA SHEET is available from your local distributor on request.

*Warning: Okadaic Acid is toxic. Gloves, mask and other protective clothing must be worn when handling okadaic acid solutions.

REFERENCES

1. Takai, A.; Bialojan, C.; Troschka, M.; Rüegg, J.C. *Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin*. *FEBS Lett*. 1987, 21781-21784.

2. Smienk H., Calvo D., Razquin P., Domínguez E. & Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins.* *Toxins*, 2012, *5*, 339-352.

3. Smienk H., Domínguez E., Rodríguez-Velasco M.L. Clarke D., Katrin K., Katikou P., Cabado A.G., Otero A., Vieties J.M. Razquin P., and Mata L. *Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study.* *Journal AOAC*, 2013. 96, 1, 77-85.

OkaTest complies with the requirements established under chapter III A (4) a, b and c from Appendix III of the European Regulation (EC) 2074/2005 and can be used as complementary method.
For further information, please visit the European Reference Laboratory website:
http://aesan.msssi.gob.es/en/CRLMB/web/otros_procedimientos/other_crlmb_standard_operating_procedures.shtml

OBJETIVO

Test para la determinación cuantitativa de Ácido Okadaico (OA) y otras toxinas del grupo del OA, incluyendo DTX1, DTX2 y DTX3. Consiste en un ensayo colorimétrico de inhibición de la actividad enzimática de una fosfatasa. Este método es aplicable a especies como mejillones, almejas, berberechos, vieiras, etc.

PRINCIPIO

Okatest es un test basado en la inhibición de la actividad enzimática de una fosfatasa (PP2A) por toxinas del grupo del ácido okadaico. En condiciones normales, la fosfatasa es capaz de hidrolizar un sustrato específico obteniéndose un producto que puede ser detectado a 405 nm. En presencia de toxina diarreica se producirá una inhibición de la actividad enzimática proporcional a la cantidad de toxina diarreica presente en la muestra. Mediante la utilización de una curva de calibrado se pueden obtener los valores de concentración de toxina presentes en la muestra analizada.

COMPONENTES DEL KIT

	Kit de 48 Tests	Kit de 96 Tests
Tiras de 8 pocillos de placa microtiter	6	12
Fosfatasa *(Phosphatase)*	2	4
Set de patrones de ácido okadaico *(Okadaic acid 0.5, 0.8, 1.2, 1.8 y 2.8 nM)*	1	1
Sustrato Cromogénico *(Chromogenic Substrate)*	1	1
Solución de Dilución de la Fosfatasa *(Phosphatase Dilution Buffer)*	1	1
Solución Tamponante *(Stock Buffer Solution)*	1	1
Solución Stop *(Stop Solution)*	1	1
Lámina adhesiva	1	2
Guión de instrucciones	1	1

MATERIAL Y REACTIVOS ADICIONALES NECESARIOS

- Micropipetas
- Homogeneizador (e.j. Ultraturax) o mortero
- Incubador a 30 ± 2ºC. (Ej. FX Incubator Ref ZE/FX, de ZEULAB)
- Lector de placas microtiter con filtro a 405 nm.
- Baño termostático 76 ± 2ºC
- Metanol (grado analítico)
- NaOH (grado analítico)
- HCl (grado analítico)
- Agua desionizada (al menos de grado 2, ISO 3696)
- Tubos de centrifuga de 50 mL
- Centrífuga
- Agitador para tubos (tipo vortex)

SOLUCIONES

1.- Estándares de Ácido Okadaico: Es muy importante agitar bien estas disoluciones justo antes de su utilización (p.e.: en vortex), para asegurar su homogeneidad
2.- Sustrato Cromogénico: esta solución contiene una resina estabilizante que no debe añadirse a los pocillos. Con este fin, se recomienda transvasar el volumen a utilizar a un vial transparente (p.e.: eppendorf o tubo de ensayo), asegurándose de no coger resina, y de ahí pipetear a los pocillos. *Nota*: no usar esta solución si la absorbancia de 90 μL es superior a 0.6.
3.- Preparación de la Fosfatasa: reconstituir el liofilizado de Fosfatasa (*Phosphatase*) en 2.0 mL de Solución de Dilución de la Fosfatasa (*Phosphatase Dilution Buffer*). Mantener la solución a temperatura ambiente (22 ± 2ºC) y con agitación suave durante 1 hora para asegurar así la correcta hidratación del liofilizado. No usar el agitador de tubos en ningún momento. Una vez reconstituido el enzima, mantenerlo en condiciones de refrigeración. No conservar la solución de Fosfatasa para su uso en días posteriores.
Cada vial de Fostatasa contiene la cantidad necesaria para 24 pocillos. Si se va a utilizar más de uno, disolver cada vial como se ha explicado anteriormente y mezclar el contenido de todos en uno único antes de usar. Agitar suavemente antes de su utilización.
Atención: el liofilizado posee una coloración azulada y al reconstituirlo se convierte en marrón. Si observa que este reactivo posee una coloración marrón antes de reconstituirlo, no usarlo, ya que podría estar dañado.
4.- Solución Tamponante x1: diluir la *Stock Buffer Solution* incluida en el kit, mezclando 1 volumen de esta solución con 9 volúmenes de agua desionizada. Preparar sólo la que se vaya a utilizar en el momento y mantener en refrigeración hasta entonces.
5.- NaOH 2.5 N: pesar 100 g de NaOH y disolver en 500 mL de agua desionizada. Seguidamente, enrasar hasta un volumen final de 1000 mL usando un matraz aforado.
6.- HCl 2.5 N: Añadir 205 mL de HCl (37 %) a 400 mL de agua desionizada. Mezclar y enrasar hasta 1000 mL con agua desionizada usando un matraz aforado.

EXTRACCIÓN DE LAS MUESTRAS

El método de preparación de muestras que se describe a continuación incluye una etapa de hidrólisis que permite la detección de todas las formas tóxicas de ácido okadaico (ácido okadaico y dinofisistoxinas).

1.- Limpiar la superficie externa del molusco con agua.
2.- Abrir los moluscos seccionando los músculos aductores.
3.- Lavar el contenido de las conchas con agua hasta conseguir eliminar todas las sustancias extrañas que puedan contener.
4.- Separar la carne de las conchas, retirando todos los músculos o tejidos que estén en contacto con ellas.
5.- Colocarlos en un papel de filtro y dejarlos secar durante unos minutos.
Se recomienda el uso de tubos calibrados para centrífuga de 50 mL durante las siguientes etapas de hidrólisis para evitar pérdidas por transvase de líquidos.
6.- Triturar el tejido hasta obtener una muestra homogénea, tomar 5 g (peso húmedo) y extraer con 25 mL de Metanol durante 2 minutos, usando un agitador para tubos.
7.- Centrifugar el homogeneizado a 2000 g durante 10 minutos a 4 ºC. Al sobrenadante lo llamaremos *extracto metanólico* y lo pasaremos a otro tubo de centrífuga por decantación.

8.- Tomar 640 µL del *extracto metanólico* y transvasarlo a un tubo para centrífuga nuevo.
9.- Añadir 100 µL de NaOH 2.5 N.
10.- Cerrar y calentar la muestra a 76 ± 2 ºC durante 40 minutos.
11.- Sin dejar enfriar, añadir 80 µL de HCl 2.5 N
12.- Añadir Solución Tamponante x1 hasta un volumen final de 20 mL.

PROCEDIMIENTO DE ENSAYO

Atención:

En este ensayo se usan reactivos en volúmenes pequeños y se debe tener especial cuidado cuando se añaden a la placa:

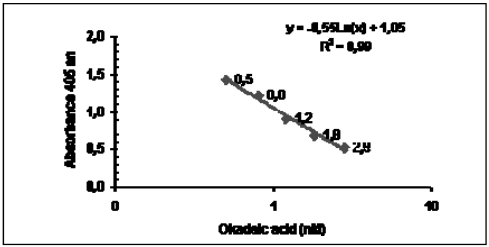
- Asegurarse de que las pipetas están calibradas antes de realizar el ensayo.
- Usar pipetas de 100 ó 200 µL de volumen máximo.
- Comprobar que la temperatura del incubador está estabilizada antes de su uso.

Es aconsejable aplicar las muestras y patrones por duplicado.

1.- Aplicar 50 µL de cada estándar o muestra.
2.- Aplicar en cada pocillo 70 µL de la Solución de Fosfatasa. Mezclar bien golpeando suavemente en el lateral de la placa.
3.- Tapar la placa con la lámina adhesiva incluida en el kit e incubar a 30 ± 2 ºC durante 20 ± 0.5 minutos.
4.- Aplicar 90 µL en cada pocillo de Sustrato Cromogénico y tapar la placa con la lámina adhesiva.
5.- Incubar a 30 ± 2 ºC durante 30 ± 0.5 minutos.
6.- Retirar la lámina adhesiva y añadir en cada pocillo 70 µL de Solución Stop.
7.– Leer la absorbancia a 405 nm en un lector de placas microtiter.

REPRESENTACIÓN Y CÁLCULO DE LOS RESULTADOS

1.- Obtener una curva de calibrado representando las absorbancias en el eje de ordenadas frente a las concentraciones de ácido okadaico en el eje de abscisas (este último en escala logarítmica). A continuación se muestra un ejemplo de curva patrón. R^2 deberá ser mayor o igual a 0.96.



2.- A partir de la curva de calibrado obtener los valores de ácido okadaico de las muestras (Cs) por interpolación o aplicando la ecuación correspondiente:

$$x = EXP\ (y - b/a)$$

x: concentración de ácido okadaico en la muestra
y: absorbancia de la muestra

*ZEULAB puede proporcionar una plantilla Excel para calcular los resultados. Para más información contacte con nosotros.

3.- Calcular la concentración de toxinas diarreicas en el tejido (Ct) a partir de la siguiente fórmula:

$$Ct\ (\mu g/kg) = \frac{Cs\ (nM) \times FD \times PM\ (g/mol) \times Ve\ (L)}{Mt\ (g)}$$

Ct: Concentración de toxinas en tejido; Cs: Concentración de toxinas de cada muestra aplicada en el pocillo; FD: Factor de dilución del extracto metanólico en la preparación de la muestra (p.e. 640 µL/20 mL → x 31.25); PM: Peso molecular ácido okadaico = 805; Ve: Volumen de extracto metanólico obtenido (0.025L); Mt: Masa de tejido pesada inicialmente (5 g).

Ej.: Para una muestra 1.5 nM de OA: 1.5 nM x 31.25 x 805 g/mol x 0.025 L / 5 g = 189 µg eq OA/kg

NOTA: Aquellas muestras cuya concentración (Cs) esté fuera del rango de trabajo (< 0.5 nM ó > 2.8 nM), los resultados se expresarán como < 0.5 nM (ó < 63 µg/Kg) ó > 2.8 nM (ó > 352 µg/kg) respectivamente.
Muestras con absorbancias inferiores a las obtenidas para el patrón 2.8 nM pueden ser analizadas de nuevo haciendo una dilución máxima de1:4 del extracto metanólico.

ESTABILIDAD Y ALMACENAMIENTO

Conservar los componentes del kit de 4 -12 ºC y en oscuridad. El kit tiene una estabilidad de 8 meses en las condiciones de conservación anteriormente indicadas.

SEGURIDAD

Se recomienda seguir unas prácticas correctas de laboratorio, así como el empleo de ropa y material de seguridad adecuados para el desarrollo del test. Evitar el contacto directo con la piel. No ingerir.

*Atención: El ácido okadaico es un producto tóxico, para su manejo es imprescindible el uso de guantes y trabajar con precaución.
Puede solicitar la hoja de seguridad del producto contactando con su distribuidor habitual o fabricante.

BIBLIOGRAFÍA

1.- Takai, A.; Bialojan, C.; Troschka, M.; Rüegg, J.C. *Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin*. *FEBS Lett*. 1987, 21781-21784.

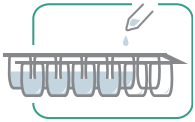
2.- .Smienk H., Calvo D., Razquin P., Domínguez E. & Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins. Toxins,* 2012, *5*, 339-352.

3.- Smienk H., Domínguez E., Rodríguez-Velasco M.L. Clarke D., Katrin K., Katikou P., Cabado A.G., Otero A., Vieties J.M. Razquin P., and Mata L. *Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal AOAC*, 2013. 96, 1, 77-85.

OkaTest cumple con los requisitos del capítulo III A (4) a, b y c del Anexo III de la Regulación Europea (EC) 2074/2005 y puede ser usado como método complementario tal y como indica el Laboratorio de Referencia Europeo en su página web: http://aesan.msssi.gob.es/en/CRLMB/web/otros_procedimientos/other_crlmb_standard_operating_procedures.shtml

FLOWCHART PROCEDURE

ESQUEMA DEL PROCEDIMIENTO

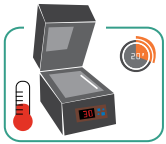


1. Add 50 µL samples/standars

1. Añadir 50 µL muestras/estándares

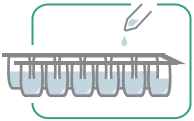
2. Add 70 µL Phosphatase Solution

2. Aplicar 70 µL Solución de Fosfatasa



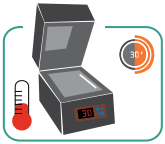
3. Incubate 20 min at 30ºC

3. Incubar 20 min a 30ºC



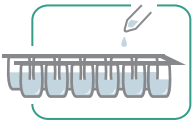
4. Add 90 µL Cromogenic Substrate

4. Añadir 90 µL Sustrato Cromogénico



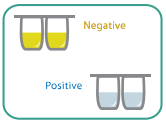
5. Incubate 30 min at 30ºC

5. Incubar 30 min a 30ºC



6. Add 70 µL Stop Solution

6. Añadir 70 µL Solución Stop



7. Read absorbance at 405 nm

7. Leer absorbancia a 405 nm

DSP PPIA kit-OkaTest

Single Laboratory Validation Report

1- EXECUTIVE SUMMARY

The **DSP PPIA (**commercial name **OkaTest)** is a test for detection of Okadaic Acid (OA) and other carboxylic toxins of the OA group including DTX1, DTX2 and DTX3 by a colorimetric phosphatase inhibition assay. It is a rapid and simple method suitable for quantitative determination of the OA- toxins group from 63 to 352 µg of OA equivalents per Kg, including the maximum limit established as 160 µg of OA equivalents /Kg in the Commission Regulation of 29 April 2004 (Regulation (EC) 853/2004). Test applicable to shellfish species such as mussels, clams, oysters and scallops.

The **OkaTest** kit was developed by ZEULAB (previous name ZEU- INMUNOTEC) based on the research work carried out by Vieytes et al. The method uses the inhibitory activity of OA and DTXs against the enzyme phosphate, which is responsible for their toxic effect, for the detection of OA-toxins group in molluscs. **OkaTest** uses a colorimetric detection system (Takai and Mieskes, 1991), while the original method (Vieytes et al., 1997) was based on fluorimetric detection.

A single laboratory validation was carried out at ZEULAB, followed by a collaborative study with 16 laboratories from 11 different countries. Both validations have been published in scientific journals; Toxins in 2012 by Smienk et al. and Journal of AOAC in 2013 by Smienk et al., respectively. Besides, OkaTest participates annually in intenational proficency exercises (Quasimeme, The Neatherlands).

OkaTest complies with the requirements established by the European Regulation (EC) 2074/2005 as complementary to the reference method.

http://www.aecosan.msssi.gob.es/en/CRLMB/web/public_documents/seccion/other_crlmb_standard_operating_procedures.htm.

Furthermore, **OkaTest** has been compared with other methodologies and using samples from the USA, UK and Argentina (Bich-Thuy et al., 2013, Turner & Goya, 2016 and Johnson et al., 2016).

This report shows the data obtained in the initial single laboratory validation (Smienk et al, 2012) that has been completed with additional information requested by the ISSC. Following a summary of the validation parameters:

Parameter	Results
Accuracy/Truness	98,00%
Measurement Uncertainty	14.92 - 31.08 µg equivalentes OA /kg
Precision	
Repeatibility:	1,4%- 3,9 % (Mean= 2,65%)
Reproducibility	0,8 %-17,7% (Mean= 6,45%)
Recovery	Okadaic acid: 78-114%
	DTX-1: 79-102%
	DTX-2: 83-94%
Working Range	63 - 352 µg equivalents OA /kg
Limit of Detection (LOD)	44 µg equivalets OA /kg
Limit of Quantification (LOQ)	56 µg equivalents OA/kg

2- METHOD PRINCIPLE AND SCOPE

DSP PPIA (OkaTest) is a protein phosphatase inhibition assay (PPIA), where the phophatase activity is ihnibited by the OA-toxins group, responsible for diarrheic shellfish poisoning (DSP). The PPIAs have been identified for a long time as an alternative for the detection of the OA-toxins, as Ser/Thr phosphatases are known to be their natural target (Bialojan & Takai, 1988). Under normal circumstances, a phosphatase enzyme is able to hydrolyse a specific substrate producing a reagent that can be detected by absorbance measurement (405 nm). Samples containing OA toxins will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample.

OkaTest is applicable to shellfish species such as mussels, clams, oysters and scallops. It is a quantitative method for determination of the OA- toxins group, where concentration of toxins present in the sample is calculated using a standard curve.

OkaTest includes five OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM), phosphatase enzyme and substrate reagents ready to use.

The test procedure is extensively described in the user manual G-COM-OA.06.

3- VALIDATION

To evaluate the performance of the OkaTest kit, accuracy, uncertainty, precision, limit of detection and quantification were calculated. The assay temperature, incubation times and other variables affecting rugedness, together with specificity and matrix effects were also evaluated. Finally, a method comparison was carried out.

3.1 Accuracy/Trueness

To estimate the accuracy of the method 20 blank mussel samples (*Mytilus edulis*) were spiked with OA at 80, 120, 160, 240 and 300 µg/kg. Percentage of recoveries were calculated and are shown in Table 1.

Table 1. Recovery values from 20 different mussels samples spiked with OA at different levels along the working range. Mean (M), standard deviation (SD) and relative standard deviation (RSD). ND < 63 µg/kg

Theoretical Spike	µg OA equivalents/kg		Recovery	Mean	SD	RDS
	Before spiked	After spiked				
80	ND	73	91.3%			
80	ND	91	113.8%			
80	ND	87	108.8%	**112.5%**	**0.18**	**16.68%**
80	ND	112	140.0%			
80	ND	87	108.8%			
120	ND	133	110.8%	**106.7%**	**0.06**	**5.52%**
120	ND	123	102.5%			
160	ND	128	80.0%			
160	ND	169	105.6%	**98.8%**	**0.13**	**12.98%**
160	ND	173	108.1%			
160	ND	162	101.3%			
200	ND	186	93.0%			
200	ND	185	92.5%	**91.3%**	**0.30**	**0.27%**
200	ND	177	88.5			
240	ND	219	91.3%			
240	ND	205	85.4%	**96.1%**	**0.21**	**21.59%**
240	ND	195	81.3%			
240	66	304	126.7%			
300	ND	250	83.3%	**82.7%**	**0.01**	**1.14%**
300	ND	246	82.0%			

3.2. Measure of Uncertainty

Measurement of uncertainty was calculated using the results obtained in the accuracy experiment considering a confidence interval of 95%. Mean and standard deviation of the difference between the concentration of the spiked sample and the spiked amount were calculated. The coefficient of confidence (Z) and maximum error (E. max) were then determined (Table 2) according to the following equation:

$$E.\ max = Z_{\alpha/2} * SD/\sqrt{n},\ \text{where}$$

E. max: maximum error, **Z:** confidence coefficient; α 95% confidence interval**, SD**: standard deviation, **n**: number of samples.

Table 2. Estimation of uncertainty based on recovery data from 20 different mussels. ABS: absolute value of differences between OA concentration in spiked samples and spike concentration. Z= coefficient of confidence. SD= standard deviation. ABS E. max=absolute value of maximum error. ND < 63 µg/kg

Sample	Spike (OA µg/kg)	Blank Sample µg OA equiv. /kg	Spiked Sample	Recovery	ABS differences	Mean	SD	ABS E. Max
1	80	ND	73	91.3%	7	13	10.87	9.53
2	80	ND	91	113.8%	11			
3	80	ND	87	108.8%	7			
4	80	ND	112	140.0%	32			
5	80	ND	87	108.8%	7			
6	120	ND	133	110.8%	13	8	7.07	6.20
7	120	ND	123	102.5%	3			
8	160	ND	128	80.0%	32			
9	160	ND	169	105.6%	9			
10	160	ND	173	108.1%	13			
11	160	ND	162	101.3%	2	14	12.83	11.25
12	200	ND	186	93.0%	14			
13	200	ND	185	92.5%	15			
14	200	ND	177	88.5%	23	17	4.95	4.34
15	240	ND	219	91.3%	21			
16	240	ND	205	85.4%	35			
17	240	ND	195	81.3%	45			
18	240	66	304	126.7%	64	41	18.08	15.85
19	300	ND	250	83.3%	50			
20	300	ND	246	82.0%	54	52	2.83	2.48
				Mean	**23**			
				SD	**18.44**			
				ABS E. Max	**8.08**			

3.3. Precision

To determine the precision of the method, relative standards devidation (RSD) for repetibility and reproducibility were calculated.

To calculate repeatibility eight replicates of two mussel samples at two levels of concentration were analysed on the same day. Mean, standard deviation and relative standard deviation were calculated. The RSD obtained for the samples tested were, 1.4 and 3.9%, respectively. These values are far below the reference value of 15% (Horwitz W., 2002).

Table 3. Repeatability of 2 different mussel samples. Mean, standard deviation (SD) and relative standard deviation (RSD).

Repetition	Sample 1 (µg OA equiv./kg)	Sample 2 (µg OA equiv./kg)
1	269	124
2	276	125
3	276	131
4	273	129
5	280	121
6	278	117
7	281	127
8	275	118
Mean	**276**	**124**
SD	**3.9**	**4.8**
RSD	**1.4%**	**3.9%**

Intermediate precision/Reproducibility

Intermediate precision was estimated by testing 13 different samples (10 mussel samples and 3 from other species) at different levels of concentration on 3 different days by the same analyst (Table 4).

Mean values, standard deviation and relative standard deviation were calculated. An average of 6.45% of RSD was calculated for all the samples with different levels of concentration. Only sample 3, at a concentration below the regulatory limit showed a RSD above 15%, which is the variability expected for this concentration range (Horwitz, 2002).

Table 4. Reproducibility of thirteen different mussel (*Mytilus edulis*), king scallop (*Pecten maximus*) and clam (*Venerupis pullastra* and *V. vomboides*) samples. Mean, standard deviation (SD), relative standard deviation (RSD) were calculated.

Sample	Matrix	Day 1	Day 2	Day 3	Mean	SD	RSD
		µ OA equivalents /kg					
1	Mussel	82	94	90	**88**	6.17	**7.0%**
2	Mussel	106	95	90	**97**	8.05	**8.3%**
3	Mussel	98	101	72	**90**	15.95	**17.7%**
4	Mussel	109	106	95	**101**	7.80	**7.8%**
5	King Scallop	125	108	117	**117**	8.20	**7.0%**
6	Mussel	122	132	113	**122**	9.57	**7.8%**
7	Mussel	196	196	215	**202**	10.57	**5.2%**
8	Mussel	211	227	187	**208**	19.84	**9.5%**
9	Clam	261	251	260	**257**	5.51	**2.1%**
10	Mussel	257	250	258	**255**	4.36	**1.7%**
11	Mussel	250	253	281	**261**	16.90	**6.5%**
12	Mussel	277	279	289	**282**	6.62	**2.4%**
13	Clam	285	285	281	**284**	2.31	**0.8%**

The intermediate precision was also further evaluated in a collaborative study with 5 samples analysed by 16 different laboratories. Values of 11.2% and 13.2% were determined as the

highest relative standard deviation for repeatability and reproducibility, respectively (Smienk et al 2013).

3.4. Recovery

Recovery was calculated by spiking mussel and scallop samples (*Mytilus edulis* and *Pecten maximus*, respectively) with okadaic acid (OA) at 0.5, 1 and 1.5 times the regulatory limit. Samples were also spiked with 80, 160 and 240 µg/kg of DTX-1 and 80 and 160 µg/kg of DTX-2. Three to five repetitions of each concentration were analysed on different days. Results are shown in Tables 5 and 6.

Table 5. Results (µg OA equivalents/kg) from recovery of OA in mussel and scallop samples at 80, 160 and 240 µg/kg. Standard deviation (SD), relative standard deviation (RSD) and recovery were calculated. ND= <63 µg/kg).

	Mussel				King Scallop			
	spiked OA (µg/kg)							
Repetition	**0**	**80**	**160**	**240**	**0**	**80**	**160**	**240**
1	86	158	230	271	ND	82	162	252
2	87	134	211	282	ND	84	142	218
3	87	178	216	257	ND	89	150	268
4	95	193	253	298	ND	102	177	268
5	95	191	257	280	ND	99	158	271
Mean	**90**	**171**	**233**	**277**	**-**	**91**	**157**	**255**
SD	4.8	25.0	20.9	15.1	-	9.0	13.3	22.2
RSD	5.4%	14.6%	8.9%	5.4%	-	9.9%	8.4%	8.7%
Recovery	**-**	**101%**	**90%**	**78%**	**-**	**114%**	**98%**	**106%**

Table 6. Results (µg OA equivalents/kg) from recovery of DTX-1 and DTX-2 in mussel and scallop samples spiked at 80, 160 and 240 µg/kg. Mean, Standard deviation (SD), relative standard deviation (RSD) and recovery were calculated. ND= <63 µg/kg).

	King scallop				Mussel					
	spiked DTX - 1 (µg/kg)				spiked DTX2 (µg/kg)					
Repetition	**0**	**80**	**160**	**240**	**0**	**160**	**0**	**80**	**0**	**160**
1	ND	63	101	211	ND	145	86	157	ND	128
2	ND	91	127	179	ND	156	101	163	ND	130
3	ND	81	132	175	ND	151	-	-	ND	124
4	ND	82	132	261	-	-	-	-	-	-
5	ND	93	140	228	-	-	-	-	-	-
Mean	**ND**	**82**	**126**	**211**	**ND**	**151**	**93.5**	**160**	**ND**	**127**
SD	-	11.9	14.8	35.6	-	5.5	-	4.2	-	2.7
RSDr	-	14.5%	11.7%	16.9%	-	3.7%	-	2.7%	-	2.1%
Recovery	**-**	**102%**	**79%**	**88%**	**-**	**94%**	**-**	**83%**	**-**	**80%**

The mean of recoveries obtained for the different concentrations tested and toxins were acceptable and ranged from 78 to 114%.

3.5. Specificity

Specificity was studied by determining the possible interferences caused by other lipophilic toxins such as Azaspirazides (AZA), Yessotoxins (YTX) and Pectenotoxins (PTX).

A mussel sample naturally contaminated was spiked, on two different days, with 160 µg/kg of AZA-1 (NRC, Institute for Marine Biosciences, Canada), 160 µg/kg PTX-2 (Cifga laboratories, Spain) and 1000 µg/kg of YTX (NRC, Institute for Marine Biosciences, Canada) and concentration of OA determined following the kits´ instructions. Results obtained for spiked and non-spiked samples were very similar and within the method variability, showing no interferences by the toxins tested.

Table 6. Results obtained from spiking a mussel sample with 160 µg/kg of azaspirazides (AZA), 160 µg/kg of pectenotoxins (PTX) and 1000 µg/kg of yessotoxins (YTX).

Spiked Mussel	Day 1	Day 2
	µg equiv. OA /kg	
0	82	82
160 (µg/kg) **PTX-2**	83	79
160 (µg/kg) **AZA-1**	82	73
1000 (µg/kg) **YTX**	82	82

3.6. Working Range and Linear Ranges

The working range is understood as the range of OA concentrations that do correctly adapt to the fitting procedure. The working range of the assay depends on the quantity and quality of the phosphatase present. Therefore, assays were performed with at least 3 different phosphatase batches and the "goodness of fit" was evaluated according to the kits´ specifications ($R^2 > 0.96$) with standard concentrations rising from 0.25 to 3.5 nM OA.

Figure 1 shows the results of three assays covering the range from 0.25 to 3.0 nM OA as this was the range that always fitted correctly (R^2>0.96). This covers sufficiently the actual range of the standards in the kit (0.5 to 2.8 nM OA).

The linearity of an assay was tested to find out whether the response of this assay is a function of the concentration of the analyte. The OkaTest assay uses a logarithmic fitting procedure.

As such the linearity of the assays' response was tested by 'backcalculation' of the standard concentration. For 'backcalculation' the equation of the standard curves used to calculate the concentration of these standards from their absorbances (Table 7).

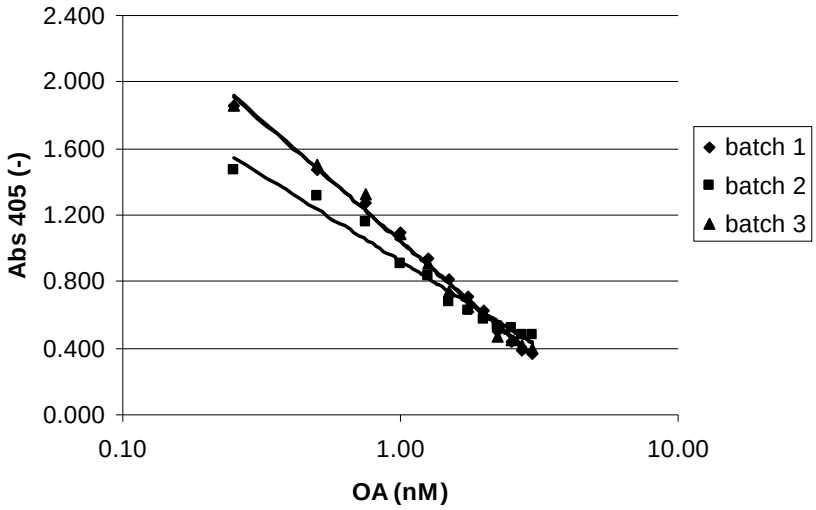


Fig 1. Working range of the assay for 3 different phosphatase batches. R^2: 0.99, 0.98 and 0.99 for batch 1, batch 2 and batch 3, respectively. Working range from 0.25 to 3.0 nM OA.

Table 7. Linearity of the assay. OA (nM) was calculated by using the standard curve of batch 1.

Standards OA (nM)	Batch 1 OA (nM)	Batch 2 OA (nM)
0.5	0.6	0.5
0.8	0.7	0.7
1.2	1.1	1.2
1.8	1.9	1.9
2.8	2.9	2.8

To check the linearity of the response, the theoretical concentration was compared to the calculated concentration for both batches (see figure 2 for the results shown in Table 1) and a linear fit was performed. The Pearson correlation coefficient (R^2) for batch 1 was 0.99 and 1.00 for batch 2.

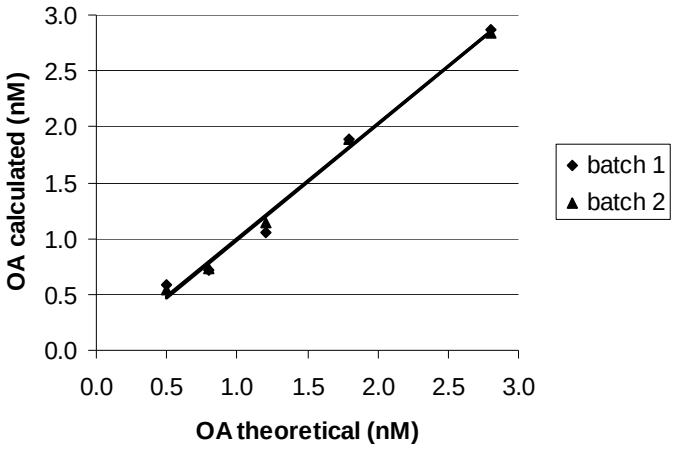


Fig 2. Comparison of the theoretical and calculated standard concentration. Concentration were `backcalculated´ by using the standard curve obtained with batch 1. R^2: 0.99 and 1.00 for batches 1 and 2, respectively.

The linearity was also determined by testing 10 blank mussel samples spiked at 80, 160, 200, 240 and 300 µg/kg (Table 8). OA concentrations obtained were divided by the spiked concentration (relative recovery). Mean of relative recovery per concentration was plot against the spiked concentration and curve equation to observe the relative response (Figure 3).

Table 8. Assay linerarity. Results obtained from 10 blank samples spiked with 80, 120, 160, 200 and 240 µg/kg to determine linearity of the assay.

Spike OA µg/kg	After spiked µg OA equiv. /kg	Relative recovery	Mean Relative Recovery
80	91	1,14	1,11
80	87	1,09	
160	169	1,06	0,96
160	162	1,01	
200	186	0,93	0,91
200	177	0,88	
240	219	0,91	0,88
240	205	0,85	
300	250	0,83	0,83
300	246	0,82	

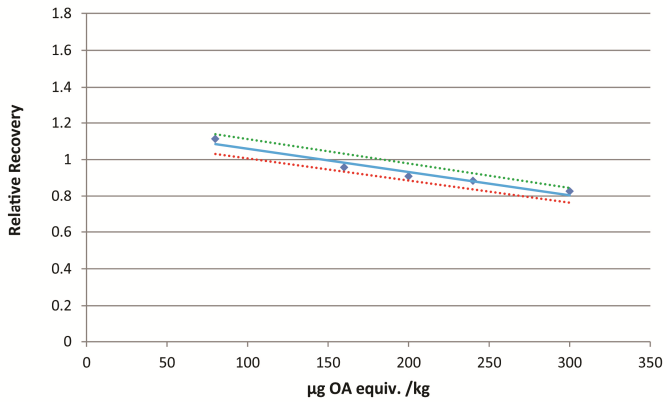


Figure 3. Assay linearity. Relative recovery data plot against spiked OA concentration µg/kg in solid blue line. Green and red dotted lines were obtained by multiplying the OA concentration by 0.95 and 1.05.

3.7. Limit of detection and Limit of quantification

To estimate the LOD and LOQ a blank mussel material was extracted ten times and analyzed according the kits' instructions. The mean and standard deviation were calculated and the limit of detection was estimated by the equation below:

$$LOD_{99\%} = X + 3SD$$

The LOQ (the lowest concentration that can be determined with an acceptable level of repeatability precision and trueness) was estimated using the same data and equation, but applying a higher factor:

$$LOQ_{99\%} = X + 10SD$$

The mean result obtained for the blank sample was 38 µg/kg. The estimated LOD and LOQ were 44 µg/kg and 56 µg/kg, respectively (Table 11).

Table 9. Quantification of the standard solvent (10 repetitions) as OA concentration equivalents (µg/kg) to estimate the LOD and LOQ. Mean, standard deviation (SD) and relative standard deviation (RSD).

Repetition	µg OA equivalents /kg
1	36
2	38
3	36
4	37
5	41
6	37
7	40
8	38
9	40
10	38
mean	38
SD	1.8
RSD	4.6%
LOD	**44**
LOQ	**56**

3.8. Ruggedness

The influence of different experimental conditions critical for the kits' performance such as assay temperature, incubation times or reaction component volumes were evaluated. The ruggedness between batches with spiked mussel samples was also evaluated.

3.8.1- Assay temperature

The hydrolysis of the substrate by the phosphatase is temperature dependent and shows the typical behaviour of an enzymatic reaction with higher reaction rates close to the optimum temperature (37°C). However, a lower assay temperature was chosen to guarantee enzyme stability during the assay and to get stable reaction rates. The assay was tested at temperatures varying from 20 to 40 °C. 30 °C was chosen as the optimum temperature. At this temperature a 2 °C variation can be expected in any incubator. So, to show the influence of this temperature variation, 3 samples were quantified performing a complete assay (standard curve and samples) at each of these temperatures (Table 10).

Table 10. Influence of the assay temperature on the results of the test. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	28 ºC	30 ºC	32 ºC	mean	SD	RSD
1	104	100	97	100	3.4	3.4%
2	176	173	176	175	1.7	1.0%
3	302	303	298	301	2.6	0.9%

Mean and relative standard deviation were calculated. For all three samples RSD were below the 15%, variation that can be expected at this concentration (Horwitz, 2002).

3.8.2- Assay incubation times

The assay consists of two different incubation steps that could affect the outcome of the test. During the first incubation the sample and the phosphatase are mixed, and the inhibition reaction should reach its endpoint. Following, the substrate is added and the plates are incubated for the second time. The main risk of this incubation step is phosphatase activity loss.

To determine the influence of time on the first incubation of the assay (normally 20 minutes), this step was varied between 18 and 24 min, while maintaining the rest of the assays' conditions according the kits' instructions. Three control samples were quantified and the variation in the relative standard deviation was evaluated. For each of the incubation conditions an independent assay was performed (Table 11).

Table 11. Influence of time on the first incubation of the assay. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	- 2 min	0	+ 2 min	+ 4 min	mean	SD	RSD
1	85	87	87	90	88	2.1	2.4%
2	152	155	161	164	158	5.7	3.6%
3	311	291	317	320	310	12.9	4.2%

In all cases the assay complied with the criterion (R^2>0.96). The relative standard deviations were comparable to those obtained when performing the test under standard conditions (highest 4.2%).

The second incubation was evaluated similarly. A 10% error from normal incubation time (30 minutes) was applied, adding some extra time (up to 20% or 6 minutes). The assay was performed as described; although no stopping solution was added to permit reading the same assay. The RSD was 2.9% at highest, a bit lower than the ones obtained for the first incubation time (Table 11).

Table 12. Influence of the incubation time (2nd incubation with the substrate) on the assay. The mean, standard deviation (SD) and relative standard deviation (RSD) were calculated.

Sample	- 3 min	0	+ 3 min	+ 6 min	mean	SD	RSD
1	89	90	89	91	90	1.0	1.1%
2	143	152	145	149	147	4.3	2.9%
3	309	321	315	313	315	5.2	1.7%

3.8.3- Influence of pipetting volumes

The OkaTest assay consists of three pipetting steps of relatively small volumes. First, 50 µL samples of standards are applied in duplicate and 70 µl of phosphatase is added. Then, after the first incubation, 80 µL of substrate and finally 70 µL of stopping solution are added. The influence of pipetting error was evaluated by introducing a 2 µL systematic error in each of the pipetting steps, e.g. a -2 µL error means pipetting 48, 68, 78 and 68 µL for samples/standard, phosphatase, substrate and stopping solution, respectively. This relatively big error (4% of the sample volume) is quite above the systematic error that can be expected in correctly calibrated pipettes (2%), but it was chosen in order to get clear results for obvious interpretation. The RSD and error were evaluated (Table 13).

Table 13. Effect of the sistematic pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSD) and error (Errormax) were calculated.

Sample	- 2 µL	0	+ 2 µL	mean	SD	RSD	E. Max*
1	83	85	93	87	4.9	5.6%	8.0 (9.4%)
2	161	148	156	155	6.7	4.3%	13 (8.8%)
3	303	289	304	299	8.5	2.8%	15 (5.1%)

*E. max = maximum difference from standard (0) conditions in µg/kg and percentage.

The RSD was at highest 5.6% and in accordance with the values normally obtained with OkaTest. The error introduced changed from 9.4 to 5.1% of the standard conditions.

The effect of a single pipetting error was evaluated by introducing a 5 µL error in one of the pipetting steps. In this case, the standard curve was performed according the kits' instructions and the error was introduced in the samples that were quantified. For example, a -5 µL error in the phosphatase means that 65 µL phosphatase was added to 50 µL sample (in duplicate) after which the assay was performed as usual. Also in this case, a relatively big error was chosen (10-6.3 % error, depending on the assay volume) (Table 14).

Table 14. Effect of a single pipetting error on the results of the test. Mean, standard deviation (SD), relative standard deviation (RSD) and error (Errormax) were calculated.

Variable	-5 µL	0	+ 5 µL	mean	SD	RSD	E Max*
Sample	132	148	173	151	20.5	13.6%	25 (17%)
Phosphatase	180	148	130	153	25.2	16.5%	32 (22%)
Substrate	167	148	159	158	9.6	6.1%	19 (13%)
Stop solution	170	148	153	157	11.6	7.4%	22 (15%)

*E. max = maximum difference from standard (0) conditions in µg/kg and percentage.

Table 14 shows that pipetting errors in sample and phosphatase volume have the biggest effect and special care have to be taken when applying these. Also the logical tendencies can be seen; when applying less samples underestimation can be expected, while with the phosphatase occurs the contrary. This is to be expected, less phosphatase means more inhibitor per amount of phosphatase and so higher estimates of the toxin concentration. Table 14 also shows that high RSDr values (above 10%, ZEULAB in-house 5%) are a good indication for pipetting error. Substrate and stop solution pipetting errors seem to be much less important RSDr < 10%.

3.8.4- Influence of phosphatase solubility

In the previous paragraph was shown that the amount of phosphatase added to each well is important for correct quantification. The phosphatase is the only component of the kit that is not ready to use. It has to be dissolved previously and insufficient solubilisation could lead to

overestimation of the toxin concentration. Therefore the solubilisation time was evaluated by dissolving three phosphatase vials of the same batch for 30, 60 and 90 minutes (normal resuspension time use is 60 minutes), and always under agitation. Three control samples were quantified and the RSD was evaluated (table 15).

Table 15. Test results after dissolving the phosphatase for 30, the normal 60 and 90 minutes. The remaining part of the assay was performed according to the kits instructions. Mean, standard deviation (SD), relative standard deviation (RSD) were calculated.

Sample	30 min	60 min	90 min	Mean	SD	RSD
1	100	95	99	98	2.5	2.5%
2	167	151	157	158	8.0	5.0%
3	317	304	318	313	8.1	2.6%

The RSD values obtained were at highest 5.0% and comparable to those obtained for within batch variability (see table 15).

3.8.5- Ruggedness between batches in samples

The ruggedness of the assay with molluscs samples was also determined. Ten blank mussel samples were spiked at 80, 120, 160, 200 and 240 µg/kg of okadaic acid and tested following the kits´ instructions in two different days and using two different batches. Differences between concentrations obtained in each batch for the different samples were calculated. Mean and standard deviation of the differences together with the experimental t-score and critical t values were also determined (Table 16).

$$t\,exp = \frac{|Mean|}{\frac{s}{\sqrt{n}}}$$

Mean ≡ mean of the difference of skewness

s ≡ Standard deviation; n ≡ number of samples

The critical value was calculated for a significance of α = 0.05 (95% confidence) for n-1 degrees of freedom. If the calculated value of experimental-t is less than the critical-t, we can affirm that the hypothesis is true, so that there is an equivalence between both methods.

The experimental t-score was smaller than the critical t-value (t exp< t crit; 1.42<2.26); and so the range of skewness was acceptable. There is not significant difference between batch 1 samples and batch 2 concentrations.

Table 16. Results from testing 10 different mussel samples spiked at different concentrations and tested with two different batches in two different days. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Sample	Batch No. 1	Batch No. 2	Differences between batches
	OA equivalents µg/kg		
1	91	74	-17
2	87	79	-8
3	133	102	-31
4	123	150	27
5	169	145	-24
6	162	177	15
7	186	177	-9
8	185	168	-17
9	219	174	-45
10	159	169	10
		Mean	**-9.9**
		SD	**22.01**
		Experimental t-score	**1.42**
		Critical t-value	**2.26**

The data was also analyzed using a Welch's test or unequal variances t-test, which is a two-sample location test used to check the hypothesis that two populations have equal means (H_0).

Therefore, considering that the null hypothesis (H_0) refers to the fact that the two batches do not show differences in the analysis of samples spiked with a known concentration of okadaic acid. Mean, variance and p-value were calculated (Table 17).

P-value (0.603) was higher than 0.05 (0.603>0.05), therefore we do not reject the null hypothesis. The observed difference between the sample´s means is not convincing enough to say that the average value between both batches differing significantly.

Table 17. Mean, variance and p-value calculated for results obtained from 10 spiked mussel samples tested with bath 1 and batch 2 of OkaTest (results from Table 16).

	Batch 1	Batch 2
Mean	151.40	141.50
Variance	1812.93	1682.50
p value	0.603	

Residual values analysis evaluates the goodness of the test. A linear relationship is confirmed when the residues have symmetry around zero and a homogeneous random dispersion. Graphical representation is the most common methodology, being a very visual and simple method to evaluate symmetry. Residual standard values were also calculated (table 18) and

the distribution plotted. The adjustment is adequate since the residual values have a random and homogeneous distribution around 0, being between ± 2 (Figure 4).

Table 18. Residual standard values obtained for OA concentration results obtained for 10 mussel samples analysed with two different batches of OkaTest.

Sample	Residual standard values
1	5.51E-05
2	-0.39
3	0.79
4	-1.67
5	0.67
6	-0.98
7	0.13
8	0.46
9	1.78
10	-0.79

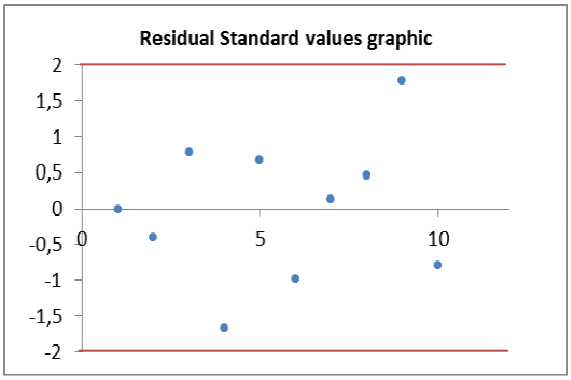


Figure 4. Distribution of residual standard values obtained for OA concentration results obtained for 10 mussel samples analysed with two different batches of OkaTest

3.9- Matrix Effects:

To determine the matrix effect 10 different molluscs' samples were tested according to the kit instructions and further diluted; where the final concentration of diluted samples was calculated multiplying by the appropriate dilution factor. Mean and SD of the differences between concentrations for diluted samples were calculated.

To evaluate if the concentrations obtained for diluted samples were within the assay variability and not due to matrix effect the experimental t-score and t-critical values were calculated (Table 19):

As the experimental t-score is smaller than the critical t-value (0.93<2.26) the skewness obtained is acceptable and does not indicate matrix effect.

Table 19. OA equivalents µg/kg for 10 mussel samples tested a two different dilutions. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Sample	Dilution 1	Dilution 2	Diferences
	OA equiv. µg/kg		
1	40	37	-3
2	980	974	-6
3	29	30	1
4	620	628	8
5	595	560	-35
6	138	104	-34
7	1192	1287	95
8	1149	1318	169
9	118	108	-10
10	85	87	2
		mean	**18.7**
		SD	**63.84**
		Experimental- t score	**0.93**
		Critical-t value	**2.26**

3.10. Method comparison

A method comparison was performed with the mouse bioassay (MBA), reference method in Europe until 2011 and LC-MS/MS (current official reference method in Europe).

To compare results from OkaTest and MBA, values obtained by OkaTest with a concentration ≥ 160 µg/kg were regarded as positive while samples with a concentration < 160 µg/kg were reported negative.

Twenty-three out of thirty-one samples tested positive for both methods and five samples were negative for both methods. However, three samples were positive for MBA and negative for OkaTest (Table 20). In all three samples OA toxins were detected, but below the regulatory limit of 160 µg/kg (144, 135 and 124 µg/kg OA toxins, respectively). Those samples were also tested by LC-MS/MS where two out of three results were above the regulatory limit as well by MBA, showing slightly higher quantifications compared to OkaTest (185, 152 and 177 OA toxins µg/kg, respectively).

OkaTest was compared with LC-MS/MS for a total of 69 samples, where results from the reference method came from two different laboratories (Tables 20 and 21).

Table 20. Results from MBA, OkaTest and LC-MS/MS. Positive results (+): ≥160 µk/kg. Negative result (-): <160 µg/kg. *HPLC-MS results were not with toxicity factors. However only 4 samples contained DTX-2. LC-MS/MS carried out at the Reference Laboratory in Vigo.

Sample	Matrix	MBA	OkaTest	OkaTest µg OA equiv. /kg	LC-MS/MS µg OA /kg
1	**Mussel**	-	-	122	ND
2	**Scallop**	-	-	ND	ND
3	**Mussel**	-	-	ND	ND
4	**Donax**	-	-	97	82
5	**Cockle**	-	-	ND	ND
6	**Mussel**	+	+	196	158
7	**Mussel**	+	+	232	502
8	**Mussel**	+	+	268	ND
9	**Scallop**	+	+	264	184
10	**Mussel**	+	+	250	177
11	**Mussel**	+	+	265	288
12	**Mussel**	+	+	196	318
13	**Mussel**	+	+	>377	604
14	**Mussel**	+	+	>377	894
15	**Mussel**	+	+	277	390
16	**Mussel**	+	+	305	658
17	**Mussel**	+	+	306	414
18	**Mussel**	+	+	310	392
19	**Mussel**	+	+	>377	444
20	**Mussel**	+	+	315	329
21	**Mussel**	+	+	270	232
22	**Mussel**	+	+	277	235
23	**Mussel**	+	-	135	152
24	**Mussel**	+	+	164	98
25	**Mussel**	+	+	211	168
26	**Mussel**	+	+	251	209
27	**Mussel**	+	+	191	113
28	**Mussel**	+	-	124	177
29	**Cockle**	+	+	252	193
30	**Mussel**	+	+	216	247
31	**Mussel**	+	-	144	185
32	**Mussel**		-	ND	ND
33	**Mussel**		+	>377	357
34	**Mussel**		-	ND	292
35	**Mussel**		-	ND	ND
36	**Mussel**		-	ND	ND
37	**Mussel**		+	304	316

A comparison of OkaTest and the reference method LC-MS/MS was made for those samples which showed a quantitative value with both methods. The samples were analyzed by paired t-test to determine the equivalence of the two analytical methods, comparing both means to determine if the difference between the expected means surpasses the one produced randomly.

The hypothetical difference of Means should be zero (Null hypothesis H_0), which means that both methods are considered equivalents.

Table 21. Analysis t Student match pairs from results OkaTest and LC-MS/MS results from table 20.

	OkaTest	LC-MS
Mean	240.33	281.71
t-statistic	1.74	
P(T≤t) value (probability value) for the t-statistic (one-tailed)	0.048	
Critical value of a t-distribution (one-tailed)	1.72	
P(T≤t) value (probability value) for the t-statistic (two-tailed)	0.097	
Critical value of a t-distribution (two-tailed)	2.09	

The null hypothesis was accepted because critical- t two-tail < t Stat < t Critical two-tail (-2.09 < -1.74 < 2.09) and p (0.097)>0.05. The observed difference between the sample means (240.33 and 281.71) was not convincing enough to say that the average value between LC-MS and Okatest differ significantly.

Besides, the test t was applied manually to the difference of values obtained for each sample. For this application, the value of the experimental t-score statistic was calculated, as well as the critical t- value:

We could affirm that the hypothesis is true because the calculated experimental-t value was smaller than the critical-t value (1.65<2.08). The skewness is acceptable and the methods Okatest and LC-MS/MS are considered to be similar (Table 22).

Table 22. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Mean	37.77
SD	107.56
Number of samples	22
Experimental t-score	1.65
Critical-t value	2.08

Table 23. Results from OkaTest and LC-MS/MS (EU harmonized SOP, v2, 2010)
Assays carried out by Jonathan Deeds from the FDA, US

Shellfish/Location	OkaTest µg equiv. OA/Kg	LC-MS/MS µg equiv. OA/Kg	OA	DTX1	DTX2
Softshell Clams (*Mya arenaria*) State: New York US East Coast Atlantic Ocean	336	373	255	118	ND
	315	325	202	105	ND
	295	307	217	108	ND
	285	260	136	69	ND
	240	205	171	89	ND
	190	155	102	53	ND
	118	75	49	26	ND
	<63	39	26	13	ND
	<63	ND	ND	ND	ND
Oysters (*Crassostrea virginica*) State: Texas US Gulf Coast Gulf of Mexico	322	563	563	ND	ND
	300	519	519	ND	ND
	245	202	202	ND	ND
	240	194	194	ND	ND
	239	221	221	ND	ND
	235	189	189	ND	ND
	198	189	189	ND	ND
	155	88	88	ND	ND
	154	97	97	ND	ND
	88	38	38	ND	ND
	<63	16	16	ND	ND
Mussels (*Mytilus edulis*) State: Washington US West Coast Pacific Ocean	>352	525	ND	525	ND
	266	272	ND	272	ND
	256	263	ND	263	ND
	171	165	ND	165	ND
	157	164	ND	164	ND
	141	131	ND	131	ND
	134	128	ND	128	ND
	127	121	ND	121	ND
	90	76	ND	76	ND
	<63	76	ND	76	ND
	<63	33	ND	33	ND
	<63	ND	ND	ND	ND

Mussels' results were statistically analyzed by applying a t-Student match pairs test to the results above the limit of quantification for each method.

The null hypothesis was accepted because the critical-t two-tail < t Stat < Critical-t two-tail (-2.37 < 0.94 < 2.37) and p (0.8) > 0.05. Therefore, we do not reject the null hypothesis. The observed difference between the sample means (167.75 and 165.00) is not convincing enough to say that the average value between LC-MS and Okatest differ significantly (Table 24).

Table 24. Analysis t Student match pairs from results OkaTest and LC-MS/MS results from table 22:

	OkaTest	LC-MS
Mean	167.75	165.00
t-statistic	0.94	
P(T≤t) value (probability value) for thet-statistic (one-tailed)	0.19	
Critical value of a t-distribution (one-tailed)	1.89	
P(T≤t) value (probability value) for the t-statistic (two-tailed)	0.38	
Critical value of a t-distribution (two-tailed)	2.36	

We applied the test t manually to the difference of values obtained for each sample. For this application the value of the experimental t-score statistic was calculated, as well as the critical-t value (Table 25).

Table 25. Mean, standard deviation (SD), experimental-t and critical-t values were calculated.

Mean	-2.75
SD	8.26
Number of samples	8
Experimental t-score	0.94
Critical-t value	2.36

We could affirm that the hypothesis is true because the calculated value of experimental-t is smaller than the critical-t (0.94<2.36). The skewness is acceptable and the values obtaines by Okatest and LC-MS/MS are considered similars (Table 23).

LITERATURE

1. Bialojan, C.; Takai, *A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases.* Biochem. J. **1988**, 256, 283-290.
2. Eberthart B.-T. L., Moore L. K., Harrington N., Adams N. G., Borchert J. and Trainer V.L. *Screening tests for the rapid detection of diarrhetic shellfish toxins in Washington State.* Marine Drugs, 11, 3718-3734. **2013**.
3. *EU-Harmonised Standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS*, version 2, July **2010.**
4. Horwitz W., *Protocol for the design, conduct and interpretation of method-performance studies.* Pure & Appl. Chem., Vol 67, No. 2, pp 331-343, **1995**.
5. Horwitz W. *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*. **2002**
6. Jonhson S., Harrison K. And Turner A.D. *Application of rapid test kits for the determination of Diarrhetic Shellfish Poisoning (DSP) toxin in bivalve molluscs from Great Britain*. Toxincon. 111, 121-129. **2016.**
7. Smienk H., Calvo D., Razquin P., Domínguez E. y Mata L. *Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins.* Toxins*, 5*, 339-352; **2012**
8. Smienk H., Domínguez E., Rodríguez-Velasco M.L. Clarke D., Katrin K., Katikou P., Cabado A.G., Otero A., Vieties J.M. Razquin P., and Mata L. *Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study. Journal AOAC*, 96, 1, 77-85, **2013**.
9. Takai A and Mieskes. *Inhibitory effect of okadaic acid on the p-nitrophenyl phosphate phosphatase activity of protein phosphatases.*, Biochem. J. 275, pp 233-239, **1991**.
10. Turner A. D. & Goya A. B. *Comparison of four rapid test kits for the detection of okadaic acid groups toxins in bivalve shellfish from Argentina. Food Control*, 59, 829-840. **2016.**
11. Vieytes M.R., Fontal O.I., Leira F., Baptista de Sousa J.M.V., and Botana L.M. *A Fluorescente microplate assay for diarrheic shellfish toxins.* Analytical Biochemistry, 248, pp 258-264, **1997**.

Toxins **2012**, *5*, 339-352; doi:10.3390/toxins4050339

OPEN ACCESS

toxins

ISSN 2072-6651

www.mdpi.com/journal/toxins

Article

Single Laboratory Validation of A Ready-to-Use Phosphatase Inhibition Assay for Detection of Okadaic Acid Toxins

Henry G. F. Smienk, Dolores Calvo, Pedro Razquin, Elena Domínguez * and Luis Mata

Zeu-Inmunotec, Polígono PLAZA, C/Bari 25 Dpdo, 50197, Zaragoza, Spain;
E-Mails: hsmienk@zeulab.com (H.G.F.S.); dcalvo@zeulab.com (D.C.);
prazquin@zeulab.com (P.R.); lmata@zeulab.com (L.M.)

* Author to whom correspondence should be addressed; E-Mail: edominguez@zeulab.com;
Tel.: +34-976731533; Fax: +34976524078.

*Received: 31 January 2012; in revised form: 4 April 2012 / Accepted: 18 April 2012 /
Published: 30 April 2012*

Abstract: A phosphatase inhibition assay for detection of okadaic acid (OA) toxins in shellfish, OkaTest, was single laboratory validated according to international recognized guidelines (AOAC, EURACHEM). Special emphasis was placed on the ruggedness of the method and stability of the components. All reagents were stable for more than 6 months and the method was highly robust under normal laboratory conditions. The limit of detection and quantification were 44 and 56 µg/kg, respectively; both below the European legal limit of 160 µg/kg. The repeatability was evaluated with 2 naturally contaminated samples. The relative standard deviation (RSD) calculated was 1.4% at a level of 276 µg/kg and 3.9% at 124 µg/kg. Intermediate precision was estimated by testing 10 different samples (mussel and scallop) on three different days and ranged between 2.4 and 9.5%. The IC_{50} values of the phosphatase used in this assay were determined for OA (1.2 nM), DTX-1 (1.6 nM) and DTX-2 (1.2 nM). The accuracy of the method was estimated by recovery testing for OA (mussel, 78–101%; king scallop, 98–114%), DTX-1 (king scallop, 79–102%) and DTX-2 (king scallop, 93%). Finally, the method was qualitatively compared to the mouse bioassay and LC-MS/MS.

Keywords: protein phosphatase inhibition assay (PPIA); protein phosphatase 2A (PP2A); validation; okadaic acid (OA); diarrheic shellfish poisoning (DSP)

1. Introduction

Diarrheic shellfish poisoning (DSP) is a consequence of the ingestion of a series of lipophilic toxins produced by dinoflagellates that can be present in shellfish for human consumption. These lipophilic toxins can be subdivided into four groups: the okadaic acid group (OA-toxins) including the dinophysistoxins (DTX), the pectenotoxin group (PTX), the yessotoxin group (YTX) and finally the azaspiracids (AZA). Only the OA-toxins and AZA are known to cause gastrointestinal problems [1,2]. For many years the mouse bioassay (MBA) has been the official method of detection for lipophilic toxins in the European Union [3], but with the publication of Commission Regulation (EU) No. 15/2011 [4], LC-MS/MS has become the reference method for their determination. This regulation also states that alternative or complementary methods can be used as long as an equivalent level of public health protection is provided, and the method performance criteria stipulated by the European Union Reference Laboratory on Marine Biotoxins (EU-RLMB) are fulfilled. Such methods should be intra-laboratory validated and successfully tested under a recognized proficiency test scheme.

Protein phosphatase inhibition assays (PPIA) have been identified for a long time as an alternative for the detection of OA-toxins, as ser/thr phosphatases are known to be their natural target [5,6]. As such, a validated phosphatase inhibition assay can be very useful in lipophilic toxin detection, complementary to the more complex, expensive and time consuming LC-MS/MS; or as an alternative when only OA-toxins are present in the samples. Different laboratories have developed in-house PPIA with good qualifications, using colorimetric or fluorimetric substrates to monitor enzyme inhibition. [7–12]. A collaborative study was also performed with a fluorimetric assay [13]. However, specific equipment, not often available in routine testing laboratories, makes difficult the use of fluorimetric assays for monitoring purposes. Besides, fluorimetric substrates are less stable than colorimetric ones and therefore less appropriate for ready-to-use kits. A standardized commercial test based on PPIA has not been available until recently. In this paper, we present a single laboratory validation of a commercial colorimetric PP2A assay (OkaTest) for the determination of OA-toxins in bivalve mollusks.

2. Materials and Methods

2.1. Reagents and Equipment

OkaTest kit (formerly Toxiline-DSP): The kit includes a 96-well microtiter plate, four vials of lyophilized protein phosphatase 2A (PP2A), purified from human red blood cells, five OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM) prepared from the OA Certified Reference Material (NRC CRM-OA-c, NRC-CNRC, Institute for Marine Biosciences), a liquid chromogenic substrate (p-Nitrophenyl phosphate), phosphatase dilution buffer and buffer solution.

Other reagents not included in the OkaTest kit: Methanol (Reagent grade, Carlo Erba), HCl (Reagent grade, 37% v/v, Carlo Erba), NaOH (Reagent grade, Scharlau), de-ionized water (type II, ISO 3696), certified Reference Materials (NRC CRM-DSP-MUS-b, NRC CRM-OA-c, NRC-CNRC, Institute for Marine Biosciences), DTX-1 (042-28661, Wako) and DTX2 (00-DTX2, Cifga).

Equipment: Ultra homogenizer (IKA werken), a water bath at 76 ± 2 °C (Raypa), a FX-incubator at 30 °C ± 2 °C (ZEU-INMUNOTEC), a microplate absorbance reader (405 nm ± 10 nm wavelength

filter, Multiskan RC, Thermo-Labsystems), roller mixer, centrifuge, micropipettes, graduated 50 mL centrifuge tubes and laboratory glassware.

2.2. Sample Preparation

Market samples were thoroughly washed, the whole mollusk tissue recovered from the shell, and then blended. Portions of 5 ± 0.1 g were prepared and used for fresh testing, or stored frozen (below −15 °C) for future analysis. The portions were extracted by adding 25 mL of methanol (100% v/v) and mixing with a vortex for 2 min. The methanolic extract was separated by centrifugation for 10 min. at 2000 × g. To perform the hydrolysis, 640 µL of the methanolic extract and 100 µL of 3 N NaOH were mixed and incubated for 40 ± 1 min. at 76 ± 1 °C. To stop the reaction, 80 µL of HCl were added and sample preparation buffer used to make up a final volume of 20 mL. For non-hydrolyzed samples, 640 µL of methanolic extract were diluted up to 20 mL with sample preparation buffer. Hydrolysis was carried out in most samples unless otherwise specified.

2.3. Assay Procedure

The phosphatase solution was prepared by adding 2 mL of dilution buffer to each vial of lyophilized PP2A. To assure full hydration of the lyophilized enzyme, it was mixed gently for 1 h ± 5 min. at room temperature (22 °C ± 2 °C) on a roller mixer. Then, 50 µL of samples or ready-to-use OA standards (0.5, 0.8, 1.2, 1.8 and 2.8 nM), and 70 µL of the prepared phosphatase solution were added in duplicate to a microwell plate. This mixture was equilibrated in an incubator for 20 ± 2 min. at 30 °C. Finally, 90 µL of the chromogenic substrate were added to each well and incubated for 30 ± 2 min. at 30 °C. The absorbance was read at 405 nm.

2.4. Calculations

The results were calculated from a standard curve by plotting the absorbance values in a linear *y axis* and the concentration of OA in a logarithmic *x axis*, and using a logarithmic fitting. As an acceptability criterion for the assay, the Pearson correlation coefficient r^2 had to be greater than or equal to 0.96. The OA concentration contained in the sample was then calculated using the following equation:

$$x = \mathrm{EXP}\ (y - \mathrm{b})/\mathrm{a}$$

where x is the OA concentration in the sample (C_s) and y the absorbance of the sample.

The OA-toxin concentration in shellfish tissue was calculated as follows:

$$C_t\ (\mu g/kg) = (C_s\ (nM) \times FD \times MW\ (g/mol) \times V_e\ (L))/M_t\ (g)$$

where C_t is the toxin concentration in tissue, expressed as equivalents of OA, *FD* is the methanolic extract dilution factor (31.25), *MW* is the OA molecular weight = 805, V_e is the methanolic extract volume (0.025 L), M_t is the tissue weight (5 g).

Samples with an OA concentration falling outside the working range (<0.5 nM or >2.8 nM) will be reported as <63 µg/kg (or <0.5 nM) or >352 µg/kg (or >2.8 nM), respectively.

2.5. Ruggedness Testing

The ruggedness testing was performed by introducing changes in the procedure and determining the effects on the sample quantification [14]. The variations used were chosen according to the values expected under normal laboratory conditions.

2.6. Spiking Procedure

Samples were spiked with OA Certified Reference Calibration Solution (NRC CRM-OA-c). The reference solution was prediluted to 2 µM in sample buffer and added accordingly. No Certified Reference Materials were available for DTX-1 and DTX-2 at the time of the performance testing. These toxins were first dissolved in methanol and diluted to 2 µM in sample buffer before adding to the samples.

A Certified Reference Material (NRC CRM-DSP-MUS-b) was also tested. However, the certified concentration of this material is far above the working range of the assay and the sample had to be diluted with blank mussel or king scallop. To do this, an amount of reference material was added as precisely as possible to 50 mL tubes, and weighed. The blank material was added on top and the mixture weighed again. Then, the amount of the mussel reference material per sample was calculated. This value was used as the theoretical spiked amount. The samples were analyzed with and without hydrolysis, as the reference material was only certified for OA and DTX-1, but ester derivates of the OA-toxins could also be present as indicated in the CRM certificate. The total recovery was calculated according to the AOAC Official methods of analysis [15].

2.7. Method Comparison

A method comparison was also carried out with OkaTest, the mouse bioassay (MBA) and LC-MS/MS, using EU harmonized protocols for the last two methods [16,17].

Shellfish samples were previously tested by a third party laboratory using mouse bioassay (MBA) and LC-MS/MS, and kindly donated to do the method comparison.

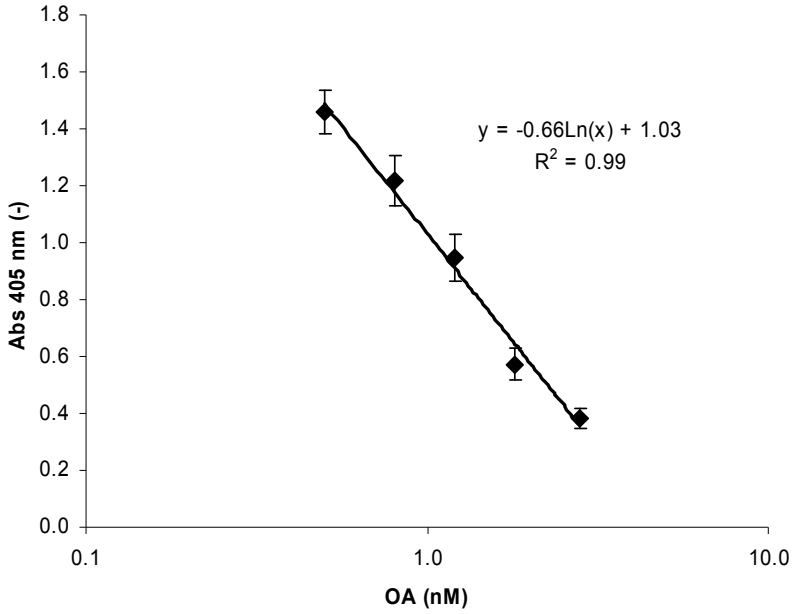
As MBA is a qualitative method, results obtained by OkaTest and LC-MS/MS were interpreted qualitatively for comparison purposes. Therefore, samples with a concentration ≥160 µg/kg were regarded as positive, while samples with a concentration <160 µg/kg were reported negative.

3. Results and Discussion

3.1. Calibration of the Assay

The assay is calibrated by five OA standards prepared by dilution from the NRC CRM-OA-c with a concentration between 0.5 and 2.8 nM OA. Following the kits sample preparation (see material and methods), this will result in a working range between 63 and 352 µg/kg. Figure 1 shows a typical calibration curve from 5 different assays using different phosphatase batches. All calibration curves were evaluated according to the Pearson correlation coefficient obtained after a logarithmic fitting procedure ($r^2 > 0.96$).

Figure 1. Typical calibration curve of OkaTest produced as the mean of 5 phosphatase batches. The Pearson correlation coefficient (r^2) of the logarithmic fit was >0.96 for each batch. The figure shows the equation and r^2 of the mean. The error bars were calculated as ±1 SD.



The bias introduced by the logarithmic fitting procedure on the calibration curve of the kit was estimated by recalculating the concentration of the OA dilutions using its own standard curve. The relative absolute difference was then calculated as the absolute difference between the theoretical and calculated OA concentration divided by the theoretical OA concentration and multiplied by 100 (Table 1). The best accuracy was found at levels around the regulatory limit (0.8% at 1.2 nM OA standards equals 151 µg OA equivalents/kg mollusk), while below that level (0.5 nM of OA), a 9.0% overestimation was calculated. Only minor deviations were calculated over the legal limit.

Table 1. Bias introduced due to the fitting procedure. Relative absolute difference was calculated from mean of 5 standard curves by relating the absolute difference to the theoretical OA concentration.

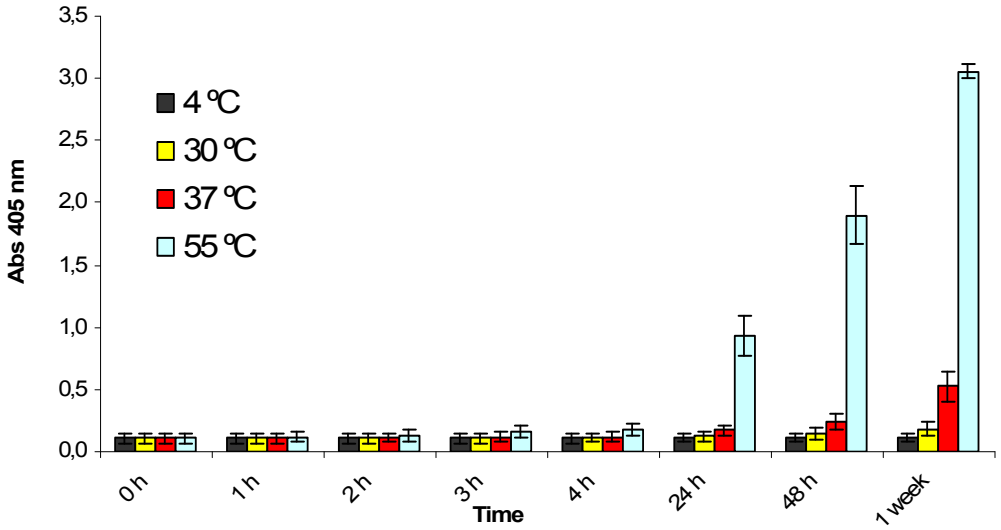
OA theoretical (nM)	OA calculated (nM)	Relative Absolute Difference
0.50	0.55	9.0%
0.80	0.83	3.8%
1.20	1.21	0.8%
1.80	1.78	1.1%
2.80	2.73	2.5%

3.2. Stability and Homogeneity of the Components

The stability and homogeneity of the critical components of the kit were studied by combining a real time and accelerated study design. Water soluble buffers such as the phosphatase dilution solution and the sample buffer were considered less critical, as sufficient internal know-how was available for these components and no stability problems were expected. Other components, such as the

ready-to-use chromogenic substrate, the PP2A or the OA standards, were specially developed for the phosphatase inhibition assay and were more extensively tested. Reagents were normally analyzed within the assay system or by performing specific tests depending on their particular characteristics. The ready-to-use substrate performed correctly in the OkaTest assay when stored for a year at temperatures between 2 and 15 °C (results not shown), as the background absorbance remained acceptable (below 0.3 absorbance units). However, accelerated studies showed that the substrate is sensitive to higher temperatures (Figure 2). After 24 h at 55 °C, the substrate was strongly hydrolyzed and after 1 week at 37 °C the absorbance of the substrate was above 0.6. Nevertheless, these results indicate that although the hydrolysis rate increases with temperature, it is very stable at temperatures below 15 °C and no problems should be expected under normal conditions of usage and storage.

Figure 2. Study of the temperature stability for the ready-to-use chromogenic substrate (p-Nitrophenyl phosphate). Absorbance at 405 nm was measured at different times and temperatures. Assays were performed in triplicate. The error bars were calculated as ±1 SD.



The OA standards and the PP2A were estimated the most critical components, as their quantity and quality establish the working range and, to a great extent, the ruggedness of the assay. The enzyme quantity determines the amount of analyte that is needed for inhibition, while the enzyme quality assures the amount of product formed per time unit [18]. Likewise, the lack of stability or impurities of the OA standards directly affect the quantification, either overestimating, in the case of degradation of the OA, or underestimating, when impurities that can inhibit the PP2A are present. Therefore, greater emphasis was put on these components and the 'between batch homogeneity' was evaluated besides the stability of the components. The between batch homogeneity was studied by taking 1 set of standards or 1 vial of phosphatase from 5 different batches. These batches were chosen along the estimated shelf life of the compounds and tested in one single assay together with internal control samples. All batches performed according to the assays' specifications ($r^2 > 0.96$) and the relative standard deviation was far below 15%, the expected value for samples assayed under repeatability conditions [19]. These results proved the stability of the enzyme for over 12 months at 4 °C and the homogeneity of between all batches tested (Table 2).

Table 2. Phosphatase stability and homogeneity. Five different phosphatase batches were tested at different stages of shelf life. Mean, standard deviation (SD) and relative standard deviation (RSDr) were calculated. Three internal control samples were used to verify correct quantification.

PP2A batch (shelf life)	Sample 1 (µg/kg)	Sample 2 (µg/kg)	Sample 3 (µg/kg)
1 (2 months)	95	160	310
2 (4 months)	100	169	304
3 (8 months)	88	162	323
4 (10 months)	94	156	300
5 (12 months)	90	144	341
mean	93	158	316
SD	5	9	17
RSDR	4.8%	6.0%	5.2%

For the OA standards, the same strategy was used. Five batches, covering 90% of the shelf life of the component (6 months), were tested in one assay to be able to single out the variation due to the standards' stability and homogeneity (Table 3). A sample shown to be blank (0 nM) was included to be able to calculate the effect of variables other than OA. The RSDr calculated from the absorbance values were all <3%, proving the stability and homogeneity of the standards over 6 months.

Table 3. OA standards stability and homogeneity. Five different batches of OA standards were tested at different stages of shelf life. The absorbances (405 nm) obtained for each of the standards are shown. Mean, standard deviation (SD) and relative standard deviation (RSDr) of these absorbances were calculated.

	Absorbance 405 nm							
Standards OA (nM)	batch 1 5 months	batch 2 4 months	batch 3 3 months	batch 4 2 months	batch 5 1 week	mean	SD	RSDr
0.0	2.042	2.100	2.064	2.073	2.120	2.079	0.031	1.5%
0.5	1.622	1.614	1.649	1.625	1.678	1.637	0.026	1.6%
0.8	1.462	1.390	1.386	1.375	1.372	1.397	0.037	2.7%
1.2	1.124	1.116	1.101	1.092	1.134	1.113	0.017	1.5%
1.8	0.772	0.792	0.769	0.822	0.809	0.793	0.023	2.9%
2.8	0.619	0.646	0.606	0.637	0.613	0.624	0.017	2.7%

3.3. Ruggedness

Enzymatic assays, such as OkaTest, can be sensitive to environmental factors, such as temperature, incubation time or reagent volume. To determine the impact of these factors, samples with concentrations around the regulatory limit were quantified at normal and suboptimal conditions (Table 4). The effect of temperature was tested by performing the OkaTest assay at three different temperatures 28, 30 and 32 °C, obtaining a RSD of 1.0%. These results showed that temperature variations of 2 °C did not affect the performance as RSDr values were lower than 10% usually obtained in the assay (Table 5).

Duration and pipetting volumes were evaluated alike and none of the variables affected the results of the test, with the exception of large pipetting errors. Pipetting errors of 5 µL in samples or phosphatase addition (errors of 10% and 7.1%, respectively) gave RSDr values of 14% and 17%, respectively. Precision in substrate addition was less critical. Pipetting samples and phosphatase are, however, the main sources of variability affecting PPIA and therefore care should be taken when adding these components.

Table 4. Ruggedness testing. The effects of variations of the normal assay conditions on sample quantification are shown.

Variable	Normal value	Variation	Mean value (µg/kg)	RSDr
Temperature	30 °C	±2 °C	175	1.0%
Pre-incubation	20 min	18, 20, 22, 24 min	158	3.6%
Incubation	30 min	27, 30, 33, 36 min	147	2.9%
Syst. pipetting error	50, 70, 90 µL	±2 µL	155	4.3%
Random pipetting error				
Sample	50 µL	±5 µL	151	14%
PP2A	70 µL	±5 µL	153	17%
Substrate	90 µL	±5 µL	158	6.1%
Phosphatase solubility time	60 ± 5 min	±30 min	158	5.0%

Table 5. Intermediate precision of ten different mussel and scallops samples. Mean, standard deviation (SD), relative standard deviation (RSDr) were calculated. < 63: below the working range of the assay (63–352 µg/kg).

Sample	Origin	Day 1 (µg/kg)	Day 2 (µg/kg)	Day 3 (µg/kg)	Mean	SD	RSDr
1	Mussel	211	227	187	208	20	9.5%
2	Mussel	122	132	113	122	10	7.8%
3	Scallop	<63	<63	<63	-	-	-
4	Mussel	82	94	90	88	6	7.0%
5	Mussel	196	196	215	202	11	5.2%
6	Scallop	<63	<63	<63	-	-	-
7	Mussel	<63	<63	<63	-	-	-
8	Scallop	125	108	117	117	8	7.0%
9	Mussel	250	253	281	261	17	6.5%
10	Mussel	277	279	289	282	7	2.4%

3.4. Applicability

There are numerous descriptions of the application of protein phosphatase inhibition assays for determination of OA and its derivatives [7–13]. However, the inhibition pattern of OA, DTX1 and DTX2 is different and is supposed to correspond to their toxicity. One way to evaluate the inhibition capacity of toxins on an enzyme is by determining the IC_{50}, the concentration of toxin able to inhibit 50% of the maximum enzyme activity. This concentration depends, among others, on the amount of enzyme and the substrate concentration present in the assay [20] and therefore the IC_{50} values published for these toxins are difficult to compare [7,8,12,18,21,22]. The IC_{50} values found in our study were 1.2 nM for both OA and DTX-2, and 1.6 nM for DTX-1 (Figure 3) and are in accordance

with the ones obtained recently by Huhn *et al*., 2009 [21]. However, these do not exactly correspond to the toxicity factors (TEF) that are used in analytical methods such as LC-MS/MS; as OA and DTX-1 have a TEF of 1, while DTX-2 has a TEF of 0.6, indicating equal toxicity for DTX-1 and OA and less toxicity for DTX-2 [2]. According to these values, our results would lead to an overestimation of the amount of DTX-2 and an underestimation of the amount of DTX-1 when compared with methods such as LC-MS/MS. However, the recovery data obtained for both DTX-1 and DTX-2 were similar to the ones obtained for OA (Table 6) suggesting that difference has a low impact in the determination of the level of toxins in shellfish samples.

Figure 3. Phosphatase inhibition curve obtained with okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2). Each point is the mean obtained from three different phosphatase batches. The standard deviation is not shown to maintain the figure legible. The IC_{50} values were 1.2 nM for both OA and DTX-2, and 1.6 nM for DTX-1.

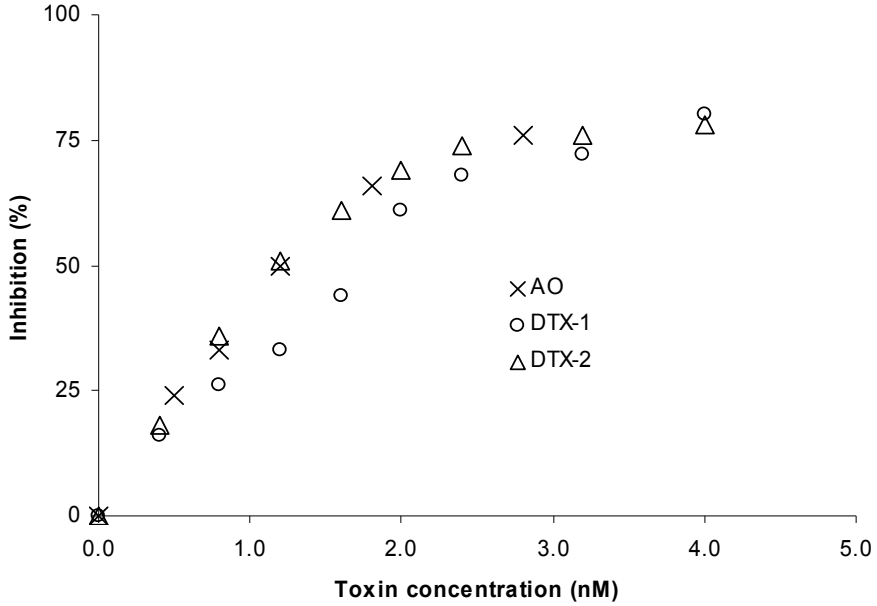


Table 6. Recovery of the different toxins was calculated testing 5 samples at 0.5, 1 and 1.5 times the regulatory limit on 3 different days. OA Certified Reference Material (NRC CRM-OA-c) was spiked on mussel and king scallop. DTX-1 and DTX-2 were spiked on king scallop. ND: not determined.

Toxin	Matrix	Recovery (RSDr)		
		80 µg/Kg	160 µg/Kg	240 µg/Kg
OA	Mussel	101% (15%)	90% (8.9%)	78% (5.4%)
	King scallop	114% (9.9%)	98% (8.4%)	106% (8.7%)
DTX-1	King scallop	102% (15%)	79% (12%)	88% (17%)
DTX-2	King scallop	93% (2.3%)	ND	ND

3.5. Limit of Detection, Limit of Quantification, Repeatability and Reproducibility

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using a blank +3 SD or blank +10 SD approach [14]. For blank mussel material, the LOD and LOQ were 44 and

56 µg/kg, respectively. These values are both below the working range of the test and sufficiently below the current European legal limit of 160 µg/kg.

To estimate the precision, the assay was tested both under repeatability and intermediate precision conditions. The repeatability characteristics were estimated by analyzing 8 fractions of two naturally contaminated mussel samples and RSDr of 1.4% with a mean of 276 µg/kg, and 3.9% with a mean of 124 µg/kg were obtained (results not shown). The intermediate precision of the test was estimated by analyzing 7 samples with OA-toxin levels covering the working range of the assay on three different days by the same analyst. For all samples, the RSDr was well below the 15% RSDr limit as calculated by Horwitz [19]. Three samples tested as negative by LC-MS/MS were included to evaluate the consistency of the negative results (Table 5).

3.6. Accuracy

The accuracy of the method was estimated by calculating recoveries for OA, DTX-1 and DTX-2 and by testing a Certified Reference Material (NRC-CNRC). Five portions containing 5 grams of mussel or king scallop were spiked with one of the three toxins at 0.5, 1 and 1.5 times the regulatory limit (80, 160 and 240 µg/kg), except for DTX-2 that was only added up to a concentration of 80 µg/kg. The five portions were analysed on three different days to determine the intermediate precision characteristics of the test. OA recoveries between 78 and 101% in mussel and 98 and 114% in king scallop were obtained. RSDr values for this toxin were below or equal to 15%. Similar recoveries were obtained for the other two toxins (Table 6). These recoveries are in agreement with the 75 to 120% range that is expected for this concentration range [19]. The RSDr results in this study were higher than the ones obtained in the precision experiments (Table 4), specially for DTX-1. This might be a consequence of the spiking. As mentioned before, the higher IC_{50} for DTX-1 compared to OA and DTX-2 had a low impact on the recovery.

Finally, four aliquotes of blank samples were spiked with the Certified Reference Material. The methanolic extract obtained was analysed with and without hydrolysis, and the recovery was estimated using the DTX-1 and OA content reported for the certified material. The recovery for the non-hydrolysed samples ranged from 71% to 98%, with a mean of 87% for mussle and 91% for king scallop (Table 7). These are acceptable recoveries and in accordance with the results showed in Table 6. However, the mean recovery of the hydrolysed samples was a 146% and 163% for mussle and king scallop, respectively. These percentages were far above the expected content of OA-toxins indicated in the reference material [23]. This could be due to the fact that the material is only certified for OA and DTX-1. Other esters of OA and DTX are reported in the certificate of anlaysis for this material.

Table 7. Recovery experiment with Certified Reference Material (NRC CRM-DSP-MUS-b). Samples were analysed with and without hydrolysis.

Matrix	Spiked level (µg/kg) (*n*)	Without hydrolysis		With hydrolysis	
		Recovery	RSDr	Recovery	RSDr
mussel	219 (4)	87%	14%	146%	12%
king scallop	180 (4)	91%	5.0%	163%	2.8%

3.7. Method Comparison

A method comparison among MBA, LC-MS/MS and OkaTest was performed with a total of 37 samples. Results were compared qualitatively for all three methods and quantitatively between OkaTest and LC-MS/MS. The 160 μg/kg regulatory limit was used to decide whether the samples were positive or negative (Table 8).

Table 8. Methods comparison. Results from OkaTest, MBA and LC-MS/MS. 31 of the 37 samples were tested by MBA. Positive results (+): ≥160 μk/kg. Negative results (−): <160 μg/kg. LOQ. Limit of quantification. NA: not available.

ID	M	MBA	LC-MS/MS	OKATEST	LC-MS/MS	OKATEST
1	**Cockle**	-	-	-	<LOQ	<LOQ
2	**Cockle**	+	+	+	193	252
3	**Donax**	-	-	-	82	97
4	**Mussel**	+	+	+	502	232
5	**Mussel**	+	-	+	<LOQ	268
6	**Mussel**	+	+	+	604	>352
7	**Mussel**	+	+	+	894	>352
8	**Mussel**	+	+	+	414	306
9	**Mussel**	+	+	+	444	>352
10	**Mussel**	NA	-	-	<LOQ	<LOQ
11	**Mussel**	NA	+	+	357	>352
12	**Mussel**	NA	-	-	<LOQ	<LOQ
13	**Mussel**	NA	-	-	<LOQ	<LOQ
14	**Mussel**	-	-	-	<LOQ	122
15	**Mussel**	+	-	+	158	196
16	**Mussel**	+	+	+	177	250
17	**Mussel**	+	+	+	288	265
18	**Mussel**	+	+	+	202	196
19	**Mussel**	+	+	+	390	277
20	**Mussel**	+	+	+	658	305
21	**Mussel**	+	+	+	392	310
22	**Mussel**	+	+	+	329	315
23	**Mussel**	+	+	+	232	270
24	**Mussel**	+	+	+	235	277
25	**Mussel**	+	-	-	152	135
26	**Mussel**	+	-	+	98	164
27	**Mussel**	+	+	+	168	211
28	**Mussel**	+	+	+	209	251
29	**Mussel**	+	-	+	113	191
30	**Mussel**	NA	+	-	292	<LOQ
31	**Mussel**	NA	+	+	316	304
32	**Mussel**	-	-	-	<LOQ	<LOQ
33	**Mussel**	+	+	-	177	124
34	**Mussel**	+	+	+	247	216
35	**Mussel**	+	+	-	185	144
36	**Scallop**	+	+	+	184	264
37	**Scallop**	-	-	-	<LOQ	<LOQ

In general, the qualitative interpretation of the results indicates that the three methods obtained equivalent results, especially taking into account that these are conceptually different methods. The OkaTest disagreed with both MBA and LC-MS/MS on two occasions (samples 33 and 35). OkaTest detected levels of OA-toxins in those two samples, but below the EU regulatory limit (124 and 144 µg/kg), while the samples were positive according to the other two methods). A third sample (25) was also identified as negative by OkaTest and positive by MBA. LC-MS/MS also gave a negative result for sample 25. The concentration of this sample determined by both methods was just below the EU regulatory limit.

The LC-MS/MS differed on four occasions: all four negative according to LC-MS/MS, but positive by the other two methods. Three of the samples (15, 26 and 29) contained OA-toxins below the EU refulatory limit, but sample 5 was quantified under the method's LOQ. Finally, one sample (30) was positive by LC-MS/MS, but under the LOQ by OkaTest. Sample 30 was not tested by MBA due to lack of material.

Quantitative results obtained by LC-MS/MS and Okatest showed some differencies. About two thirds of the samples gave similar results (±25%) with both methods, but the rest of the samples did not show a clear tendency. There is no evident explanation for this and further investigation would be required.

4. Conclusions

A colorimetric phosphatase inhibition assay for determination of OA-toxins, OkaTest, was single laboratory validated according to international methods validation guidelines. The limit of quantification of the method is well below the EU regulatory limit and the method permitted the easy quantification of up to 43 samples within one hour, excluding sample preparation. The method is robust, with very good precision characteristics, adequate specificity and accuracy.

This colorimetric phosphatase inhibition assay could be used as a complementary assay to the reference method for determination of lipophilic toxins, once a collaborative study has been completed and it has been successfully tested under recognized proficiency tests. This assay could be applied for monitoring purposes when OA-toxins are identified to be responsible for a bloom.

Acknowledgements

We thank María Rodríguez Velasco, from the EURLMB (European Reference Laboratory for Marine Biotoxins), for revising the data, providing the mollusk material and coordinating the method comparison. We also want to thank Adriano Rodriguez from the EURLMB for performing the LC-MS/MS determination and Anfaco-Cecopesca for the MBA and mollusk material.

Note: Collaborative efforts between the EURLMB and ZEU-INMUNOTEC do not amount to an endorsement of the firm's products.

References

1. Gerssen, A.; Pol-Hofstad, I.E.; Poelman, M.; Mulder, P.P.J.; van Den Top, H.J.; de Boer, J. Marine Toxins: Chemistry, toxicity, occurrence and detection with special reference to the Dutch situation. *Toxins* **2010**, *2*, 878–904.

2. EFSA. Marine biotoxins in shellfish-okadaic acid and analogues—Scientific Opinion of the Panel on Contaminants in the Food Chain. *EFSA J*. **2008**, *589*, 1–62.
3. European Commission. EC Commission Regulation (EC) No. 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No. 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No. 854/2004 of the European Parliament and of the Council and Regulation (EC) No. 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No. 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No. 854/2004. *Off. J. Eur. Comm.* **2005**, *338*, 27–59.
4. European Commission. EC Commission Regulation (EC) No. 15/2011 of 10 January 2011 amending Regulation (EC) No. 274/2005 as regards recognized testing methods for detecting marine biotoxins in live bivalve molluscs. *Off. J. Eur. Comm.* **2011**, *6*, 3–6.
5. Takai, A.; Bialojan, C.; Troschka, M.; Rüegg, J.C. Smooth muscle myosin phosphatase inhibition and force enhancement by black sponge toxin. *FEBS Lett*. **1987**, 21781–21784.
6. Bialojan, C.; Takai, A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. *Biochem. J*. **1988**, *256*, 283–290.
7. Tubaro, A.; Florio, C.; Luxich, E.; Sosa, S.; Della Loggia, R.; Yasumoto, T. A protein phasphatase 2A inbhition assay for a fast and sensitive assessment of okadaic acid contamination in mussels. *Toxicon* **1996**, *34*, 743–752.
8. Honkanen, R.E.; Stapleton, J.D.; Bryan, D.E.; Abercrombie, J. Development of a protein phosphatase-based assay for the detection of phosphatase inhibitors in crude whole cell and animal extracts. *Toxicon* **1996**, *34*, 1385–1392.
9. Mountfort, D.O.; Kennedy, G.; Garthwaite, I.; Quilliam, M.; Truman, P.; Hannah, D.J. Evaluation of the fluorimetric protein phosphatase inhibition assay in the determination of okadaic acid in mussels. *Toxicon* **1999**, *37*, 909–922.
10. Ramstad, H.; Shen, J.L.; Larsen, S.; Aune, T. The validity of two HPLC methods and a colorimetric PP2A assay related to the mouse bioassay in quantification of diarrheic toxins in blue mussels (*mytilus edulis*). *Toxicon* **2001**, *39*, 1387–1391.
11. Simon, J.F.; Vemoux, J.P. Highly sensitive assay of okadaic acid using protein phosphatase and paranitrophenyl phosphate. *Nat. Toxins* **1994**, *2*, 293–301.
12. Vieytes, M.R.; Fontal, O.I.; Leira, F.; Baptista de Sousa, J.M.V.; Botana, L.M. A fluorescent microplate assay for diarrheic shellfish toxins. *Anal. Biochem*. **1997**, *248*, 258–264.
13. Gonzalez, J.C.; Leira, F.; fontal, O.I.; Vieytes, M.R.; Arévalo, F.F.; Vieites, J.M.; Bermúdez-Puente, M.; Muñiz, S.; Salgado, C.; Yasumoto, T.; *et al*. Inter-laboratory validation of the fluorescent protein phosphatase inhibitions assay to determine diarrheic shellfish toxins: Intercomparison with liquid chromatography and mouse bioassay. *Anal. Chim. Acta* **2002**, *466*, 233–246.
14. EURACHEM. *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics*; EURACHEM: Prague, Czech Republic, 1998.
15. AOAC. *Appendix D: Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis*; AOAC International: Gaithersburg, MD, USA, 2002.

16. EURLMB. EU-Harmonised Standard Operating Procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS. In *EU Harmonised SOP LIPO LCMSM Version 1*; EURLMB: Vigo, Spain, 2006;
17. EURLMB. EU Harmonised Standard Operating Procedure for detection of lipophilic toxins by mouse bioassay. In *EU Harmonised SOP MBA Lipophilic Version 4*; EURLMB: Vigo, Spain, 2007;
18. Takai, A.; Murata, M.; Torigoe, K.; Isobe, M.; Mieskes, G.; Yasumoto, T. Inhibitory effect of okadaic acid derivatives on protein phosphatases. *Biochem. J*. **1992**, *284*, 539–544.
19. Horwitz, W. *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals*; AOAC International: Gaithersburg, MD, USA, 2002; pp. 12–19.
20. Engel, P.C. *Enzymology Labfax*; Academic Press: San Diego, CA, USA, 1996.
21. Huhn, J.; Jeffrey, P.D.; Larsen, K.; Rundberget, T.; Rise, F.; Cox, N.R.; Arcus, V.; Shi, Y.; Miles, C.O. A structural basis for the reduced toxicity of dinophysistoxin-2. *Chem. Res. Toxicol*. **2009**, *22*, 1782–1786.
22. Aune, T.; Larsen, S.; Aasen, J.A.B.; Rehman, N.; Satake, M.; Hess, P. Relative toxicity of dinphysistoxin-2 (DTX-2) compared with okadaic acid, based on acute intraperitoneal toxicity in mice. *Toxicon* **2007**, *49*, 1–7.
23. Perez, R.A.; Crain, S.M.; Walter, J.A.; Quilliam, M.A.; Melanson; J.E. *NRC CRM-OA-c*, *Certified Calibration Solution for Okadaic Acid*; Technical Report for CRMP: Halifax, Canada, 2008.

FOOD CHEMICAL CONTAMINANTS

Quantitative Determination of the Okadaic Acid Toxins Group by a Colorimetric Phosphatase Inhibition Assay: Interlaboratory Study

HENRY SMIENK and ELENA DOMÍNGUEZ[1]
ZEU-INMUNOTEC, C/Bari 25 Dpdo. 50197, Zaragoza, Spain
MARÍA L. RODRÍGUEZ-VELASCO
EURLMB-AESAN, Estación Marítima S/N, Muelle de Trasatlánticos, 36200 Vigo, Spain
DAVID CLARKE
Marine Institute, Rinville, Oranmore, Co. Galway, Ireland
KATRIN KAPP
BFR—Federal Institute for Risk Assessment, Thielallee 88-92, 14195, Berlin, Germany
PANAGIOTA KATIKOU
NRL Marine Biotoxins, Institute of Food Hygiene, 3A Limnou St, 54627 Thessaloniki, Greece
ANA G. CABADO, ALBERTO OTERO, and JUAN M. VIEITES
ANFACO CECOPESCA, Ctra. Colexio Universitario, 16, Lagoas, 36310, Vigo, Spain
PEDRO RAZQUIN and LUIS MATA
ZEU-INMUNOTEC, C/Bari 25 Dpdo., 50197, Zaragoza, Spain

An interlaboratory collaborative study to validate a colorimetric phosphatase inhibition assay for quantitative determination of the okadaic acid (OA) toxins group in molluscs, OkaTest, was conducted. Eight test materials, including mussels, scallops, clams, and cockles, were analyzed as blind duplicates. Blank samples and materials containing different OA toxin levels ranging from 98 to 275 µg/kg OA equivalents were included. The study was carried out by a total of 16 laboratories from 11 different countries. Values obtained for repeatability relative standard deviations (RSD_r) ranged from 5.4 to 11.2% (mean 7.5%). Reproducibility RSD (RSD_R) values were between 7.6 and 13.2% (mean 9.9%). The Horwitz ratio (HorRat) values ranged between 0.4 and 0.6. A recovery assay was also carried out using a sample spiked with OA. A mean recovery of 98.0% and an RSD of 14.5% were obtained. The results obtained in this validation study indicate that the colorimetric phosphatase inhibition assay, OkaTest, is suitable for quantitative determination of the OA toxins group. OkaTest could be used as a test that is complementary to the reference method for monitoring the OA toxins group.

Okadaic acid (OA) and its analogs dinophysistoxin-1 and -2 (DTX1, DTX2), together with their ester forms, are known as the OA toxins group. These lipophilic and heat stable toxins are produced by dinoflagellates and can be found in various species of shellfish, mainly in filter-feeding bivalve molluscs.

OA toxins causes diarrheic shellfish poisoning, which is characterized by symptoms, such as diarrhea, nausea, vomiting, and abdominal pain. These symptoms may occur in humans shortly after consumption of contaminated bivalve molluscs, such as mussels, clams, scallops, or oysters. Inhibition of serine/threonine phosphoprotein phosphatases (PPs) is assumed to be responsible for these toxic effects. These compounds are also involved in tumor promotion (1). Therefore, these toxins are regulated by European Union law.

Regulation (EC) No. 853/2004 (2) states that live bivalve molluscs placed on the market for human consumption must not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed 160 µg of OA equivalents/kg for OA, dinophysistoxins, and pectenotoxins together.

Commission Regulation (EC) No. 15/2011 (3) indicates that in the case of lipophilic toxins including OA toxins, LC/MS/MS is the reference method for routine testing of official controls or any checks done by food operators. This regulation has recently amended the Commission Regulation (EC) No. 2074/2005 (4), in which biological methods (mouse and rat bioassay) were considered the reference. From now on, they will only be used for a transitional period of time (until the end of 2014) or in special circumstances.

Both regulations (No. 2074/2005 and No. 15/2011) contemplate other methods for routine testing of lipophilic toxins, providing they are intralaboratory-validated and successfully tested under a recognized proficiency test scheme. Those methods should detect, either alone or in combination with others, all of the lipophilic toxin analogs (OA, pectenotoxins, yesotoxins, and azaspiracids group toxins). The protein phosphatase inhibition assay (PPIA) is specifically mentioned in these regulations as an alternative or complementary method, considering that the PPs are known to be OA-toxins natural targets (5, 6). In-house PPIAs using different phosphatase sources and colorimetric or fluorometric substrates have been previously developed (7–12). Later improvements to detect all OA derivatives by hydrolysis of samples were also suggested

Received October 28, 2011. Accepted by AP May 23, 2012.
[1] Corresponding author's e-mail: edominguez@zeulab.com
DOI: 10.5740/jaoacint.11-465

Table 1. Details of matrixes and species origin of test materials used in this study

Code	Matrix/Species	Origin
A	Mussel (*M. galloprovincialis*)	Galicia (NW Spain)
D	Clam (*V. pullastra*)	Food & Agricultural Organization, 37 Mediterranean Sea
E	Mussel (*M. galloprovincialis*)	Galicia (NW Spain)
F	Scallop (*P. maximus*)	FAO 27 NE Atlantic
G	Clam (*V. decussatus*)	Galicia (NW Spain)
K	Clam (*V. romboides*)	Galicia (NW Spain)
L	Cockle (*C. edulis*)	Portugal and Galicia (NW Spain)
N	Mussel (*M. edulis*)	Ireland
BM	Scallop (*P. maximus*)	Scotland

(13), and a collaborative study was also performed with a fluorometric PPIA (14). However, none of those assays was commercially available for routine analysis, nor were they demonstrated to comply with the legislation requirements.

ZEU-INMUNOTEC (Zaragoza, Spain) has developed a commercial kit (OkaTest, formerly Toxiline-DSP) based on a colorimetric PP2A inhibition assay for quantification of the OA toxins group in molluscs (15).

The PPIA described in this study uses a human PP2A purified by ZEU-INMUNOTEC that has showed higher sensitivity than other commercial and genetic engineering produced enzymes (16). PP2A was stabilized by freeze-drying to obtain a standardized assay with shelf life of up to 12 months at 4°C (15). Colorimetric substrate was chosen over a fluorometric one as the latter is less stable and, therefore, less appropriate for ready-to-use kits. Besides, fluorometric assays require specific equipment not often available in routine testing laboratories; therefore, they are difficult to use for monitoring purposes.

The robustness and performance of OkaTest were evaluated by the manufacturer in a single-laboratory validation according to AOAC and Eurachem guidelines (15). All of the results obtained showed that the OkaTest kit is robust and accurate, and, therefore, suitable for an interlaboratory study.

Interlaboratory Study

A colorimetric PPIA, OkaTest, was interlaboratory-validated for quantification of the OA toxins group. The main purpose of this study was to determine repeatability and between-laboratory reproducibility. A recovery assay was also carried out, and accuracy of the method confirmed.

A validation management team (David Clarke, Elena Domínguez, Katrin Kapp, Panagiota Katikou, and María Luisa Rodríguez) was appointed to supervise, advise on the accomplishment of the study, and ensure its independence. A total of 16 laboratories from 11 different countries in Europe and South America participated in the study.

The study plan including details of the test method, experimental design, preparation of test materials, instructions for participants, key personnel, schedule, and data analysis was prepared and agreed to by the validation management team. Participants were fully informed of the study design prior to distribution of testing materials.

Eight different test materials, as blind duplicates, were analyzed by each laboratory on 2 different days. Five materials contained different OA toxin levels, all naturally contaminated except for one that was partially spiked. Three of the test materials were blank samples. An additional blank material (BM) was used in the recovery study. The test materials comprised four different genera of molluscs (*Mytilus spp, Pecten spp., Venerupis spp.,* and *Cerastoderma spp*.) and seven different species. Details of the materials used are shown in Table 1. The materials were prepared by the Spanish Association of Seafood Products Manufacturers (ANFACO-CECOPESCA; Vigo, Spain) as explained below.

All participants sent back an electronic copy of a tailor-made Excel reporting sheet for each day of analysis with raw data and final results for each test material. The reporting sheets were checked upon receipt for obvious errors in sample codes and calculations.

Participants also completed a questionnaire with details of the equipment used and preparation of reagents and samples, as well as feedback on the assay.

Preparation of Test Materials

Materials A and E (mussel) and D and K (clam) were purchased from the retail market fresh and alive. They were thoroughly cleaned outside and inside with fresh water to remove sand and any other foreign materials. Tissues were removed from the shell, transferred to strainers, and drained for 5 min before homogenization (blender and Ultraturrax®; IKA, Staufen, Germany). The homogenate (at least 450 g) was then distributed into plastic containers (5.0 ± 0.1 g), frozen, and stored at –20 ± 2°C until analysis or the day of shipment.

Materials F (scallop) and G (clam) were purchased frozen

Table 2. Total concentration of OA toxins group (µg/kg) determined by OkaTest, and toxins profile by LC/MS/MS

Test material[a]	Matrix/species	Total OA equivalents, µg/kg[b]	OA toxins content[c]
BM	Scallop (*P. maximus*)	<LOD	—
A	Mussel (*M. galloprovincialis*)	<LOD	—
F	Scallop (*P. maximus*)	<LOD	—
G	Clam (*V. decussatus*)	<LOD	—
E	Mussel (*M. galloprovincialis*)	79 ± 5	OA
L	Cockle[d] (*C. edulis*)	168 ± 11	OA, DTX1, and DTX2
D	Clam (*V. pullastra*)	240 ± 9	OA
K	Clam (*V. romboides*)	250 ± 6	OA
N	Mussel[e] (*M. edulis*)	276 ± 6	OA and DTX2

[a] Samples presented in increasing order of concentration.

[b] Determined by OkaTest; LOD = 44 OA equivalents µg/kg.

[c] Determined by LC/MS/MS.

[d] Artificially contaminated with DTX1 and mixed with blank material.

[e] Mixed with blank material.

Table 3. Results from homogeneity study for test materials for the determination of OA (µg OA total equivalents/kg)

Test material	Variance of sums, Vs	Analytical variance, s_{an}^2	Allowable sampling variance, σ_{all} ^2	Sampling variance, S_{sam}^2	Critical value, c	Test for homogeneity result
D	166	90.7	36.8	116	310	S_{sam}^2 < c
E	84.7	8.09	19.8	11.1	29.1	S_{sam}^2 < c
K	139	19.6	32.5	126	257	S_{sam}^2 < c
L	356	46.9	85.7	55.6	152	S_{sam}^2 < c
N	124	24.2	28.4	154	314	S_{sam}^2 < c

from the retail market. They were thawed at room temperature, cleaned, and prepared as described above.

Material L (cockle) was provided cleaned, blended, and frozen by the European Reference Laboratory for Marine Biotoxins (EURLMB, Vigo, Spain). The sample contained OA, DTX2, and traces of DTX1. In order to achieve a suitable toxin profile, the sample was mixed with fresh cockle from the same species (*C. edulis*) without toxin prior to being spiked with DTX1 (Wako Chemicals, Neuss, Germany). The sample was thawed at room temperature, mixed with the cockle blank material (purchased in Porto, Portugal), and spiked. Then, it was distributed into plastic containers (5.0 ± 0.1 g), frozen, and stored at –20 ± 2°C until the day of shipment.

Material N (mussel) was provided cleaned, blended, and frozen by the National Reference Laboratory of Ireland, Galway, Ireland. The sample contained a high level of OA toxins, so it was mixed with mussel (*M. edulis*) without toxin (purchased in a retail market in Ireland) to achieve a suitable toxin concentration. The sample was thawed at room temperature, mixed, and distributed into plastic containers (5.0 ± 0.1 g). The material was then frozen and stored at –20 ± 2°C until the day of shipment.

The BM (scallop) was provided blended and homogenized by Integrin Advanced Bioscience (Oban, Scotland) and stored frozen at approximately –20 ± 2°C until the day of shipment.

Homogeneity and stability of test materials were studied according to the International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories (17). Ten containers of 5 g were randomly selected for each material. The content of each container was homogenized and extracted, and two test portions (from the sample extract) were analyzed to estimate the analytical variance. A total of 20 portions/material were tested under repeatability conditions and in a random order using the OkaTest kit.

To ensure the stability of the materials during shipment to participants and the study duration, aliquots of each material were taken randomly and split into two subsets, each of them containing five samples. One subset was used as control and stored at –18 ± 1°C. The second was stored under experimental conditions of 9.0 ± 1°C for 5 days. Samples of both subsets were randomized before testing and analysis simultaneously using the OkaTest kit under repeatability conditions. The test materials were also analyzed by LC/MS/MS (18, 19) to determine the OA toxin profile.

The test materials were blind coded by EURLMB and distributed by ANFACO-CECOPESCA to the participants. The codes were securely kept by EURLMB until statistical analysis was carried out.

The materials were shipped in isothermal boxes with dry ice and were received within the following 2 days by most participants. Materials sent to South American countries were delivered more than a week after the dispatch date, as they have long customs check up procedures. Samples were, however, reported to have been kept frozen while stored at customs. Two laboratories informed that the box containing the samples did not arrive in good conditions, and six reported that samples were cold, but defrosted.

PPIA

Principle

OkaTest is an enzymatic test based on a colorimetric PPIA for quantitative determination of OA and other toxins of the OA group, including DTX1, DTX2, and their ester forms.

Table 4. Results obtained for the stability assays conducted for materials D, E, K, L, and N

	Storage conditions						
	–18 ± 1°C	9.0 ± 1°C					
	Mean						
Test material	Total OA equivalents, µg/kg		Absolute difference D	Variance *F*-test	*t*-test	Test criterion C	D < C
D	265 ± 10	262 ± 15	3.02	0.54	0.71	34.5	Pass
E	84.0 ± 4	85.1 ± 3	–1.19	0.45	0.62	10.9	Pass
K	255 ± 8	257 ± 7	–1.57	0.87	0.75	33.2	Pass
L	171 ± 7	169 ± 8	1.63	0.79	0.73	22.2	Pass
N	343 ± 24	355 ± 32	–13.0	0.58	0.49	44.6	Pass

Table 5. Calibration curve parameters obtained by each laboratory every day of the study

	R^2		Slope		Absorbance 405 nm, lowest standard 0.5 nM		Absorbance 405 nm, highest standard 2.8 nm	
Lab	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
A	0.99	0.98	–0.12	–0.45	0.734	1.287	0.524	0.505
B	0.99	0.99	–0.50	–0.65	1.157	1.425	0.334	0.339
C	0.98	0.98	–0.64	–0.44	1.530	1.177	0.496	0.468
D	0.98	0.98	–0.67	–0.58	1.537	1.402	0.430	0.459
E	0.97	0.98	–0.51	–0.48	1.222	1.221	0.409	0.436
F	1.00	0.99	–0.72	–0.74	1.684	1.726	0.482	0.491
G	0.98	1.00	–0.79	–0.58	1.781	1.411	0.462	0.423
H	0.99	0.99	–0.78	–0.73	1.644	1.609	0.366	0.414
I	0.99	0.99	–0.76	–0.68	1.661	1.486	0.409	0.357
J	0.97	0.98	–0.41	–0.45	1.164	1.204	0.498	0.458
K	0.99	0.98	–0.77	–0.74	1.712	1.690	0,438	0,485
L[a]	0.93	0.96	–0.63	–1.13	1.488	2.588	0.425	0.709
M	0.99	0.99	–0.78	–0.65	1.697	1,464	0.419	0.390
N	0.99	0.98	–0.54	–0.65	1.273	1,497	0.384	0.444
O	0.97	0.98	–0.49	–0.32	1.188	0,992	0,396	0.470
P	0.97	0.99	–0.27	–0.58	1.015	1.474	0.549	0.520

[a] Standard curve obtained by Laboratory L on Day 1 was rejected as R^2 criterion was not met. Assay could not be repeated due to time issues.

This method is applicable to shellfish species, such as mussels, clams, cockles, and scallops.

The toxicity of the OA toxins group is directly related to its inhibitory activity against a family of structurally related PPs, in particular PP1 and PP2A. OkaTest uses this strong inhibitory activity to determine the OA content in shellfish using the PP2A with a chromogenic substrate for this enzyme. After the substrate's hydrolysis by the enzyme, the product can be measured at 405 nm by a microplate reader. As the ability of the PPs to hydrolyze the substrate depends on the amount of OA and analogs in the samples, the toxin concentration can be calculated by using a standard curve.

Apparatus

(**a**) *Micropipets*.—Adjustable 100, 200, and 1000 µL (Thermo Labsystems, Helsinki, Finland).

(**b**) *Ultra homogenizer*.

(**c**) *Block heater or incubator*.—For 30 ± 2°C (ZEU-INMUNOTEC, Zaragoza, Spain).

(**d**) *Microwell absorbance reader*.—405 ± 10 nm wavelength filter (Thermo Labsystems).

(**e**) *Water bath*.—Set at 76 ± 2°C (Raypa, Barcelona, Spain).

(**f**) *Centrifuge tubes*.—Graduated 50 mL.

(**g**) *Laboratory glassware*.

Reagents

(**a**) *Extraction solvent*.—Methanol, reagent grade, 100% (v/v; Sharlab, Barcelona, Spain).

(**b**) *HCl*.—Reagent grade, 37% (v/v; Sharlab).

(**c**) *NaOH*.—Reagent grade (Sharlab).

(**d**) *Deionized water*.—Type II, ISO 3696 (Ellix 5; Millipore, Germany).

(**e**) *OkaTest kit*.—From ZEU-INMUNOTEC containing:

(*1*) 96-well microtiter plate and plate adhesive film.

(*2*) Lyophilized PP2A purified from human blood cells.

(*3*) Ready-to-use OA Standards of 0.5, 0.8, 1.2, 1.8, and 2.8 nM, prepared from the OA reference solution (NRC CRM-OA-c, Institute for Marine Biosciences, Halifax, Canada).

(*4*) Chromogenic substrate.

(*5*) Phosphatase dilution buffer.

(*6*) Stock buffer solution.

(*7*) OA Spiking solution (2 µM) prepared from the OA reference solution (NRC CRM-OA-c, Institute for Marine Biosciences).

Spiking Procedure

Due to the limited experience on the homogeneity and stability of spiked samples with OA toxins, each participant prepared a spiked sample on the day of the assay. A BM and an OA solution of known concentration (2 µM, to prepare a final concentration of 161 µg/kg) were provided to each participant.

A blank sample was spiked with OA solution for the recovery study as follows:

(**a**) Mix 500 µL OA spiking solution (2 µM) with 5.0 ± 0.1 g homogenous blank sample.

(**b**) Add 25 mL extraction solvent [methanol, 100% (v/v)] to the mixture and shake for 2 min by vortexing. Proceed with the extraction procedure described below under point (**b**).

Sample Extraction

(**a**) Thaw each aliquot with 5.0 ± 0.1 g homogenized mollusc at room temperature (22 ± 2°C). Add 25 mL extraction solvent [methanol, 100% (v/v)]; then mix for 2 min using an ultra homogenizer.

(**b**) Centrifuge at 2000 g for 10 min at 4°C. The supernatant is called "methanolic extract."

(**c**) Pipet 640 µL methanolic extract into a 50 mL graduated centrifuge tube and add 100 µL 2.5 M NaOH.

(**d**) Seal the test tube and heat at 76 ± 2°C for 40 min in a water bath.

(**e**) Do not cool the sample; add 80 µL 2.5 M HCl immediately.

(**f**) Add 19.18 mL buffer solution with a glass pipet up to a total volume of 20 mL.

Assay Procedure

(**a**) Rehydrate the lyophilized phosphatase (PP2A) by adding 2.0 mL phosphatase dilution buffer to the vial and mix gently for 60 ± 5 min at room temperature (22 ± 2°C) on a roller mixer or a shaker (maximum 60 rpm) (both from JP Selecta, Barcelona, Spain).

(**b**) Add 50 µL each sample extract or standard to wells. Samples and standards have to be analyzed in duplicate.

(**c**) Add 70 µL phosphatase solution to each well. Cover the plate with the adhesive film provided in the kit, and mix by gentle tapping on the side.

(**d**) Incubate at 30 ± 2°C for 20 ± 0.5 min.

(**e**) Remove the adhesive film and add 90 µL chromogenic substrate to each well and mix by tapping gently on the side. Incubate at 30 ± 2°C for 30 ± 0.5 min.

(**f**) Read the absorbance of samples and standards at 405 ± 10 nm.

Calculations

The results were calculated from a standard curve by plotting the absorbance values on a linear y axis and the concentration of OA on a logarithmic x axis, and using a logarithmic fitting. As an acceptability criterion for the assay, the Pearson correlation coefficient R^2 had to be equal to or greater than 0.96. The OA concentration contained in the sample was then calculated using the following equation:

$$x = EXP\ (y - b)/a$$

where x is the OA concentration in the sample (Cs), y the absorbance of the sample, a is the slope, and b is the y-intercept.

The OA toxin concentration in shellfish tissue was calculated as follows:

$$Ct,\ \mu g/kg = [Cs\ (nM) \times FD \times MW\ (g/mol) \times Ve\ (L)]/\ Mt\ (g)$$

where Ct is the toxin concentration in tissue expressed as equivalents of OA, FD is the methanolic extract dilution factor, MW of OA = 805, Ve is the methanolic extract volume (0.025 L), and Mt is the tissue weight (5 g).

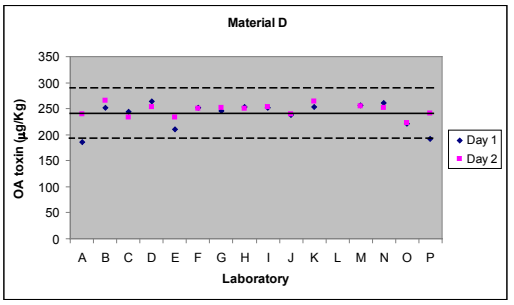
Samples with an OA concentration falling outside the working range (<0.5 nM or >2.8 nM) will be reported as <63 µg/kg (or <0.5 nM) or >352 µg/kg (or >2.8 nM), respectively.

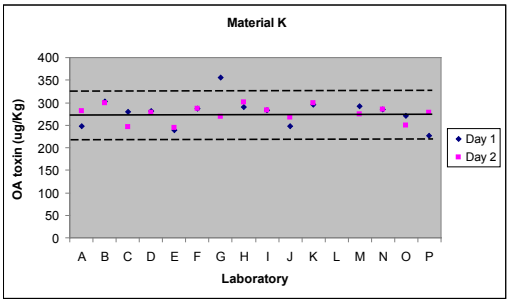
Results were recorded by each participant in a tailor-made Excel spreadsheet with which the results were automatically calculated when the absorbance values were entered. All participants sent back an electronic copy of the reporting sheet for each day of analysis.

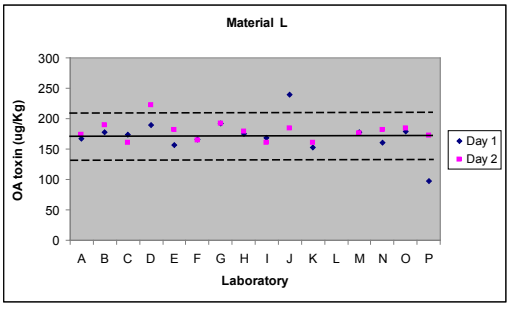
Table 6. Individual results (µg OA total equivalents/kg) reported from laboratories A to P for Materials A, D, E, F, G, K, L, and N on Days 1 and 2. Invalid or incorrect results are those in bold type.

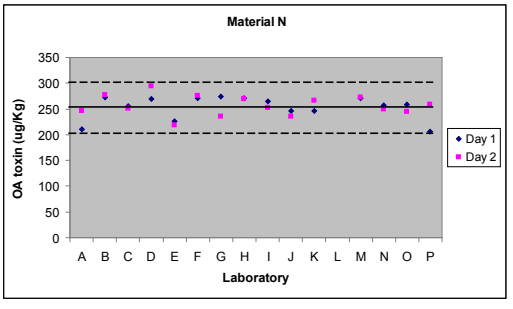
	µg OA total equivalents/kg															
	Material															
	A		D		E		F		G		K		L		N	
	Day															
Lab	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
A	<63	<63	186	239	97	102	<63	<63	<63	<63	248	281	167	174	210	247
B	<63	<63	251	266	100	101	<63	<63	<63	<63	302	299	177	190	273	277
C	<63	<63	244	233	96	87	<63	<63	<63	<63	279	246	174	160	256	251
D	<63	<63	264	253	125	100	<63	<63	<63	<63	282	277	189	223	269	295
E	<63	<63	210	233	101	120	<63	<63	<63	<63	239	244	156	181	226	219
F	<63	<63	252	250	113	116	<63	<63	<63	<63	287	286	166	165	271	275
G	<63	<63	246	252	89	100	<63	<63	<63	<63	356[a]	269[a]	192	192	274	236
H	<63	<63	253	250	90	99	<63	<63	<63	<63	291	301	175	179	271	270
I	<63	<63	252	254	95	87	<63	<63	<63	<63	284	283	169	161	265	253
J	70[a]	98[a]	238	239	163[a]	102[a]	<63	<63	78[a]	67[a]	248	268	239	184	246	235
K	<63	<63	253	264	81	81	<63	<63	<63	<63	295	300	152	160	247	266
L	**—**	**<63**	**—**	**242**	**—**	**145**	**—**	**<63**	**—**	**—**	**—**	**266**	**—**	**202**	**—**	**182**
M	<63	<63	257	255	101	104	<63	<63	<63	<63	292	274	177	176	271	272
N	<63	<63	261	251	98	101	<63	<63	<63	<63	285	285	161	181	257	250
O	<63	<63	221	223	91	94	<63	<63	<63	<63	270	249	179	184	259	244
P	<63	<63	192	241	69[a]	153[a]	<63	<63	<63	<63	226	278	97	173	206	259

[a] Outlier.









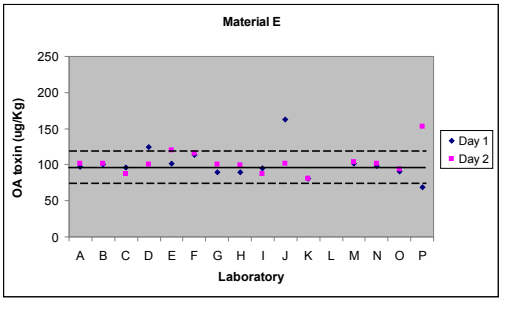


Figure 1. Individual results for each test material obtained per lab and per day of analysis (including outliers). The solid line shows the assigned mean value calculated in this study for each material. The dashed lines indicate the theoretical reproducibility SD determined for each material in this study ($PRSD_R$).

Statistics

Analysis of Valid Data and Outliers

Statistical data analysis was carried out following the approach described in the AOAC/IUPAC guidelines (17, 20). Submitted results were initially reviewed to remove invalid data. Results from assays with calibration curves with a $R^2 < 0.96$ and results outside the working range or showing deviations from the Standard Operating Procedure were considered invalid.

The valid data were first analyzed for possible outliers applying the Cochran and Grubbs tests. Then, precision parameters, HorRat values, and recovery were calculated.

The Cochran test was applied to remove laboratories showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material. A 1-tail test at a probability value of 2.5% was applied (17, 20).

The Grubbs test was used to remove results from laboratories with extreme averages (17, 20). This test was applied to the remaining values from the Cochran test. A single value test (two-tail, $P = 2.5\%$) was first applied, followed by a pair value test (two values at the highest end, two at the lowest end, and one at each end, at an overall $P = 2.5\%$).

Precision

To estimate the precision of the method, the within-laboratory repeatability and between-laboratory reproducibility were determined by calculating s_r (repeatability SD), s_R (reproducibility SD), RSDs (RSD_r and RSD_R), repeatability and reproducibility limits (r and R), and HorRat values. These parameters were calculated following the AOAC guidelines (20).

Recovery

For recovery calculations, the marginal recovery was calculated as follows:

$$\text{Recovery, \%} = 100\,(C_f - C_u)/C_A),$$

where C_f is the amount found for the spiked concentration, C_u is the amount present originally for the unspiked concentration, and C_A is the amount added.

Results and Discussion

Test Material Results

The test materials were first analyzed by OkaTest and LC/MS/MS to determine the content and profile of OA toxins. Results obtained by both methods for samples A, F, and G showed concentration for OA toxins below their LOD (44 and 40 µg/kg, respectively). The BM was tested by LC/MS/MS (19) at EURLMB, and no peaks were detected for this group of toxins (LOD for this method is 15 µg/kg). Therefore, materials A, F, G, and BM were considered blank; therefore, no homogeneity or stability studies were carried out.

Analyses by LC/MS/MS were used to identify the toxin profile and to ensure that all toxins belonging to the OA group were present in the materials. Table 2 shows concentration

Table 7. Details of the test materials, number of results submitted, and results after removing outliers, together with performance values of precision (repeatability and reproducibility) obtained for the colorimetric OkaTest[a]

							Repeatability[c]			Reproducibility[c]			
							µg total equiv.OA/kg						
Test material	Matrix	Runs/lab	No. labs submitting results	No. labs after invalid/incorrect results	No. of labs after outliers[b]	Mean (µg total equivalent OA/kg)[c]	S_r	r	RSD_r, %	S_R	R	RSD_R, %	HorRat
A	Mussel *M. galloprovincials*	2	16	14	—	<63	—	—	—	—	—	—	—
D	Clam *V. pullastra*	2	16	15	15 (0)	242	14.7	41.2	6.1	19.4	54.4	8.0	0.4
E	Mussel *M. galloprovincialis*	2	16	15	13 (2)	98.8 (102)	7.32 (20.8)	20.5 (58.4)	7.4 (20.5)	10.7 (19.6)	30.0 (54.8)	10.7 (19.2)	0.5 (0.8)
F	Scallop *P. maximus*	2	16	15	—	<63	—	—	—	—	—	—	—
G	Clam *V. decussatus*	2	16	14	—	<63	—	—	—	—	—	—	—
K	Clam *V. romboides*	2	16	15	14 (1)	275 (277)	14.9 (21.4)	41.8 60.1)	5.4 (7.7)	21.0 (25.0)	58.7 (70.1)	7.6 (9.0)	0.4 (0.5)
L	Cockle *C. edulis*	2	16	15	15 (0)	175	19.6	55.0	11.2	23.2	64.9	13.2	0.6
N	Mussel *M. edulis*	2	16	15	15 (0)	255	15.6	43.7	6.1	20.7	58.1	8.1	0.4

[a] S_r = Repeatability SD, S_R = reproducibility SD, RSD_r = repeatability RSD, RSD_R = reproducibility RSD, r = repeatability limit, R = reproducibility limit.

[b] Number of laboratories remaining after removal of outliers (number of outliers).

[c] Mean, repeatability, and reproducibility (values obtained including outliers).

in OA equivalents determined by OkaTest and toxins profile of the different materials used. All test materials were found to be stable for the duration of the study and with sufficient homogeneity (Tables 3 and 4).

Interlaboratory Study Results

All participants who received test materials reported results. The sample concentration was calculated by standard curves obtained by each laboratory every day of analysis. Fit parameters of each standard curve are shown Table 5. Although the slopes show differences depending on the laboratory and day, the calculated samples concentration was not affected. The data obtained by each laboratory per test material and day of analysis are shown in Table 6.

All individual values obtained per material, day and laboratory were also plotted. One graph per material is shown in Figure 1. The solid lines represent the assigned mean value obtained for each material in this study (Table 7). The area between the dashed lines demonstrates the range of deviation from the mean value based on the theoretical reproducibility SD ($PRSD_R$).

Two laboratories reported one of the assays with $R^2 < 0.96$; one (Laboratory A) repeated the analysis obtaining R^2 within the required criterion. Laboratory L, however, could not repeat the assay on time, and those results were considered invalid and removed for statistical analysis.

Materials A, F, and G were not statistically analyzed, as they were blank samples. However, Laboratory J reported values within the working range of the test for Materials A and G. These values are considered incorrect according to the AOAC guidelines (20), as they are positive values found for a blank material. All the other laboratories in the study identified the blank materials below the working range of the test.

The valid data from the contaminated test materials (D, E, K, L, and N) were then analyzed for identification of outliers applying Cochran and Grubbs tests (20). Results from Laboratory L could not be included in the statistical analysis, as only one value per material was available.

The Cochran test showed Laboratory G for Material K and Laboratory P for Material E as outliers. This test was applied again after these outliers were removed. Laboratory J for Material E was also excluded in a second round. The Grubbs single and pair values tests were then applied; no further outliers were identified.

The mean values assigned for OA-toxins for the test materials were 98.8, 175.4, 242.8, 255.0, and 275.0 µg total equivalents OA/kg for Materials E, L, D, N, and K, respectively (Table 7).

Values obtained for repeatability SD (S_r) ranged from 7.3 µg/kg for Material E to 19.6 µg/kg for Material L, with repeatability RSDs (RSD_r) from 5.4% for Material K to 11.2% for Material L (Table 7). The reproducibility SD (S_R) calculated for the five test materials ranged from 10.7 to 23.2 µg/kg, with reproducibility RSD (RSD_R) values from 7.6 to 13.2% for Materials K and L, respectively (Table 7).

The HorRat values obtained were 0.4 for Materials D, K, and N, 0.5 for Material E, and 0.6 for Material L (Table 7), indicating a very good performance of the method. These values are just at the lower limit of the range considered as normally expected for a good reproducibility of a method ($0.5 < HorRat \leq 1.5$), according to the AOAC guidelines (20). HorRat values between 0.64 and 2.61 for OA-toxins group (21), 0.3 and 2.0 for paralytic

Table 8. Results from the recovery experiment carried out during Day 2 of the interlaboratory study

	µg OA total eq./kg			
Lab code	BM[a]	Spiked concn.	BM + OA[b]	Recovery, %
A	—	161	172	107.1
B	—	161	162	100.7
C	—	161	155	96.3
D	—	161	115	71.6
E	—	161	124	77.3
F	—	161	138	85.5
G	—	161	162	100.7
H	—	161	131	81.1
I	—	161	152	94.4
J	—	161	197	122.3
K	—	161	152	94.4
L	—	161	196	121.6
M	—	161	153	95.0
N	—	161	174	108.3
O	—	161	155	96.3
P	—	161	185	114.7
Mean recovery, %				98.0
SD				14.2
RSD, %				14.5

[a] BM = Blank material. No OA toxins were detected; therefore, a concentration of zero was considered for calculation purposes.

[b] BM + OA = Concentration of the samples spiked with 161 µg/kg.

shellfish toxins (22) and 1.1 to 2.4 for domoic acid (23) were previously described for other methods.

The statistical analysis was also carried out including outliers (Table 7). Although there were some differences when including outlier values, repeatability and reproducibility remained satisfactory and within the expected values for this type of interlaboratory study.

Although the main objective of the validation study was to determine the repeatability and between-laboratory reproducibility of the OkaTest kit, a recovery assay was also carried out. A scallop blank sample (BM) was spiked with OA by each laboratory, and the recovery of OkaTest calculated. Recovery values from all participants ranged from 71.6 to 122.3%. The mean and RSD were 98.0 and 14.5%, respectively (Table 8). These recoveries met the criteria set in the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals (24).

Comments from Participants

Most participants reported that the SOP for the method provided all the information they needed to perform the assay and that they did not have difficulties understanding any part of it. Some comments were made about the phosphatase preparation. Those led to the conclusion that the use of a nonorbital shaker does not always guarantee full dissolution of this reagent. Manual mixing, longer preparation, and a final visual check of the solution should be included in the SOP. Other minor comments were made, and were answered or resolved by the study director.

Conclusions

The precision and recovery values determined in this study for OkaTest can be considered satisfactory for this type of methodology and the concentration range required. The colorimetric PPIA, OkaTest, could be used as an assay complementary to the reference method for determination of the OA toxins group in molluscs according to the Commission Regulations (EC) No. 2074/2005 and No. 15/2011. Additional methods have to be implemented in a laboratory to analyze all regulated lipophilic marine biotoxins.

Acknowledgments

We thank the following collaborators for their participation, time, effort, and comments towards this study:

Dolores Calvo, ZEU-INMUNOTEC, Spain;

Monica Campàs, IRTA, Spain;

Guntis Cepurnieks, Institute of Food Safety, Animal Health and Environment, Latvia;

David Clarke, Marine Environment and Food Safety Services, Ireland;

Jorge Correa, INTECMAR, Spain;

Lorena A. Delgado, Public Health Institute, Chile;

Alejandra Goya A, SENASA, Argentina;

Katrin Kapp, BfR (Federal Institute for Risk Assesment), Germany;

Panagiota Katikou, Ministry of Rural Development and Food Centre of Veterinary Institutions of Thessaloniki Institute of Food Hygiene, Greece;

Mirslaw Michalski and Kataryna Graziel, National Veterinary Research Institute, Poland;

María José Chapela, ANFACO-CECOPESCA, Spain;

Sonia Piñero, European Reference Laboratory for Marine Toxins, Spain;

Vlad Serafim, The Institute for Diagnosis and Animal Health, Romania;

Ulrich Schwank, Bavaria's policies on health and consumer protection (LGL), Germany;

Andrew Turner and Clothilde Brunet, CEFAS, UK; and

Paulo Vale and Susana Rodrigues, IPIMAR, Portugal.

Note: Collaborative efforts among the European Reference Laboratory for Marine Toxins (EURLMB), NRLs, and ZEU-INMUNOTEC does not amount to an endorsement of the firm's products.

References

(1) Xing, Y., Xu, Y., Chen, Y., Jeffrey, P.D., Chao, Y., Lin, Z., Li, Z., Strack, S., Stock, J.B., & Shi, Y. (2006) *Cell* **127**, 341–353. http://dx.doi.org/10.1016/j.cell.2006.09.025

(2) Regulation (EC) No. 853/2004 (2004) *Off. J. Eur. Union* **L139**, 55–205

(3) Commission Regulation (EC) No. 15/2011 (2011) *Off. J. Eur. Union* **L6**, 3–6

(4) Commission Regulation (EC) No. 2074/2005 (2005) *Off. J. Eur. Union* **L338**, 27–59

(5) Takai, A., Bialojan, C., Troschka, M., & Rüegg, J.C. (1987)

FEBS Lett. **217**, 81–84. http://dx.doi.org/10.1016/0014-5793(87)81247-4

(6) Bialojan, C., & Takai, A. (1988) *Biochem. J.* **256**, 283–290

(7) Simon, J.F., & Vemoux, J.-P. (1994) *Nat. Toxins* **2**, 293–301. http://dx.doi.org/10.1002/nt.2620020508

(8) Honkanen, R.E., Stapleton, J.D., Bryan, D.E., & Abercrombie, J. (1996) *Toxicon* **34**, 1385–1392. http://dx.doi.org/10.1016/S0041-0101(96)00095-5

(9) Tubaro, A., Florio, C., Luxich, E., Sosa, S., Della Loggia, R., & Yasumoto, T. (1996) *Toxicon* **34**, 743–752. http://dx.doi.org/10.1016/0041-0101(96)00027-X

(10) Vieytes, M.R., Fontal, O.I., Leira, F., Baptista de Sousa, J.M.V., & Botana, L.M. (1997) *Anal. Biochem*. **248**, 258–264. http://dx.doi.org/10.1006/abio.1997.2127

(11) Mountfort, D.O., Kennedy, G., Garthwaite, I., Quilliam, M., Truman, P., & Hannah, D.J. (1999) *Toxicon* **37**, 909–922. http://dx.doi.org/10.1016/S0041-0101(98)00222-0

(12) Ramstad, H., Shen, J.L., Larsen, S., & Aune, T. (2001) *Toxicon* **39**, 1387–1391. http://dx.doi.org/10.1016/S0041-0101(01)00097-6

(13) Mountfort, D.O., Suzuki, T., & Truman, P. (2001) *Toxicon* **39**, 383–390. http://dx.doi.org/10.1016/S0041-0101(00)00144-6

(14) Gonzalez, J.C., Leira, F., Fontal, O.I., Vieytes, M.R., Arévalo, F.F., Vieites, J.M., Bermúdez-Puente, M., Muñiz, S., Salgado, C., Yasumoto, T., & Botana, L.M. (2002) *Anal. Chim. Acta* **466**, 233–246. http://dx.doi.org/10.1016/S0003-2670(02)00597-4

(15) Smienk, H.G.F., Calvo, D., Razquin, P., Domínguez, E., & Mata, L. (2012) *Toxins* **5**, 339–352. http://dx.doi.org/10.3390/toxins4050339

(16) Sassolas, A., Catanante, G., Hayat, A., & Marty, J.-L. (2011) *Anal. Chim. Acta* **702**, 262–268. http://dx.doi.org/10.1016/j.aca.2011.07.002

(17) Thompson, M., Ellison, S.L.R., & Wood, R. (2006) *Pure App. Chem.* **78**, 145–196. http://dx.doi.org/10.1351/pac200678010145

(18) Capela, M.J., Reboreda, A., Vieites, J.M., & Cabado, A.G. (2008) *J. Agric. Food Chem*. **56**, 8979–8986. http://dx.doi.org/10.1021/jf801572j

(19) Villar-González, A., Rodriguez-Velasco, M.L., & Gago, A. (2011) *J. AOAC Int*. **94**, 909–922

(20) *AOAC Official Methods of Analysis, Interlaboratory Collaborative Study* (2002) AOAC INTERNATIONAL, Gaithersburg, MD, Appendix D, p. 9

(21) Van den Top, H.J., Gerssen, A., & Van Egmond, H.P. (2011) *Report on Quantitative Determination of Liphophhilic Toxins in Shellfish by LC/MS/MS*, http://www.edepot.wur.nl/180890

(22) Van de Riet, J., Gibbs, R.S., Muggah, P.M., Rourke, W.A., & MacNeil, J.D. (2011) *J. AOAC Int*. **94**, 1154–1176

(23) Kleivdal, H., Kristiansen, S.I., Nilsen, M.V., & Gokoyr, V. (2007) *J. AOAC Int.* **90**, 1011–1027

(24) *AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals* (2002) AOAC INTERNATIONAL, Gaithersburg, MD

Proposal No. **13-114**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Darcie Couture
Affiliation	Resource Access International
Address Line 1	710 River Road
Address Line 2	
City, State, Zip	Brunswick, ME 04011
Phone	207-266-8984
Fax	None
Email	darcie.couture@att.net
Proposal Subject	Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination
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 This submission presents the 'Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination' for consideration as an NSSP Approved Limited Use Method. 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). Results from those studies, and additional data, are included in this proposal submission for the RBA to be considered for approval as an NSSP Approved Limited Use Method for Marine Biotoxin Testing.
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 Limited Use Method for PSP toxicity determination 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	The estimated cost for a full 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitative results would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced. Further, the filter plates used in the RBA differ from ELISA plates in that all reagents are added to each well as needed rather than already being a component of the plate, making it more practical and cost-effective to analyze samples when there is less than a full plate.
Action by 2013 Laboratory Methods and Quality Assurance Review Committee	1. Recommended approval of this method as an alternative to the mouse bioassay for PSP in mussels. 2. Recommended approval of this method for Limited Use for clams and scallops for the purpose of screening and precautionary closure for PSP. 3. Recommended referral of this proposal to an appropriate committee as determined by the Conference Chairman to address this method in oysters. 4. Recommended Executive Office sends a letter to submitter to request a checklist for evaluation of labs using this method with said checklist to be submitted within three (3) months.
Action by 2013 Task Force I	Recommended adoption of Laboratory Method Review and Quality Assurance Committee recommendation on Proposal 13-114.
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-114.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-114.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 13-114 to an appropriate committee as determined by the Conference Chair until additional data for oyster matrix are received.
Action by 2015 Task Force I	Recommended adoption of Laboratory Methods Review Committee recommendation on Proposal 13-114.
Action by 2015 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-114.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-114.
Action by 2017 Laboratory Committee	Recommended referral of Proposal 13-114 to an appropriate committee as determined by the Conference Chair.
Action by 2017 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 13-114.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-114.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 13-114.

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	Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination
Name of the Method Developer	Dr. Fran Van Dolah
Developer Contact Information	Tel: (843) 725-4864 Email: Fran.vandolah@noaa.gov

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.	Y	Paralytic shellfish poisoning (PSP) is the human intoxication that results from the consumption of seafood, primarily bivalve molluscs, contaminated with natural, algal-derived toxins known as paralytic shellfish toxins (PSTs) or the saxitoxins (STXs). This family of neurotoxins binds to voltage-gated sodium channels, thereby attenuating action potentials by preventing the passage of sodium ions across the membrane. Symptoms include tingling, numbness, headaches, weakness, and difficulty breathing. Medical treatment is to provide respiratory support, without which the prognosis can be fatal. To protect human health, seafood harvesting bans are implemented when toxins exceed a safe guidance level (80 μg STX equivalents per 100 g tissue or 800 μg STX equivalents per kg). Successful monitoring and management programs are attributed with minimizing the number of PSP cases and associated deaths. The mouse bioassay (MBA) has long-served as the gold standard method for detecting PSP in regulatory environments. Even though the MBA is an NSSP Approved Method for Marine Biotoxin Testing, there are numerous reasons for considering alternative methods for PSP detection. Disadvantages of the MBA include high variability and the use of live animals. Given these limitations of the MBA, particularly the ethical concerns of using live animals, there have been great strides in method development and validation for alternative approaches. Recently, the post-column oxidation liquid chromatographic method (PCOX) for PSP detection was accepted as an NSSP Approved Limited Use Method, providing an alternative to the MBA. While some laboratories are in the process of transitioning to this

		method, implementation requires costly instrumentation and skilled personnel. Furthermore, the PCOX method identifies and quantifies individual PSP toxins. Toxicity equivalency factors must then be taken into consideration to calculate the expected overall toxicity in μg STX equivalents per 100 g tissue. The proposed receptor binding assay (RBA) addresses the major shortcomings of the PCOX and MBA by quantitatively measuring the overall PSP toxicity and doing so without the need of live animals, respectively. The RBA relies on the interaction of the toxins with the native receptor site (i.e., voltage-gated sodium channels). In this functional assay toxins bind to their receptors according to their affinity, yielding an integrated toxic potency. The RBA is more sensitive than the MBA, allowing monitoring laboratories earlier warning capabilities as toxins become elevated. 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). This proposal provides data from the AOAC studies as well as additional data to seek consideration for the RBA to be an NSSP Approved Limited Use Method.
2. What is the intended purpose of the method?	Y	This method is intended for use as an NSSP Approved Limited Use Method for screening for PSP toxicity in shellfish. Applications include: (1) Growing Area Survey & Classification and (2) Controlled Relaying. 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.
3. Is there an acknowledged need for this method in the NSSP?	Y	Yes, there is an acknowledged need for this method in the NSSP. Even though the MBA and PCOX 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, and (2) the PCOX method, which requires costly equipment and skilled personnel and offers low throughput.
4. What type of method? i.e. chemical, molecular, culture, etc.	Y	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 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	Y	Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination
Method Scope	Y	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 Limited Use Method for the purposes of screening for PSP toxicity.

References	Y	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	Y	This assay is based on the interaction between the toxins and their native receptor, the voltage-gated sodium channels. All PSTs bind to site 1 of the voltage-gated sodium channels according to their potency, resulting in a measure of integrated potency (independent of knowing which toxin congeners are present) similar to mouse intraperitoneal potency. In the RBA, tritiated saxitoxin (^{3}H-STX) competes with unlabeled PSTs in the homogenized and extracted shellfish sample for a finite number of available receptor sites in a rat brain membrane preparation. After a binding equilibrium is reached, unbound ^{3}H-STX is removed by filtration and the remaining ^{3}H-STX is measured with a scintillation counter (as counts per minute or CPM). The amount of ^{3}H-STX present is indirectly related to the amount of unlabeled PSTs in the sample. Scintillation counting can be conducted using traditional scintillation counters or microplate counting. However, the microplate format is preferred as it minimizes sample handling and the amount of radioactivity used.
Any Proprietary Aspects	N	None. All reagents can be prepared or purchased.
Equipment Required	Y	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 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 (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

		Additional supplies when using a traditional scintillation counter (as opposed to a microplate counter): (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) Blender or homogenizer for sample homogenization (u) Pipets (v) Centrifuge tubes (15 ml, conical, plastic) (w) pH meter or pH paper (x) Hot plate or water bath (y) Graduated centrifuge tubes (15 ml) (z) Centrifuge and rotor for 15 ml tubes For rat brain isolation: (aa) Teflon/glass homogenizer (Motorized tapered Teflon pestle and glass tune (15 ml) (bb) Motorized tissue homogenizer (Polytron or small handheld blender) (cc) High-speed centrifuge and fixed angle rotor (20 000 x g rcf) (dd) Centrifuge tubes (12-15 ml, rated for 20 000 x g) (ee) plastic cryovials (2 ml) (ff) Graduated beaker (300 or 500 ml) (hh) Pipets (5-10 ml, disposable) (ii) Forceps (jj) Ice bucket and ice (kk) top loading balance
Reagents Required	Y	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] (b) ^{3}H-STX (0.1 mCi per ml, ≥10 Ci per mmol, ≥90% radiochemical purity; available through 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]) For microplate counter only: (e) Ultima Gold liquid scintillation cocktail (PerkinElmer Inc.; Waltham, MA; Cat. No. 6013321 [or equivalent]) For traditional counter only: (f) Scintiverse BD liquid scintillation cocktail (Fisher Scientific; Waltham, MA; Cat. No. SX-18 [or equivalent]) For sample extraction: (g) Hydrochloric acid (HCl; 1.0 and 0.1 M) (h) Sodium hydroxide (0.1 M) (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, St. Louis, MO; Cat. No. M3183-500G [or equivalent]) (l) Choline chloride (100 mM; Sigma; Cat. No. C7527-500G [or equivalent]) (m) Phenyl methylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO: Cat. No. P7626) (n) Isopropanol (o) Micro bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL)
Sample Collection, Preservation and Storage Requirements	Y	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 ^{3}H-STX must be stored at -80 °C. The rat brain preparation can be produced in bulk, partitioned into aliquots, and stored long-term at -80 °C until use.
Safety Requirements	Y	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. Please see Appendix C for further safety requirements.
Clear and Easy to Follow Step-by-Step Procedure	Y	The protocol is very clear and easy to follow. Please see the detailed protocol below in Appendix A.
Quality Control Steps Specific for this Method	Y	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 IC_{50} (inhibitory concentration at which CPM is 50% max) is out of the acceptable range (2.0 nM ± 30%), the data should be considered unacceptable and the assay should be rerun. (d) A QC sample should always be included 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 should be within 30%. 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 $\leq 30\%$. These quality control criteria are also stated in section H in Appendix IV.
C. Validation Criteria		
1. Accuracy / Trueness	Y	Validation data presented in Section C are from both the SLV (Van Dolah et al. 2009) and the collaborative study (Van Dolah et al. 2012). Nine laboratories from six countries completed the collaborative study. There were a total of 21 shellfish homogenates tested in three different assays on independent days. Different shellfish species from a range of geographical locations were used in the study: 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 surfclam (*Spisula solidissima*) from the U.S. east coast, butter clam (*Saxidomus gigantea*) from the U.S. west coast, almeja clam (*Venus antiqua*) from Chile, and Atlantic sea scallop (*Placopecten magellanicus*) from the U.S. east coast. Samples included those that were naturally contaminated, those that were spiked, and another that served as a negative control. Accuracy was evaluated based on recovery. As also stated under Section C. 4., Recovery of the QC check sample (3 nM in-well solution) was 99.3% (Appendix II). During the SLV recovery was evaluated for STX standard spiked into mussel tissue at concentrations below, at and above the regulatory guidance level. Recovery for the nominal spike at 40 μg STX eq 100 g^{-1} was 115%. At 80 μg STX eq 100 g^{-1}, recovery was found to be 129%. At a nominal spike of 120 μg STX eq 100 g^{-1}, recovery was 121% (Appendix II). During the collaborative study, recovery of PSTs from shellfish was found to be 84.4% (when spiked with 20 μg STX eq 100 g^{-1}), 93.3% (when spiked with 50 μg STX eq 100 g^{-1}), and 88.1% (when spiked with 120 μg STX eq 100 g^{-1}). See Appendix III.
2. Measurement Uncertainty	Y	ND

3. Precision Characteristics (repeatability and reproducibility)	Y	Repeatability (RSD_r) was determined during the SLV on six naturally contaminated shellfish samples on five independent days and was found to be 17.7%. See Appendix II. The reproducibility (RSD_R) during the collaborative study was found to be 33.2% for all laboratories. However, upon removing the results from the one laboratory that had no previous RBA experience, the RSD_R was 28.7%. If data from routine users of the RBA were evaluated, the RSD_R was 23.1%. See Appendix III. Repeatability (RSD_r) during the collaborative study ranged from 11.8-34.4%. For routine users of the RBA, the average RSD_r = 17.1%, consistent with the RSD_r obtained during the SLV. See Appendix III.
4. Recovery	Y	Recovery of the QC check sample (3 nM in-well solution) was 99.3% (Appendix II). During the SLV recovery was evaluated for STX standard spiked into mussel tissue at concentrations below, at and above the regulatory guidance level. Recovery for the nominal spike at 40 μg STX eq 100 g^{-1} was 115%. At 80 μg STX eq 100 g^{-1}, recovery was found to be 129%. At a nominal spike of 120 μg STX eq 100 g^{-1}, recovery was 121% (Appendix II). During the collaborative study, recovery of PSTs from shellfish was found to be 84.4% (when spiked with 20 μg STX eq 100 g^{-1}), 93.3% (when spiked with 50 μg STX eq 100 g^{-1}), and 88.1% (when spiked with 120 μg STX eq 100 g^{-1}). See Appendix III.
5. Specificity	Y	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	Y	The dynamic range of the assay was determined to be 1.2-10.0 nM in-well concentration (Appendix II). Linearity assessment was conducted with three calibration standards (1.5, 3.0, and 6.0 nM STX in -well concentration) on five independent days. The linear regression yielded a slope of 0.98 and an r^2 = 0.97 (Appendix II). During the collaborative study, the assay was set for the critical range of shellfish toxicities below, near and just above the regulatory guidance level (~15-240 μg STX eq 100 g^{-1} or ~150-2400 μg STX eq kg^{-1}). Appendix III.
7. Limit of Detection	Y	The LOD, as determined in the collaborative study, is 4.5 μg STX eq 100 g^{-1} or 45 μg STX eq kg^{-1} See Appendix III.
8. Limit of Quantitation / Sensitivity	Y	The limit of quantitation (LOQ) was empirically determined as the concentration in a 10-fold diluted sample that resulted in a in a B/B0 of 0.7 (more conservative than the 0.8 typically used as the cut off for such assays). The LOQ was determined to be 5.3 μg STX eq 100 g^{-1} during the SLV (Appendix II).

		The LOQ of the RBA is 12.6 μg STX eq 100 g^{-1} or 126 μg STX eq kg^{-1}, as compared to the MBA LOQ of ~40 μg STX eq 100 g^{-1} (or ~400 μg STX eq kg^{-1}). See Appendix III.
9. Ruggedness	Y	Ruggedness was addressed and critical steps were noted that could affect precision and accuracy. 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. 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. For more detail please refer to Appendix II and Appendix III.
10. Matrix Effects	Y	No matrix effects were reported. Minimum dilutions of shellfish extracts were 10-fold and were found to be sufficient to eliminate matrix effects. See Appendix III.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	The RBA was compared to the MBA and the pre-column oxidation (Pre-COX) liquid chromatography with fluorescence detection (LC-FD) approach during the SLV. RBA results compared well to those obtained by the MBA in two separate studies. In one component of the SLV, six naturally contaminated samples (clams, mussels, and sea scallops) were tested by RBA and MBA. Between-assay RSDs ranged from 9 to 25% (mean 17.7%). An r^2 = 0.98 was obtained, with a slope of 1.29. In the second component of the SLV, which included 110 naturally contaminated shellfish, an r^2 = 0.88 and a slope of 1.32 was obtained (Appendix II). Nine naturally contaminated samples (six blue mussels and three scallops) were extracted and analyzed by RBA and Pre-COX. Samples were analyzed using the RBA following the typical extraction (0.1 M HCl), but also following the extraction procedure used for the Pre-COX method (1% acetic acid). A good correlation was found between the two methods for both extraction methods. When the RBA samples were extracted with HCl, the RBA compared to the Pre-COX yielded an r^2 = 0.98 and a slope of 1.39. When samples were extracted the same for both methods (acetic acid), the correlation was slightly improved with an r^2 = 0.99 and a slope of 1.32 (Appendix II). During the collaborative study, ten laboratories from seven countries performed the RBA. Additionally three of the laboratories conducted the MBA, and one laboratory tested the samples using the Pre-COX LC-FD. The MBA and RBA data comparison yielded an r^2 = 0.84 and a slope of 1.63. The LC-FD and RBA data comparison yielded an r^2 = 0.92 and a slope of 1.20. Both RBA and LC-FD methods generally report higher toxicity in shellfish, especially at or near the guidance level, relative to the MBA. This provides a conservative measure and allows for an earlier warning of developing

		toxicity. See Appendix III.
D. Other Information		
1. Cost of the Method	Y	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.
2. Special Technical Skills Required to Perform the Method	Y	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	Y	A microplate scintillation counter is needed and the cost is ~$60-100K for a new counter, depending on the brand and number of simultaneous detectors. However, used instruments can be purchased for ~$13K.
4. Abbreviations and Acronyms Defined	Y	A list of abbreviations and acronyms is provided below in Appendix I.
5. Details of Turn Around Times (time involved to complete the method)	Y	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.
6. Provide Brief Overview of the Quality Systems Used in the Lab	Y	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 (See Appendix V). 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 VI).
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.

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.

Appendix A: RBA Step-by-Step Procedure

A. Sample Extraction
   a. The extraction detailed below represents a small scale MBA extraction 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: ^{3}H-STX
      i. Calculate the concentration of ^{3}H-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 ^{3}H-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 ^{3}H-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. 6×10^{-6} [100 μl 268.8 μM STX + 4.38 ml 0.003 M HCl]
        2. 6×10^{-7} [500 μl 6×10^{-6} M STX + 4.5 ml 0.003 M HCl]
        3. 1.8×10^{-7} [1.5 ml 6×10^{-7} M STX + 3.5 ml 0.003 M HCl]
        4. 6×10^{-8} [500 μl 6×10^{-7} M STX + 4.5 ml 0.003 M HCl]
        5. 1.8×10^{-8} [500 μl 1.8×10^{-7} M STX + 4.5 ml 0.003 M HCl]
        6. 6×10^{-9} [500 μl 6×10^{-8} M STX + 4.5 ml 0.003 M HCl]
        7. 6×10^{-10} [500 μl 6×10^{-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 (1.8×10^{-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 ^{3}H-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. $y = \text{min} + (\text{max-min})/1+10^{(\text{x-log IC50})\text{Hill slope}}$
    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; IC50 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 ligand 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 ^{3}H-STX in CPM in the sample and B_0 represents the max bound ^{3}H-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

A. 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.
B. 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.
C. 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.

D. 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 ^{3}H-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 I. Abbreviations and Acronyms

^{3}H-STX	Tritiated saxitoxin
AOAC	Association of Analytical Communities
ARC	American Radiolabeled Chemicals
B	Bound CPM
B_o	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
QC	Quality control
QS	Quality System
RBA	Receptor binding assay
RSD	Relative standard deviation
SLV	Single laboratory validation
STX	Saxitoxin

FOOD CHEMICAL CONTAMINANTS

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

FRANCES M. VAN DOLAH, TOD A. LEIGHFIELD, and GREGORY J. DOUCETTE
NOAA National Ocean Service, Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412
LAURIE BEAN
Maine Department of Natural Resources, McKown Point, W. Boothbay Harbor, ME 04575
BARBARA NIEDZWIADEK and DOROTHEA F.K. RAWN
Health Canada, Food Research Division, Sir Frederick Banting Research Centre, Ottawa, ON, Canada K1A 0L2

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.

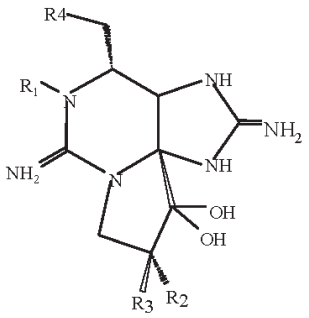
Received March 9, 2009. Accepted by AP May 10, 2009.
Corresponding author's e-mail: fran.vandolah@noaa.gov

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 Duropore 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

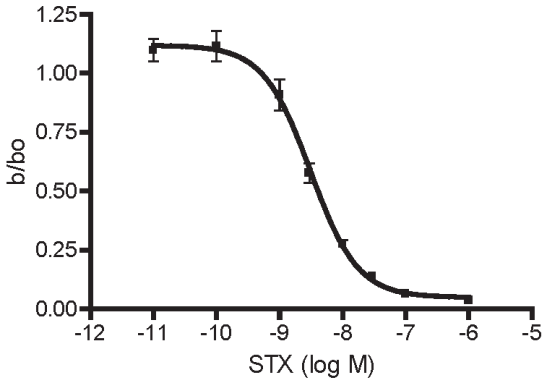


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

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

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:

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

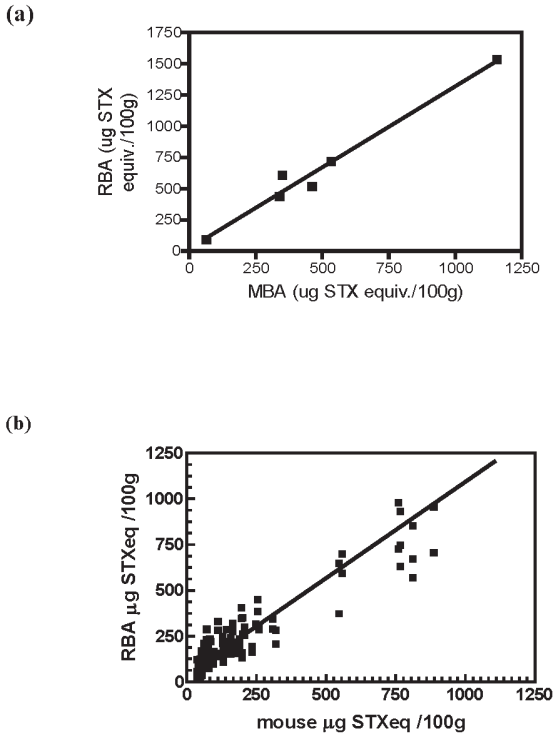


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days (r^2 = 0.98, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with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

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

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

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.

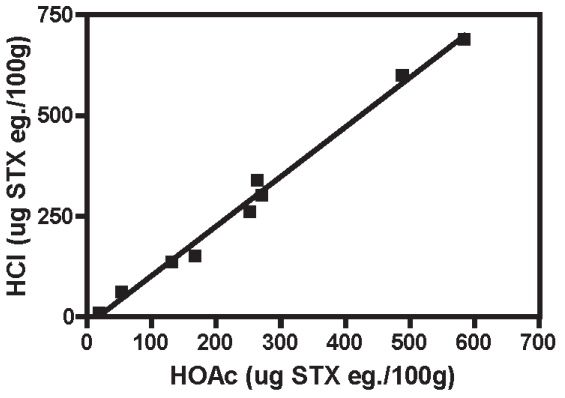


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

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.

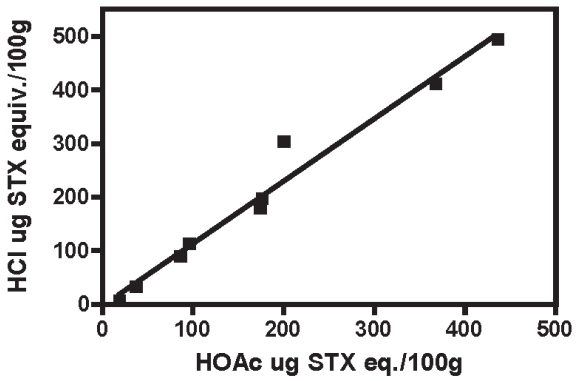


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

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.

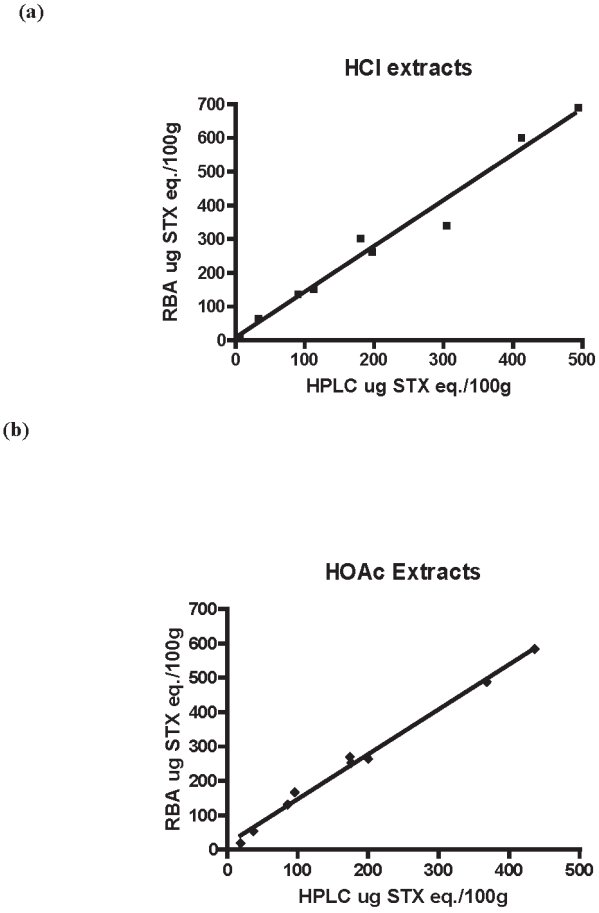


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

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.

Acknowledgments

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

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2006) *J. AOAC Int.* **88**, 1714–1732

(3) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(4) Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., & Lees, D.N. (2009) *J. AOAC Int.* **92**, 190–207

(5) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425

(6) Hall, S., Strichartz, G., Moczydlowshi, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origins, Structure, and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(7) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636

(8) Van Dolah, F.M., Finley, E.L., Haynes, B.L., Doucette, G.J., Moeller, P.D., & Ramsdell, J.S. (1994) *Nat. Toxins* **2**, 189–196

(9) Powell, C.L., & Doucette, G.J. (1999) *Nat. Toxins* **7**, 393–400

(10) Suarez-Isla, B.A., & Valez, P. (2000) in *Seafood and Freshwater Toxins,* L.M. Botana (Ed.), Marcel Dekker Inc., New York, NY, pp 187–202

(11) Llewellyn, L.E., Doyle, J., Jellett, J., Barrett, R., Alison, C., Bentz, C., & Quilliam, M. (2001) *Food Addit. Contam.* **18**, 970–980

(12) Ruberu, S.R., Liu, Y.-G., Wong, C.T., & Perera, S.K. (2003) *J. AOAC Int.* **86**, 1–9

(13) Usup, G., Leaw, C.-P., Cheah, M.-Y., Ahmad, A., & Ng, B.-K. (2004) *Toxicon* **44**, 37–43

(14) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667

(15) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

FOOD CHEMICAL CONTAMINANTS

Appendix III

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

FRANCES M. VAN DOLAH, SPENCER E. FIRE, TOD A. LEIGHFIELD, CHRISTINA M. MIKULSKI, and GREGORY J. DOUCETTE
Center for Coastal Environmental Health and Biomolecular Research, NOAA Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412

Collaborators: Å. Andersson; L. Bean; D. Couture; S. DeGrasse; A. DeLeon; V. Dell'Ovo; L. Flewelling; P. Holland; G. Langlois; R. Lewis; M. Masuda; P. McNabb; C. Mikulski; B. Niedzwiadek; B. Porntepkasemsan; D. Rawn; E. Sombrito; K. Srisuksawad; B. Suarez; S. Subsinserm; A. Tubaro

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.

Submitted for publication December 3, 2011.
The recommendation was approved by the Method Committee on Paralytic Shellfish Toxins as First Action. *See* "Methods News," (2011) *Inside Laboratory Management*, January/February issue.

Corresponding author's e-mail: Fran.vandolah@noaa.gov
DOI: 10.5740/jaoacint.CS2011_27

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:

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

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%.

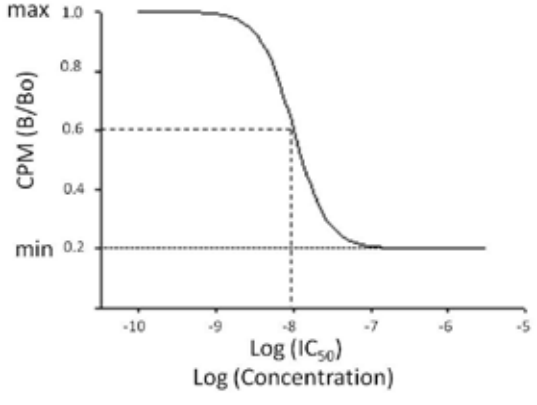


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

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

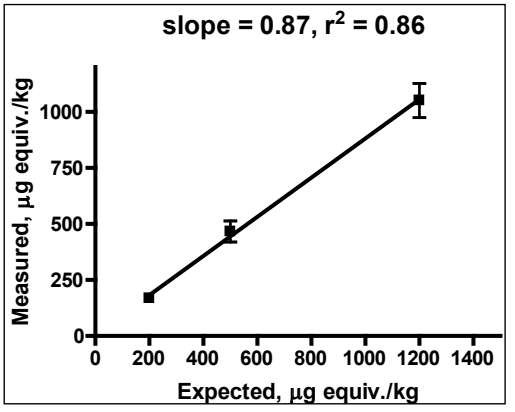


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

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

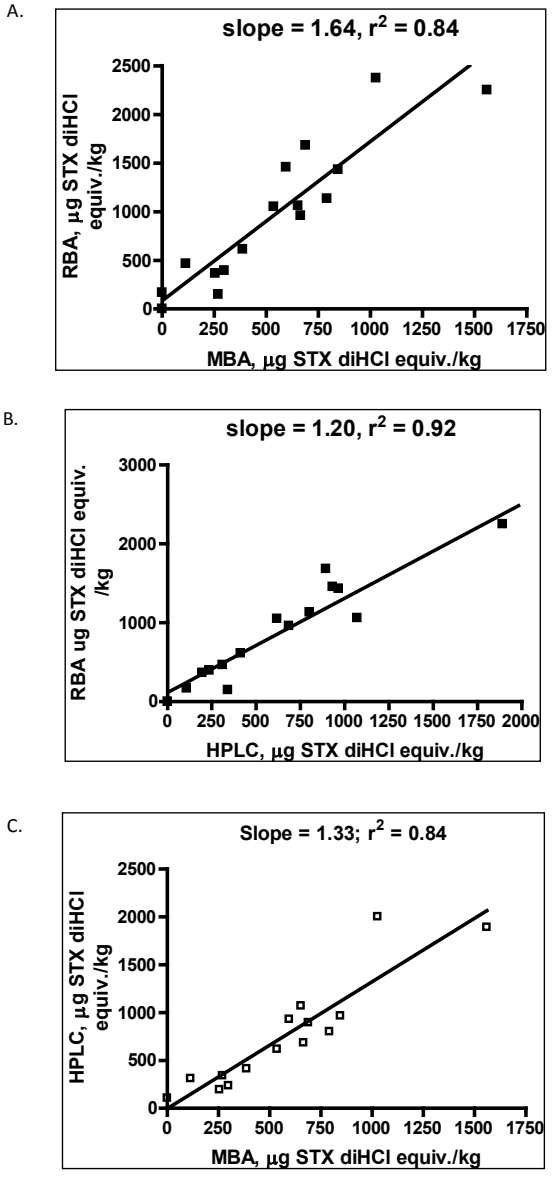


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

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.

Acknowledgments

We would like to thank Laurie Bean (Maine Department of Marine Resources), Stacey DeGrasse (U.S. Food and Drug Administration), Patrick Holland (Cawthron Institute), Gregg Langlois (California Department of Health Services), Bob Lona (Washington State Department of Health), and Benjamin Suarez (University of Chile) for providing the shellfish material used in the study. We are grateful to Barbara Niedzwiadek and Dorothea Rawn (Health Canada) for HPLC analyses and to Laurie Bean and Darcie Couture (Maine Department of Marine Resources), Benjamin Saurez, and Supanoi Subsinserm (Thailand Department of Fisheries) for performing mouse bioassays. We are indebted to the following collaborators for their dedication and perseverance in carrying out the RBAs for this study:

Leanne Flewelling, Florida Wildlife and Marine Research Institute, St. Petersburg, FL

Stacey DeGrasse, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD

Patrick Holland and Paul McNabb, Cawthron Institute, Nelson, New Zealand

Gregg Langlois and Melisa Masuda, California Department of Public Health, Richmond, CA

Richard Lewis and Åsa Andersson, University of Queensland, Queensland, Australia

Christina Mikulski, NOAA Marine Biotoxins Program, Charleston, SC

Elvira Sombrito and Aileen DeLeon (Philippine Nuclear Research Institute, Manila, the Philippines

Kanitha Srisuksawad and Boonsom Porntepkasemsan, Thailand Nuclear Research Institute, Bangkok, Thailand

Aurelia Tubaro and Valeria Dell'Ovo, Dipartimento dei Materiali e Risorse Naturali, Trieste, Italy

This project was funded by the International Atomic Energy Agency Interregional Project INT7017 9001 and NOAA Marine Biotoxins Program Project 02E0017. FDA saxitoxin diHCl used for tritiation by IICH and STX diHCl reference standard (NIST RM 8642) were provided by Sherwood Hall, FDA Office of Seafoods.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) LeDoux, M., & Hall, S. (2000) *J. AOAC Int.* **83**, 305–310

(3) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005) *J. AOAC Int.* **88**, 1714–1732

(4) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(5) *Official Methods of Analysis* (2011) AOAC INTERNATIONAL, Gaithersburg, MD, Method **2011.02**

(6) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425. http://dx.doi.org/10.1016/S0041-0101(02)00153-8

(7) Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origin, Structure and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(8) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667. http://dx.doi.org/10.1021/tx050277i

(9) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636. http://dx.doi.org/10.1016/S0041-0101(96)00189-4

(10) Van Dolah, F.M., Leighfield, T.A., Doucette, G.J., Bean, L., Niedzwiadek, B., & Rawn, D.F.K. (2009) *J. AOAC Int.* **92,** 1705–1713

(11) McFarran, E.F. (1959) *J. AOAC Int.* **42**, 263–271

(12) EU Reference Laboratory for Marine Biotoxins (2010) *Report*

on the EURLMB 2010 proficiency testing on saxitoxin group (PSP) toxins determination, Vigo, Spain

(13) Eurachem Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics,* http://www.eurachem.org/index.php/publications/guides/mv

(14) Kleivdahl, H., Kristiansen, S.-I., Nilson, M.A., Goksoyr, A., Briggs, L., Holland, P., & McNabb, P. (2007) *J. AOAC Int.* **90**, 1011–1027

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.

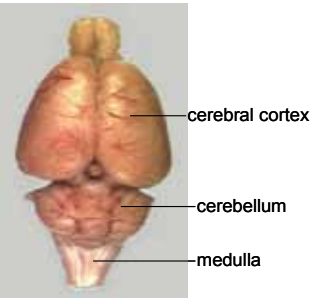


Figure 1. Rat brain.

(*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.

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

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.

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

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

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.

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

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).]

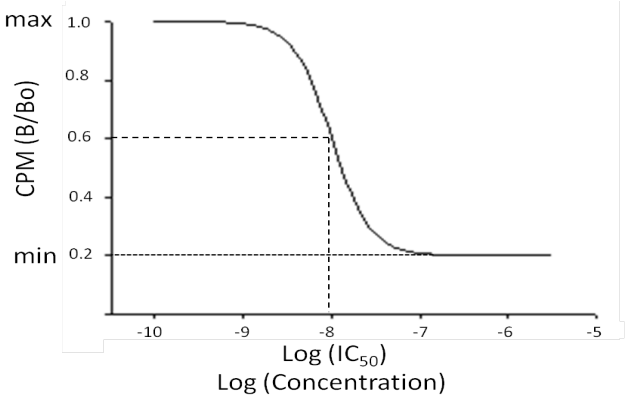


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC50.

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)

National Institute of Standards & Technology

Report of Investigation

Reference Material 8642

FDA Saxitoxin Dihydrochloride Solution

This Reference Material (RM) is intended for use in calibrating the mouse bioassay used in AOAC International Official Method 959.08 Paralytical Shellfish Poison [1] and for other similar uses. RM 8642 FDA Saxitoxin Dihydrochloride Solution was prepared by the U.S. Food and Drug Administration's (FDA's) Center for Food Safety and Applied Nutrition (CFSAN), where it was identified as Lot 089. The RM is saxitoxin dihydrochloride (CAS No. 35554-08-6) in a solution containing a hydrochloric acid concentration of 5 mmol/L in 20 % ethanol in water (volume fraction). A unit of RM 8642 consists of ten amber, borosilicate glass ampoules, each containing approximately 1.2 mL of solution.

Reference Mass Fraction Value: The reference value for the mass fraction of saxitoxin hydrochloride in solution in RM 8642, identified by FDA as lot 089, is 103 µg/g with an expanded uncertainty of 4 µg/g. Reference values are noncertified values that are estimates of the true value; however, the values do not meet the NIST criteria for certification and are provided with associated uncertainties that may reflect only measurement precision, may not include all sources of uncertainty, or may reflect a lack of sufficient statistical agreement among multiple analytical methods [2]. The reference mass fraction value is based on the gravimetric preparation of a stock solution and gravimetric dilution to produce the final material, and uncertainties associated with the associated weighings. The uncertainty is expressed as an expanded uncertainty, $U = ku_c$, at the 95 % level of confidence, $k = 2$, and includes a 2 % Type B purity uncertainty component as well as the gravimetric uncertainty [3]. Values are reported on an "as-received" basis in mass fraction units [4].

Expiration of Value Assignment: The reference value for RM 8642 is valid, within the measurement uncertainty specified, until **01 July 2013**, provided the RM is handled and stored in accordance with instructions given in this report (see "Instructions for Use"). This report is nullified if the RM is damaged, contaminated, or otherwise modified.

Maintenance of RM: NIST will monitor this RM over the period of its validity. If substantive technical changes occur that affect the value assignment before the expiration of this report, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The technical and support aspects involved in the preparation and issuance of this Reference Material were coordinated through K.E. Sharpless of the NIST Analytical Chemistry Division and M.P. Cronise of the NIST Measurement Services Division.

The solution was prepared and characterized by S. Hall of the Division of Bioanalytical Chemistry, Office of Regulatory Science, CFSAN, FDA.

Statistical analysis was provided by J.H. Yen of the NIST Statistical Engineering Division.

Support aspects involved in the issuance of this SRM were coordinated through the NIST Measurement Services Division.

Stephen A. Wise, Chief
Analytical Chemistry Division

Robert L. Watters, Jr., Chief
Measurement Services Division

Gaithersburg, MD 20899
Report Issue Date: 09 December 2010
Report Revision History on last page.

NOTICE AND WARNING TO USERS

Warning: For laboratory use only.

Storage: Unopened ampoules should be stored upright under normal laboratory conditions inside the original container supplied by NIST.

INSTRUCTIONS FOR USE

Gently tap the ampoule prior to opening to allow any solution in the tip to drain into the body of the ampoule.

Prepare a working solution as follows: On a top-loading balance, record the tare weight of an appropriate plastic bottle to 0.1 g or better. To the bottle, add approximately 100 mL water that has been acidified to pH 3 with hydrochloric acid. To minimize error due to evaporation, be prepared to immediately transfer the RM solution to this bottle after opening the ampoule. To open, hold the ampoule steady and grasp the stem at the metallic band with thumb and forefinger; **minimal** thumb pressure should be applied to the stem to snap it. Correctly done, the stem should break easily where pre-scored. Aspirate the RM solution into a dry, clean, disposable plastic syringe, 2 mL to 5 mL capacity, fitted with a suitable needle (such as 18 G × 1 ½″), weigh the syringe and its contents to 1 mg or better, and dispense the solution into the bottle of acidified water. Do not rinse the syringe. Reweigh the emptied syringe to determine the mass of RM solution transferred to the bottle. Add sufficient acidifed water (pH 3, HCl) to adjust the concentration to 1 µg/g. Weigh the bottle and its contents to determine the mass of solution prepared and the exact concentration of the working solution.

Because of the volatility of ethanol, the reference value is not applicable to material in ampoules that have been previously opened. The concentration of the working solution should be stable for more than one month if the solution is protected from evaporation. Dilution by mass is preferred but, if dilution by volume must be performed, the density of the solution is 0.971 g/mL and the concentration of this standard is 100 µg/mL with an expanded uncertainty of 4 µg/mL. This uncertainty is calculated as described above.

Source and Preparation of Material: Saxitoxin was extensively purified on three low-pressure preparative columns, each containing a different stationary phase. The saxitoxin was converted to the dihydrochloride form by passage through an ion exchange resin in the chloride form. Purity was assessed at FDA by proton nuclear magnetic resonance spectroscopy, combustion analysis, and optical rotation. RM 8642, identified by FDA as lot 089, was prepared by dissolving the saxitoxin dihydrochloride in a solution of hydrochloric acid (5 mmol/L) in 20 % ethanol in water (volume fraction).

REFERENCES

[1] AOAC International; *Official Methods of Analysis of AOAC International*, 18th Edition, Gaithersburg, MD (2005).

[2] May, W.; Parris, R.; Beck II, C.; Fassett, J.; Greenberg, R.; Guenther, F.; Kramer, G.; Wise, S.; Gills, T.; Colbert, J.; Gettings, R.; MacDonald, B.; *Definition of Terms and Modes Used at NIST for Value-Assignment of Reference Materials for Chemical Measurements*; NIST Special Publication 260-136 (2000); available at http://ts.nist.gov/MeasurementServices/ReferenceMaterials/PUBLICATIONS.cfm (accessed Nov 2010).

[3] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (ISO GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at http://www.bipm.org/utils/common/documents/jcgm/JCGM_100_2008_E.pdf (accessed Nov 2010); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at http://www.nist.gov/physlab/pubs/index.cfm (accessed Nov 2010).

[4] Thompson, A.; Taylor, B.N.; *Guide for the Use of the International System of Units (SI)*; NIST Special Publication 811; U.S. Government Printing Office: Washington, DC (2008); available at: http://ts.nist.gov/WeightsAndMeasures/Metric/mpo_pubs.cfm (accessed Nov 2010).

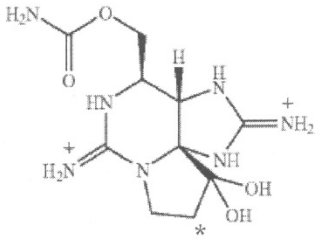
Report Revision History: 09 December 2010 (Extension of the period of validity; editorial changes.); 09 June 2010 (Original report date).

Users of this RM should ensure that the Report of Investigation in their possession is current. This can be accomplished by contacting the SRM Program: telephone (301) 975-2200; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.

American
Radiolabeled
Chemicals, Inc.

101 ARC Dr.
St. Louis, MO 63146 U.S.A.
Ph. (314) 991-4545 or (800) 331-6661
Fax (314) 991-4692 or (800) 999-9925
Web: http://www.arc-inc.com
E-mail: arcinc@arc-inc.com

TECHNICAL DATA SHEET
ART 1301 Saxitoxin [11-^{3}H]



LOT SPECIFIC TECHNICAL DATA:

Lot number: 120814
Specific activity: estimated 20-30 Ci/mmol
Solvent: Methanol
Radioactive concentration: 0.05 mCi/ml
Molecular weight: 299.2

PACKAGING INFORMATION:

ART 1301 is packaged as a solution in methanol in a sealed ampoule. It is shipped in dry ice.

STABILITY AND STORAGE RECOMMENDATIONS:

A working stock of 1/50 dilution in methanol can be stored at 4°C. Long-term storage should be carried out at -80°C, based on the previous commercially available Saxitoxin [^{3}H], which was not stable at -20°C. The rate of degradation at -80°C is approximately 0.3-1% for the first month.

RADIOCHEMICAL AND CHEMICAL PURITY:

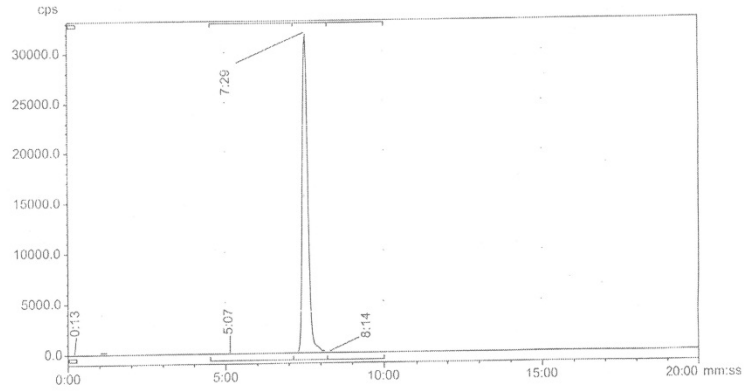
Radiochemical Purity: 99.56%

Column: Zorbax SB-AQ (250 x 3.0mm)

Mobile Phase: water:isopropanol: heptafluorobutyric acid (99.5 : 0.5 : 0.005)

Flow Rate: 0.5 ml/min

Detector: β-RAM [^{3}H]



Name	Start (mm:ss)	End (mm:ss)	Retention (mm:ss)	Height (cps)	Area (Counts)	%ROI (%)
Bkg 1	0:02	0:16	0:13	183.0		
Region 1	4:31	7:08	5:07	158.0	1375.5	0.36
Region 2	7:08	8:11	7:29	32767.0	384313.4	99.56
Region 3	8:11	9:58	8:14	136.0	320.3	0.08
3 Peaks					386009.1	100.00

Proposal No. **13-116**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Florida Department of Agriculture and Consumer Services
Affiliation	Florida Department of Agriculture and Consumer Services
Address Line 1	1203 Governor's Square Blvd.
Address Line 2	Suite 501
City, State, Zip	Anchorage, Alaska 99507
Phone	850-488-4033
Fax	850-410-0893
Email	Kimberly.Norgren@freshfromflorida.com
Proposal Subject	Shellfish Quarantine Guidance Document
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.04 Marine Biotoxin Control Section IV. Guidance Documents Chapter II. Growing Areas .02 Guidance for Developing Marine Biotoxin Contingency Plans
Text of Proposal/ Requested Action	Model Ordinance Chapter IV. Shellstock Growing Areas @.04 Marine Biotoxin Control Section A. (4) describes agreements or memoranda of understanding between the Authority and individual shellfish harvesters or individual shellfish dealers, to allow harvesting during marine Biotoxin closures under specific, controlled conditions. The State of Florida has successfully implemented such an agreement to address Neurotoxic Shellfish Poisoning (NSP) for over a decade. This pilot project, developed in consultation with FDA, has resulted in zero cases of NSP in commercially harvested shellfish from Florida waters. NSP may affect any Gulf or South Atlantic state and therefore Florida wishes to provide ISSC member states with a proven quarantine protocol template for incorporation into the Model Ordinance Section IV. Guidance Documents. Guidance Documents Chapter II. Growing Areas .02 Guidance for Developing Marine Biotoxin Contingency Plans. Text of the proposed guidance is as follows: Example Protocol for Quarantine Harvest of Shellfish from Aquaculture Leases During *Karenia brevis* Closures: A. Closure of an entire shellfish growing area due to *Karenia brevis* shall be in accordance with Model Ordinance Chapter IV. @.04 C. (1). B. When a shellfish growing area is closed due to *Karenia brevis*, the Authority may allow harvest of shellfish from selected aquaculture leases within a specific zone by authorized harvesters and subsequent controlled quarantine at a certified shucker packer or shellstock shipper. This option would not be

available if any Authority collected water samples in the specific zone exceeded 200,000 cells per liter of *Karenia brevis*. Zone is defined as an Authority delineated geographic area within a Conditionally Approved or Approved classified shellfish growing area.

Controlled quarantine conditions:

The Authority will determine and plot the specific zones. Certified processors possessing a valid shellfish processing plant certification license must have written permission from the Authority to engage in this activity. To be eligible for participation in the quarantine program, the certified processor must:

(1) Provide the Authority with written and signed agreements the processor has with shellfish aquaculture leaseholders who would be supplying the shellfish and;
(2) Notate on their application letter which FDA-approved marine Biotoxin laboratory will be used to conduct the approved mouse bioassay and;
(3) Provide the Authority with the cooler capacity, physical address and current certification number of the facility to be used for controlled quarantine of shellfish. All quarantine coolers must be non-mobile, secure from unauthorized access and equipped with warning signs in a language readily understood by all employees.

Participation in each week's quarantine program is only possible for certified processors who:

(1) Have written permission on file with the Authority and are on an Authority-controlled document listing current approved quarantine program processors and;

(2) Possess emailed permission granted by the Authority the day before harvest for that one specific quarantine and;

(3) Propose harvesting a quantity of shellfish that meets the Authority established minimum number but does not exceed the maximum allowed number of shellfish of one specific species for that day.

Under no circumstances may any approved processor participate in any quarantine until they possess written (emailed) documentation sent by the Authority before each specific quarantine event.

- The authorization email sent by the Authority shall explicitly state the permissible species that may be harvested by that approved processor.
- The Authority will notify the appropriate law enforcement entity in charge of patrol of shellfish growing areas with a list of participants in that specific day's harvest.
- Persons harvesting a species not authorized for that day's harvest will be subject to seizure of that harvest by the Authority. In addition, the Authority will immediately seize and destroy product

which is improperly tagged, violates any National Shellfish Sanitation Program (NSSP) Model Ordinance regulations, state laws or is from non-authorized participants.

- Co-mingling of species is not allowed to make up an individual lot.

Violation of the terms of this protocol may result in the termination of the participant's future eligibility in the quarantine program, as determined by the Authority.

Prior to being considered for participation in any specific quarantine event, approved processors shall be contacted by the Authority and asked to provide the name of the species they plan to harvest and the quantity they plan on harvesting. Quantities shall be described as approximate total number by species in addition to total number of baskets, containers, bags, etc. with specific weights (if applicable) for those baskets, containers, bags, etc.

Eligible processors should be aware that daily implementation of this program is contingent on marine Biotoxin laboratory availability as well as Authority staffing considerations given staff time necessary to fulfill the requirements of the program.

Regulatory considerations on behalf of the Authority and staffing considerations on behalf of the marine Biotoxin lab necessitate an Authority developed maximum number of samples that could be potentially tested on any given week.

The Authority may implement a lottery, random rotation or similar procedure to ensure a fair distribution of testing opportunities among the eligible processors. It is suggested that the Authority develop this procedure with industry involvement.

Once specific permission is received from the Authority, the processor:

(2) May receive properly tagged shellfish from eligible aquaculturists only as indicated in the Authority's authorization email;

(3) Must upon receipt of shellfish, separate and maintain the shellfish into specific lots [A Lot is defined as shellfish of one species from no more than one day's harvest from a specific zone within a shellfish growing area];

(4) Must place shellfish under proper controls and quarantine; Proper controls and quarantine are defined by bold, clear, warning signage signaling the properly tagged and segregated shellfish within the processor's cooler are under quarantine and must not be moved until Authority permission is obtained pending outcome of laboratory testing. The signage should be such that it is clear to anyone entering the cooler (including facility employees and/or regulatory inspectors) that the affected shellfish are under quarantine. Wrapping of the entire lot with a single bright red or yellow ribbon or equivalent attached to the bold warning sign will

	further reinforce the warning message. (5) Must allow the Authority to take two (2) random samples [minimum of twenty (20) shellfish per each sample] from each lot and deliver to the approved laboratory for approved mouse bioassay; (6) Must hold all shellfish in quarantine at the approved processor's certified facility until receiving official written test result notice from the Authority via email or fax that the shellfish are cleared for sale; (7) Must either return shellfish to aquaculture lease(s) in the zone(s) from where harvested if any sample in a lot is 20 Mouse Units / 100 grams or greater or destroy the shellfish, both activities of which must be witnessed and documented by the Authority; (8) Must cease this activity if any Authority collected red tide cell counts in the specific zone exceeds 200,000 cells per liter of *Karenia brevis*; and (9) Must document all of the requirements listed above in the approved facility HACCP plan. C. If cell counts in all water samples fall to 5,000 cells/L or less Karenia brevis in the entire area, the Authority will collect shellfish meat samples for toxicity testing and the entire Shellfish Harvesting Area will be reopened if results of all samples are <20 MU/100g. I _____ (print name) have received a copy of this quarantine protocol and I agree to abide by all terms and conditions. I understand I am bound by the terms of this agreement during the period of time that I am processing shellfish from a shellfish growing area that is currently in the closed status due to Karenia brevis. _____ _____ Signed Date
13. Public Health Significance	Closures of shellfish growing areas due to Neurotoxic Shellfish Poisoning (NSP) may occur at any time in the Gulf of Mexico and to a lesser degree, the Atlantic coast. Well established procedures for detecting and responding to *Karenia brevis* blooms have safeguarded public health. Clear early warning signs, a cell count action level with a high factor of safety and established sampling networks provide excellent public health protection. A very real impact of *Karenia brevis* blooms is the resulting long-term closures of shellfish growing areas and severe economic impact to commercial shellfish operations. Florida addressed this issue after studying years of water quality samples and mouse bioassay results from shellfish growing areas. Hydrodynamic studies linked to water samples obtained from fixed stations over an extended period of time established clear patterns in distribution of *Karenia brevis*. Working in conjunction with harmful algal bloom researchers, shellfish growing area managers, FDA and industry, Florida developed a NSP quarantine protocol that has resulted in the retention of a shellfish industry in one of the most severely impacted HAB regions of the Gulf while protecting public health as required by the Model Ordinance. An enormous amount of data has been generated and reviewed during the years this protocol has been used. Repeated mouse bioassay testing on shellfish exposed to different levels of *Karenia brevis*

	has provided Florida with sufficient data to refine the protocol into a powerful management tool. Florida's experience pre-quarantine protocol was unfortunate, as several fledgling businesses failed due to repeated NSP closures. It was this economic damage that spurred the aforementioned collaborative effort between leading edge HAB researchers, shellfish growing area managers, FDA and industry. If adopted, shellfish producing states impacted by *Karenia brevis* could reference this protocol in the Guidance Document and use it to effectively manage NSP closures.
Cost Information	The estimated cost for a full 96-well plate assay is ~$95.00. Including standards and samples with triplicate measurements (as well as three dilutions per sample to ensure the unknown samples fall within linear range of assay), the cost per sample for quantitative results would be ~$13.60. If running multiple plates or in screening mode, sample costs would be reduced. Further, the filter plates used in the RBA differ from ELISA plates in that all reagents are added to each well as needed rather than already being a component of the plate, making it more practical and cost-effective to analyze samples when there is less than a full plate.
Action by 2013 Task Force I	Recommended referral of Proposal 13-116 to an appropriate committee as determined by the Conference Chairman
Action by 2013 General Assembly	Adopted recommendation of 2013 Task Force I on Proposal 13-116.
Action by FDA May 5, 2014	Concurred with Conference action on Proposal 13-116.
Action by 2015 Biotoxin Committee	Recommended adoption of Proposal 13-116 with substitute language as follows: (4) The plan may include agreements or memoranda of understanding, between the Authority and individual shellfish harvesters or individual shellfish dealers, to allow harvesting in designated parts of a state growing area while other parts of the same ~~the~~ growing area are placed in the closed status. Such controlled harvesting shall be conducted with strict assurances of safety. In state growing areas or designated portions of state growing waters that are closed, the authority may allow for harvesting if an end product testing program is developed and~~, such as by batch release of shellfish lots only after~~ samples of each lot are tested and found to be below the action levels specified in Section C. The program must include at a minimum: i. Establishment of appropriate pre-harvest screening levels; ii. Establishment of appropriate screening and end product testing methods; iii. Establishment of appropriate laboratories/analysts to conduct screening and end product testing methods; iv. Establishment of representative sampling plan for both i. and ii. above; and v. Other controls as necessary to ensure that shellstock are not released prior to meeting all requirements of the program. Should the above amended proposal be adopted by the conference, then the Biotoxin Committee should develop a Guidance Document that includes guidance for development of end-product testing programs to address biotoxins in closed state waters.
Action by 2015 Task	Recommends adoption of Biotoxin Committee recommendation on Proposal 13-

Force I	116.
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 13-116.
Action by 2017 Task Force I	Recommended the Biotoxin Committee should develop a Guidance Document that includes guidance for development of end-product testing programs to address Biotoxins in closed State waters.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 13-116.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 13-116.

Proposal No. **15-109**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ 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
Address Line 1	194 McKown Point Road and 5251 Dr. MLK Jr., Avenue
Address Line 2	
City, State, Zip	West Boothbay Harbor, ME 04575 and Anchorage, AK 99507
Phone	207-633-9401 and 907-375-8229
Fax	207-633-9579 and 907-929-7335
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	The PCOX method was developed to provide a rapid, high throughput chemical

Significance	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 2015 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.

Proposal No. **15-112**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting

a. ☒ Growing Area
b. ☐ Harvesting/Handling/Distribution
c. ☐ Administrative

Submitter	Executive Board
Affiliation	Interstate Shellfish Sanitation Conference (ISSC)
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Direct Plating Method for trh
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	This method was developed by Jessica Jones (FDA Gulf Coast Seafood Laboratory) and is being submitted by the ISSC Executive Board. The Executive Board granted interim approval to this method on March 13, 2015. The Executive Board is submitting this proposal to comply with Article V. Section 1. of the ISSC Constitution, Bylaws, and Procedures.

Submitted by method developer Jessica Jones (FDA Gulf Coast Seafood Laboratory)

5. Approved Methods for Vibrio Enumeration

	Vibrio Indicator Type:	Application: PHP Sample Type: Shucked	Applicatio Reopenin
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	
Direct Plating[6]	*trh+ Vibrio parahaemolyticus (V.p.)*	X	X

Footnotes:

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

[2] 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 labeled gene probe (vvhA).

[3] MPN format with confirmation by biochemical analysis, gene probe methodology as listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State can demonstrate is equivalent.

[4] PCR methods as they are listed in Chapter 9 of the FDA Bacteriological Analytical Manual, 7th Edition, May 2004 revision, or a method that a State

	can demonstrate is equivalent. [5]*Vibrio vulnificus*, ISSC Summary of Actions 2009. Proposal 09-113, Page 123. [6]Direct plating method for *trh* as described in Nordstrom et al., 2006.
Public Health Significance	Scientific evidence suggests that the presence of the *trh* gene in *V. parahaemolyticus (V.p.)* is correlated with higher virulence. Additionally, at the 2013 conference, proposal 13-202 was adopted which requires testing for the presence of trh prior to reopening of growing areas closed as a result of *V.p.* illnesses [Chapter II @.01.F(5)]. Currently, there are no NSSP approved methods for enumeration of *trh*. This method is a needed option for testing following *V.p.* illness closures.
Cost Information	This method costs ~$5 per test for laboratory consumables, supplies, and reagents. Most equipment needed for testing is standard microbiology equipment, but purchase of a specialized water bath or environmental chamber may be necessary at a cost of ~$3,000-$5,000. Additional costs for a laboratory would vary based on their operational overhead and labor.
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 15-112 to an appropriate committee as determined by the Conference Chair to further review the data submitted.
Action by 2015 Task Force I	Recommended adoption of 2015 Laboratory Methods Review Committee recommendation on Proposal 15-112.
Action by 2015 General Assembly	Adopted recommendation of Task Force I on Proposal 15-112
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-112.
Action by 2017 Laboratory Committee	Recommended referral of Proposal 15-112 to an appropriate committee as determined by the Conference Chair.
Action by 2017 Task Force I	Recommended adoption of Lab Committee recommendation on Proposal 15-112.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 15-112.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 15-112.

Validation Data for Direct plating method for trh

Name of Method Submitter: Jessica L. Jones, Ph.D.

Specific purpose or intent of the method for use in the NSSP: Seeking approval for this method as an approved limited use method that can be used as appropriate for PHP validation and verification testing, as well as environmental testing such as that which may be required for the re-opening of growing areas closed due to illness.

Validation Criteria Data: For evaluation of all validation criteria below, PHP oysters were obtained in the best effort to find samples free of the target organism. A different lot of PHP oysters was used for each sample. For each sample, a minimum of 10 animals were used to prepare a homogenate. The homogenate was then aliquoted and appropriate aliquots spiked with a *tdh+/trh+ Vibrio parahaemolyticus* (unless otherwise noted), while one aliquot was left unioculated (sample blank). Spike levels were determined by spread plating dilution of the culture in triplicate onto TSA+2% NaCl. Appropriate aliquots of spiked samples were spread plated onto T1N3 agar and colony lifts hybridized with an alkaline phosphatase-labeled probe specific for *trh*.

1. Accuracy/Trueness: Using the data from Table 1, the average of plate counts was 3.80 log and the average from DNA probe was 3.62 log. The *Accuracy/Trueness of the method is 95%.*

Table 1. Data for determination of Accuracy/Trueness and Measurement Uncertainty.

Sample	Plate Count (log CFU)	Probe Result (log CFU/g)
1-2X	5.18	4.76
2-2X	5.18	4.65
3-4X	3.15	2.90
4-4X	3.15	2.85
5-6X	1.23	1.48
6-6X	1.23	1.00
7-2X	5.76	5.59
8-2X	5.76	5.64
9-4X	3.68	3.59
10-4X	3.68	3.72

2. Measurement Uncertainty: Using the data from Table 1 above, *measurement uncertainty is 0.11*.

3. Precision: Using the data from Table 2, there *was no significant difference between the plate counts and the values generated with DNA probe* (p=0.58). The difference in variance is not significant (p=0.48) for any platform/gene target combination.

4. Recovery: The average of plate counts was 3.40 log the average (adjusted for sample blanks) from DNA probe was 3.65 log. Using this data, the *Recovery of the methods was determined to be 107% on both platforms for both gene targets.*

Table 2. Data for determination of Precision and Recovery			
Sample	**Aliquot**	**Plate Count (log CFU)**	**Probe Result (log CFU/g)**
1	**Blank**	N/A	<1.00
1	**2X**	5.18	4.76
1	**2Z**	5.18	5.38
1	**4X**	3.18	2.48
1	**4Z**	3.18	2.85
1	**6X**	1.18	<1.00
1	**6Z**	1.18	1.00
3	**Blank**	N/A	<1.00
3	**2X**	5.15	4.65
3	**2Z**	5.15	4.76
3	**4X**	3.15	2.90
3	**4Z**	3.15	3.04
3	**6X**	1.15	<1.00
3	**6Z**	1.15	1.78
5	**Blank**	N/A	1.85
5	**2X**	5.23	3.54
5	**2Z**	5.23	3.84
5	**4X**	3.23	2.70
5	**4Z**	3.23	3.00
5	**6X**	1.23	1.48
5	**6Z**	1.23	1.30
7	**Blank**	N/A	1.00
7	**2X**	5.76	5.59
7	**2Z**	5.76	5.23
7	**4X**	3.76	3.57
7	**4Z**	3.76	3.64
7	**6X**	1.76	1.78
7	**6Z**	1.76	1.7
9	**Blank**	N/A	<1.00
9	**2X**	5.68	5.25
9	**2Z**	5.68	5.11
9	**4X**	3.68	3.59
9	**4Z**	3.68	3.54
9	**6X**	1.68	2.94
9	**6Z**	1.68	1.48

5. Specificity: Samples were prepared as above and the interfering organism was spiked at an ~4 log higher concentration than *Vibrio parahaemolyticus*. Using the data from Table 3, the average *Specificity of the method is 1.38,* which is within the 95% confidence interval of the method (0.44) from 1.

Table 3. Data for determination of Specificity.	
	Probe Result

	(log CFU/g)	
Sample	**Spiked with Vp only**	**Spiked with Vp and Vv**
6-Blank	<1.00	---
6-6T	1.60	1.30
6-6U	1.30	<1.00
6-6W	1.48	1.48
6-6X	1.00	1.00
6-6Z	1.48	1.48

6. Working and Linear Range: Based on the data presented in Table 4, there is a significant correlation between the plate counts and CFU values by DNA probe ($p<0.001$). The *correlation coefficient is 0.96*, demonstrating the linearity of the method.

Table 4. Data for determination of Working and Linear Range, Limit of Detection, and Limit of Quantitation/Sensitivity

Sample	**Aliquot**	**Plate Count (log CFU)**	**Probe Result (log CFU/g)**
1	**1X**	6.18	5.36
1	**1Z**	6.18	6.18
1	**2X**	5.18	4.76
1	**2Z**	5.18	5.38
1	**4X**	3.18	2.48
1	**4Z**	3.18	2.85
1	**6X**	1.18	<1.00
1	**6Z**	1.18	1.00
1	**7X**	0.18	<1.00
1	**7X**	0.18	<1.00
3	**1X**	6.15	6.29
3	**1Z**	6.15	6.09
3	**2X**	5.15	4.65
3	**2Z**	5.15	4.76
3	**4X**	3.15	2.90
3	**4Z**	3.15	3.04
3	**6X**	1.15	<1.00
3	**6Z**	1.15	1.78
3	**7X**	0.15	1.00
3	**7Z**	0.15	<1.00
5	**1X**	6.23	5.57
5	**1Z**	6.23	5.64
5	**2X**	5.23	3.54
5	**2Z**	5.23	3.84
5	**4X**	3.23	2.70
5	**4Z**	3.23	3.00
5	**6X**	1.23	1.48

5	**6Z**	1.23	1.30
5	**7X**	0.23	1.30
5	**7Z**	0.23	1.48
7	**1X**	6.76	6.68
7	**1Z**	6.76	6.37
7	**2X**	5.76	5.59
7	**2Z**	5.76	5.23
7	**4X**	3.76	3.57
7	**4Z**	3.76	3.64
7	**6X**	1.76	1.78
7	**6Z**	1.76	1.70
7	**7X**	0.76	1.00
7	**7Z**	0.76	<1.00
9	**1X**	6.68	6.44
9	**1Z**	6.68	4.70
9	**2X**	5.68	5.25
9	**2Z**	5.68	5.11
9	**4X**	3.68	3.59
9	**4Z**	3.68	3.54
9	**6X**	1.68	2.94
9	**6Z**	1.68	1.48
9	**7X**	0.68	<1.00
9	**7Z**	0.68	<1.00

7. Limit of Detection: The *Limit of Detection of the method is 10 CFU/g*. This is reliant upon the amount of sample (0.1g) that can be tested by the spread plate method.

8. Limit of Quantification/ Sensitivity: The limit of quantification/sensitivity is also reliant upon the amount of sample that can be tested.

9. Ruggednes: Replicate spiked aliquots from each sample were processed with different batches of media/ lots of reagents at the same time. Different samples were processed on different days. Using the data in Table 5, there was *no significant difference (p=0.94) between batches/lots* of media and reagents.

Table 5. Data for determination of Ruggedness.

Sample	**Probe Result (log CFU/g)**	
	Replicate 1 (X)	**Replicate 2 (Z)**
2	4.78	4.88
4	2.85	2.78

6	1.00	1.48
8	5.64	5.73
10	3.72	3.57

10. ***Matrix Effects:*** Effects of oyster matrix on the performance of the method was taken into consideration in testing all of the above criteria by using the sample blank.

11. Additional Data: *Inclusivity/Exclusivity*. Control filters with the isolates listed below were prepared and tested as outlined above. All isolates. All isolates gave the expected reaction, demonstrating 100% *Inclusivity/Exclusivity*.

Species	**Number of Strains Tested**	**Number *trh*-positive**
V. parahaemolyticus[†]	43	43
*V. parahaemolyticus**	39	0
V. cholerae	25	0
V. vulnificus	13	0
V. metschnikovii	12	0
V. fluvialis	6	0
V. hollisae	5	0
V. algenolyticus	2	0
Salmonella spp.	20	0
Listeria spp.	20	0
Other non-*Vibrio* species	15	0

[†] *V. parahaemolyticus* strains previously determined to be *trh*-positive.

***** *V. parahaemolyticus* strains previously determined to be *trh*-negative.

Step-by-step procedure including equipment, reagents and safety requirements necessary to run the method:

1. Special Equipment, Media, and Reagents
   1.1. Special Equipment and Materials Required
      1.1.1.Shaking water bath(s) (42°C and 54°C)
      1.1.2.Orbital shaker

1.1.3. Microwave
1.1.4. Plastic tubs with lids (300-500 ml capacity)
1.1.5. Whatman 541 filters, 85mm
1.1.6. Sterile spread rods
1.1.7. Sterile inoculating loops
1.1.8. Sterile toothpicks
1.1.9. Whirl-Pak bags (4.5”x9”)

1.2. Media and Reagents
1.2.1. Alkaline peptone water (APW)
1.2.2. Phosphate buffered saline (PBS)
1.2.3. Thiosulfate citrate bile salts sucrose (TCBS) agar
1.2.4. T_1N_3 agar
1.2.5. Lysis solution
1.2.6. 2M ammonium acetate
1.2.7. 20X SSC and 1X SSC
1.2.8. 1X SSC/SDS
1.2.9. Proteinase K
1.2.10. Hybridization solution
1.2.11. NBT/BCIP tablets
1.2.12. AP-labeled DNA probes (DNA Technology)

2. Outlined Procedure
2.1. Preparation of shellfish
2.1.1. Hands of examiner must be scrubbed thoroughly with soap and potable water; latex or nitrile gloves should be worn while cleaning oysters.
2.1.2. Scrape off growth and loose material from shell, and scrub shell stock with sterile stiff brush under running water.
2.1.3. Place clean shellstock on clean towels or absorbent paper.
2.1.4. Change gloves and brushes between samples.
2.1.5. Protective chain mail glove can be used under a latex glove; outer gloves should be changed between samples.
2.1.6. Tare a sterile blender.
2.1.7. Using a sterile oyster knife, insert the point between the shells on the ventral side, about ¼ the distance from the hinge to the bill; alternately, knife can be inserted after making small opening with sterile bone cutting forceps.
2.1.8. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.
2.1.9. The upper shell can then be pried loose at hinge and discarded.
2.1.10. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.
2.1.11. A minimum of 12 animals or 200g is required.
2.1.12. Blend without adding diluent for 60-120 sec at 14,000 rpm.

2.2. Preparation of spread plates
2.2.1. Prepare 10-fold serial dilutions of shellfish homogenate in PBS
2.2.2. Inoculate 100µl of appropriate dilutions onto pre-dried T_1N_3 agar plates
2.2.3. Spread inoculum gently into agar until completely absorbed
2.2.4. Invert plates and incubate at 30-37°C overnight.

2.2.5. Alternately, this method can be utilized with suspect isolates replicated to T_1N_3 agar from 96 well plates obtained from a standard MPN method

2.3. DNA Probe Colony Hybridization

2.4. Alkaline phosphatase-labeled oligonucleotide probes (AP-*tlh*, AP-*tdh*, and AP-*trh*) can be stored in the refrigerator (4°C) for 1-2 years; do not freeze.

2.5. Filter Preparation

2.5.1. Label #541 Whatman filters with sample number, date, analyst initials, and probe to be hybridized with (*tlh, tdh,* or *trh*). Make sure orientation of filter is noted so that positive spots can be correlated to the appropriate well in the microtiter plate. A dot near the A1 well is sufficient.

2.5.2. Place each filter label-side down on appropriate T_1N_3 plate; apply gentle pressure to ensure contact with each colony. Allow labeled filter to sit at RT for 1-30 min. Transfer each filter with colony-side up to a plastic or glass petri dish lid containing 1 ml of lysis solution.

2.5.3. Microwave filters in petri dishes (full power) for 15-20 sec/filter depending on wattage of microwave; rotate dishes with filters and repeat microwaving. Filters should be hot and almost completely dry but not brown.

2.5.4. Transfer filters to a plastic wash container (up to 30 filters can be combined in one container) and neutralize with ammonium acetate (4 ml/filter) for 5 min on shaker at RT.

2.5.5. Decant ammonium acetate and rinse filters 2 times with 1X SSC buffer (10 ml/filter), for 2 min each time. (Filters can be air dried and stored at this point.)

2.6. Proteinase K (proK) treatment

2.6.1. Prepare proK solution (this is made by adding 10 ml/filter of 1X SSC and 20 µl/filter of proK stock solution) for the appropriate number of filters. Place filters (up to 30) in plastic wash container of proK solution. Incubate for 30 min in a 42°C water bath with shaking (50 rpm) to destroy naturally occurring alkaline-phosphatase and digest bacterial protein.

2.6.2. Decant proK solution. Rinse filter 3 times in 1X SSC (10 ml/filter) for 10 min at RT with shaking at 50 rpm. (Filters can be air dried by placing on paper towels and stored when completely dry.)

2.7. Hybridization

2.7.1. Place up to 5 proK-treated filters (either dried or straight from treatment) in a Whirl-Pak bag. Add 10 ml of pre-warmed hybridization buffer and close bag to exclude air. Avoid trapping air bubbles. Incubate filters for 30 min at 54°C in a shaking (50 rpm) water bath.

2.7.2. Pour off buffer from bag and add 10 ml fresh pre-warmed buffer/bag. Add probe (final conc. is 0.5 pmol/ml) to bag with filters. Reseal bag, excluding air, and incubate 1 h in a 54°C water bath with shaking. The temperature is critical for hybridization and washing steps.

2.7.3. Remove filters from hybridization bags and place in plastic wash container(s).

2.7.4. Add 10 ml/filter 1X SSC/1% SDS . Incubate in a 54°C water bath with shaking for 10 min. Repeat wash a second time.

2.7.5. Rinse filter 5 times for 5 min each in 1X SSC at RT on an orbital shaker, 100 rpm.

2.8. Color development

2.8.1. In petri dish, add 20 ml of NBT/BCIP solution. Add filters (5 or fewer) to dish and incubate with gentle shaking at 35-37°C; cover to omit light. Check development of positive control every 30 min.

2.8.2. Transfer filters to a plastic wash container and add tap water (10 ml/filter). Rinse filters at RT with shaking for 10 min. Repeat rinse 2 additional times to stop color development. Do not expose filters to light as they will continue to develop. Consider purple or brown spots positive.

Validation Data for Direct Plating Method for *trh*, Proposal 15-112

Name of Method Submitter: Jessica L. Jones, Ph.D.

Specific purpose or intent of the method for use in the NSSP: Seeking approval for this method as an approved limited use method that can be used as appropriate for PHP validation and verification testing of oysters, as well as environmental testing such as that which may be required for the re-opening of growing areas closed due to illness.

Validation Criteria Data: For evaluation of all validation criteria below, PHP oysters were obtained in the best effort to find samples free of the target organism. A different lot of PHP oysters was used for each sample. For each sample, a minimum of 10 animals were used to prepare a homogenate. The homogenate was then aliquoted and appropriate aliquots spiked with a *tdh+/trh+ Vibrio parahaemolyticus* culture (unless otherwise noted), while one aliquot was left unioculated (sample blank). Spike levels were determined by spread plating dilutions of the culture in triplicate onto TSA+2% NaCl. Appropriate aliquots of spiked samples were spread plated onto T1N3 agar and colony lifts hybridized with an alkaline phosphatase-labeled probe specific for *trh* as detailed in the step-by-step procedure. Data were analyzed as described in the "SLV Documents for Marine Biotoxin and Non-MPN Based Microbiological Methods" on the ISSC website.

Table 1. Data for determination of Accuracy/Trueness and Measurement Uncertainty

Sample	Plate Count (log CFU/g)	Sample Blank Probe Result (log CFU/g)	Spiked Sample Probe Result (log CFU/g)
1-2X	5.18	<1.00	4.76
2-2X	5.18	<1.00	4.78
3-4X	3.15	<1.00	2.90
4-4X	3.15	<1.00	2.85
5-6X	1.15	1.85	1.48
6-6X	1.15	<1.00	1.00
7-2X	5.76	1.00	5.59
8-2X	5.76	<1.00	5.64
9-4X	3.68	<1.00	3.59
10-4X	3.68	<1.00	3.72
11-6X	1.60	<1.00	1.70
12-6X	1.60	<1.00	1.85
13-2X	5.72	<1.00	5.70
14-2X	5.72	<1.00	5.44
15-4X	3.62	<1.00	3.49
16-4X	3.62	<1.00	3.53
17-6X	1.74	1.00	1.70
18-6X	1.62	1.00	2.04
19-2X	5.74	<1.00	5.45
20-6X	1.62	1.00	1.78

1. Accuracy/Trueness: Using the data from Table 1, the average of plate counts was 3.52 log and the average from DNA probe (after adjustment with sample blank results) was 3.48 log. The *Accuracy/Trueness of the method is 99%.*

Measurement Uncertainty: Using the data from Table 1 above, *measurement uncertainty is 0.13* log CFU/g.

Table 2. Data for determination of Ruggedness

Sample	Probe Result (log CFU/g)	
	Replicate 1 (X)	Replicate 2 (Z)
2	4.78	4.88
4	2.85	2.78
6	1.00	1.48
8	5.64	5.73
10	3.72	3.57
12	1.85	1.85
14	5.44	5.29
16	3.53	3.71
18	2.04	2.08
20	1.78	1.78

2. Ruggedness: Replicate spiked aliquots from each sample were processed with different batches of media/ lots of reagents at the same time. Different samples were processed on different days. Using the data in Table 2, a left skew of both sets of data was observed, with a variance ratio of 1.09 (not significant, p=0.91), so a paired t-test was used to compare the results. There was *no significant difference (p=0.94) between batches/lots* of media and reagents.

Table 3. Data for determination of Precision and Recovery

Sample	Aliquot	Plate Count (log CFU/g)	Sample Blank Probe Result (log CFU/g)	Spiked Sample Probe Result (log CFU/g)
1	**2x**	5.18	<1.00	4.76
1	**2z**	5.18	<1.00	5.38
1	**4x**	3.18	<1.00	2.48
1	**4z**	3.18	<1.00	2.85
1	**6x**	1.18	<1.00	<1.00
1	**6z**	1.18	<1.00	1.00
3	**2x**	5.15	<1.00	4.65
3	**2z**	5.15	<1.00	4.76
3	**4x**	3.15	<1.00	2.90
3	**4z**	3.15	<1.00	3.04
3	**6x**	1.15	<1.00	<1.00
3	**6z**	1.15	<1.00	1.78
5	**2x**	5.16	1.85	3.54
5	**2z**	5.16	1.85	3.84
5	**4x**	3.16	1.85	2.70
5	**4z**	3.16	1.85	3.00

5	**6x**	1.15	1.85	1.48
5	**6z**	1.15	1.85	1.30
7	**2x**	5.76	1.00	5.59
7	**2z**	5.76	1.00	5.23
7	**4x**	3.76	1.00	3.57
7	**4z**	3.76	1.00	3.64
7	**5x**	2.76	1.00	2.58
7	**5z**	2.76	1.00	2.57
7	**6x**	1.76	1.00	1.78
7	**6z**	1.76	1.00	1.70
9	**2x**	5.68	<1.00	5.25
9	**2z**	5.68	<1.00	5.11
9	**4x**	3.68	<1.00	3.59
9	**4z**	3.68	<1.00	3.54
9	**6x**	1.68	<1.00	2.94
9	**6z**	1.68	<1.00	1.48
11	**2x**	5.60	<1.00	5.54
11	**2z**	5.60	<1.00	5.46
11	**4x**	3.60	<1.00	3.48
11	**4z**	3.60	<1.00	3.71
11	**6x**	1.60	<1.00	1.70
11	**6z**	1.60	<1.00	1.00
13	**2x**	5.72	<1.00	5.70
13	**2z**	5.72	<1.00	5.08
13	**4x**	3.72	<1.00	3.36
13	**4z**	3.72	<1.00	3.36
13	**6x**	1.72	<1.00	1.48
13	**6z**	1.72	<1.00	1.30
15	**2x**	5.62	<1.00	5.53
15	**2z**	5.62	<1.00	4.98
15	**4x**	3.62	<1.00	3.49
15	**4z**	3.62	<1.00	3.48
15	**6x**	1.62	<1.00	1.30
15	**6z**	1.62	<1.00	1.00
17	**2x**	5.74	1.00	5.51
17	**2z**	5.74	1.00	5.42
17	**4x**	3.74	1.00	3.57
17	**4z**	3.74	1.00	3.64
17	**6x**	1.74	1.00	1.70
17	**6z**	1.74	1.00	1.90
19	**2x**	5.74	<1.00	5.45

19	**2z**	5.74	<1.00	5.64
19	**4x**	3.74	<1.00	3.51
19	**4z**	3.74	<1.00	3.45
19	**6x**	1.74	<1.00	1.78
19	**6z**	1.74	<1.00	1.70

3. Precision: Using the data from Table 3, there was no significant difference ($p>0.05$) in the variance ratio across the range of concentrations, with a coefficient of variance of 45% for the method. Additionally, there *was no significant difference between the plate counts and the values generated with DNA probe* ($p=0.68$).

Recovery: The average of plate counts was 3.51 log CFU/g the average (adjusted for sample blanks) from DNA probe was 3.28 log CFU/g. Using this data, the *Recovery of the method was determined to be 93%.*

Table 4. Data for determination of Specificity

Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Vv)**	**Log CFU/g**
6	**6T**	1.60	**VVT**	1.30
6	**6U**	1.30	**VVU**	<1.00
6	**6W**	1.48	**VVW**	1.48
6	**6X**	1.00	**VVX**	1.00
6	**6Z**	1.48	**VVZ**	1.48
Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Vc)**	**Log CFU/g**
12	**6T**	1.60	**VCT**	1.48
12	**6U**	2.00	**VCU**	<1.00
12	**6W**	1.70	**VCW**	1.60
12	**6X**	1.85	**VCX**	1.48
12	**6Z**	1.85	**VCZ**	1.00
Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Vf)**	**Log CFU/g**
18	**6T**	1.95	**VFT**	<10.00
18	**6U**	2.11	**VFU**	<10.00
18	**6W**	2.11	**VFW**	<10.00
18	**6X**	2.04	**VFX**	<10.00
18	**6Z**	2.08	**VFZ**	<10.00
Sample	**Aliquot (Vp only)**	**Log CFU/g**	**Aliquot (Vp and Va)**	**Log CFU/g**
20	**6T**	1.70	**VAT**	<1.00
20	**6U**	1.48	**VAU**	<1.00
20	**6W**	1.78	**VAW**	<1.00
20	**6X**	1.78	**VAX**	<1.00
20	**6Z**	1.78	**VAZ**	<1.00

4. Specificity: Interfering organisms tested were *V. vulnificus* (Vv)*, V. cholerae* (Vc)*, V. fluvialis* (Vf)*, and V. alginolyticus* (Va). Using the data from Table 4, the overall average *Specificity Index of the method is 1.43,* which is within the 95% confidence interval of the method (0.44) from 1.

However, significant differences between the average specificity indices and 1 were observed when examining the data from each interfering organism. Differences were significant for *V. fluvialis* ($p<0.001$) and *V. alginolyticus* ($p<0.001$), as well as *V. cholerae* ($p=0.05$). This significance is likely due to the differences in spike levels, rather than the specific organism as the ratio of *V. parahaemolyticus* to interfering organism was 1:13000, 1:6000, 1:1500, and 1:240 for *V. fluvialis, V. alginolyticus, V. cholerae,* and *V. vulnificus*, respectively. Additionally, it should be noted that filters were lifted from plates with more colonies than recommended from the samples with the interfering organisms to give the best likelihood of enumerating *V. parahaemolyticus*. Together, this indicates that the method specificity is dependent on the ratio of target to interfering organism, where ratios of 1:1000 may cause interference, but lower ratios do not. In real world samples, this ratio of *trh+ V. parahaemolyticus* to other *Vibrio* species, is unlikely, supporting the fit-for-purpose of this method.

Table 5. Data for Working/Linear Range

Sample	Plate Count	Replicate 1 (X)	Replicate 2 (Z)
1-1	6.18	5.36	6.18
1-2	5.18	4.76	5.38
1-4	3.18	2.48	2.85
1-5	2.18	2.00	1.85
1-6	1.18	<1.00	1.00
1-7	0.18	<1.00	<1.00
3-1	6.15	6.29	6.09
3-2	5.15	4.65	4.76
3-4	3.15	2.90	3.04
3-5	2.15	2.23	2.68
3-6	1.15	<1.00	1.78
3-7	0.15	1.00	<1.00
5-1	6.16	5.57	5.64
5-2	5.16	3.54	3.84
5-4	3.16	2.70	3.00
5-5	2.16	2.20	2.38
5-6	1.15	1.48	1.30
5-7	0.15	1.30	1.48
7-1	6.76	6.68	6.37
7-2	5.76	5.59	5.23
7-4	3.76	3.57	3.64
7-5	2.76	2.58	2.57
7-6	1.76	1.78	1.70

7-7	0.76	1.00	<1.00
9-1	6.68	6.44	4.70
9-2	5.68	5.25	5.11
9-4	3.68	3.59	3.54
9-5	2.68	2.49	2.79
9-6	1.68	2.94	1.48
9-7	0.68	<1.00	<1.00
11-1	6.60	6.40	6.44
11-2	5.60	5.54	5.46
11-4	3.60	3.48	3.71
11-5	2.60	3.06	2.84
11-6	1.60	1.70	1.00
11-7	0.60	<1.00	<1.00
13-1	6.72	6.71	5.55
13-2	5.72	5.70	5.08
13-4	3.72	3.36	3.36
13-5	2.72	2.48	2.38
13-6	1.72	1.48	1.30
13-7	0.72	<1.00	<1.00
15-1	6.62	6.22	6.40
15-2	5.62	5.53	4.98
15-4	3.62	3.49	3.48
15-5	2.62	1.85	1.60
15-6	1.62	1.30	1.00
15-7	0.62	<1.00	<1.00
17-1	6.74	6.33	6.39
17-2	5.74	5.51	5.42
17-4	3.74	3.57	3.64
17-5	2.74	2.60	2.72
17-6	1.74	1.70	1.90
17-7	0.74	1.30	<1.00
19-1	6.74	6.36	6.32
19-2	5.74	5.45	5.64
19-4	3.74	3.51	3.45
19-5	2.74	2.72	2.53
19-6	1.74	1.78	1.70
19-7	0.74	1.30	<1.00

5. Working and Linear Range: Based on the data presented in Table 4, the linear range of the method is 50 to 100,000 CFU/g. There is a significant correlation between the plate counts and CFU values by DNA probe ($p<0.001$), with a *correlation coefficient is 0.93*.

Limit of Detection: The theoretical LOD based on the data above is 4.7 CFU/g. However, this is reliant upon the amount of sample (0.1g) that can be tested by the spread plate method. Therefore, the *Limit of Detection of the method is 10 CFU/g*.

Limit of Quantitation/ Sensitivity: The limit of quantification/sensitivity is also reliant upon the amount of sample that can be tested. As such, the *Limit of Quantitation of the method is 10 CFU/g*.

Additional Data: *Inclusivity/Exclusivity*. Control filters with the isolates listed below were prepared and tested as outlined above. All isolates. All isolates gave the expected reaction, demonstrating 100% *Inclusivity/Exclusivity*.

Species	**Number of Strains Tested**	**Number *trh*-positive**
V. parahaemolyticus†	43	43
*V. parahaemolyticus**	39	0
V. cholerae	25	0
V. vulnificus	13	0
V. metschnikovii	12	0
V. fluvialis	6	0
Grimontia hollisae	5	0
V. alginolyticus	2	0
Salmonella spp.	20	0
Listeria spp.	20	0
Other non-*Vibrio* species	15	0

† *V. parahaemolyticus* strains previously determined to be *trh*-positive.

***** *V. parahaemolyticus* strains previously determined to be *trh*-negative.

Step-by-step procedure including equipment, reagents and safety requirements necessary to run the method:

1. Special Equipment, Media, and Reagents
   1.1. Special Equipment and Materials Required
      1.1.1.Shaking water bath(s) (42°C and 54°C)

1.1.2. Orbital shaker
1.1.3. Microwave
1.1.4. Plastic tubs with lids (300-500 ml capacity)
1.1.5. Whatman 541 filters, 85mm
1.1.6. Sterile spread rods
1.1.7. Sterile inoculating loops
1.1.8. Sterile toothpicks
1.1.9. Whirl-Pak bags (4.5”x9”)

1.2. Media and Reagents
1.2.1. Alkaline peptone water (APW)
1.2.2. Phosphate buffered saline (PBS)
1.2.3. Thiosulfate citrate bile salts sucrose (TCBS) agar
1.2.4. T_1N_3 agar
1.2.5. Lysis solution
1.2.6. 2M ammonium acetate
1.2.7. 20X SSC and 1X SSC
1.2.8. 1X SSC/SDS
1.2.9. Proteinase K
1.2.10. Hybridization solution
1.2.11. NBT/BCIP tablets
1.2.12. AP-labeled DNA probes (DNA Technology)

2. Outlined Procedure
2.1. Preparation of shellfish
2.1.1. Hands of examiner must be scrubbed thoroughly with soap and potable water; latex or nitrile gloves should be worn while cleaning oysters.
2.1.2. Scrape off growth and loose material from shell, and scrub shell stock with sterile stiff brush under running water.
2.1.3. Place clean shellstock on clean towels or absorbent paper.
2.1.4. Change gloves and brushes between samples.
2.1.5. Protective chain mail glove can be used under a latex glove; outer gloves should be changed between samples.
2.1.6. Tare a sterile blender.
2.1.7. Using a sterile oyster knife, insert the point between the shells on the ventral side, about ¼ the distance from the hinge to the bill; alternately, knife can be inserted after making small opening with sterile bone cutting forceps.
2.1.8. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.
2.1.9. The upper shell can then be pried loose at hinge and discarded.
2.1.10. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.
2.1.11. A minimum of 12 animals or 200g is required.
2.1.12. Blend without adding diluent or with equal weight of diluent (APW or PBS) for 60-120 sec at 14,000 rpm.

2.2. Preparation of spread plates
2.2.1. Prepare 10-fold serial dilutions of shellfish homogenate in PBS
2.2.2. Inoculate 100µl of appropriate dilutions onto pre-dried T_1N_3 agar plates
2.2.3. Spread inoculum gently into agar until completely absorbed

2.2.4. Invert plates and incubate at 30-37°C overnight.

2.2.5. Alternately, this method can be utilized with suspect isolates replicated to T_1N_3 agar from 96 well plates obtained from a standard MPN method

2.3. DNA Probe Colony Hybridization

2.4. Alkaline phosphatase-labeled oligonucleotide probes (AP-*tlh*, AP-*tdh*, and AP-*trh*) can be stored in the refrigerator (4°C) for 1-2 years; do not freeze.

2.5. Filter Preparation

2.5.1. Label #541 Whatman filters with sample number, date, analyst initials, and probe to be hybridized with (*tlh, tdh,* or *trh*). Make sure orientation of filter is noted so that positive spots can be correlated to the appropriate well in the microtiter plate. A dot near the A1 well is sufficient.

2.5.2. Place each filter label-side down on appropriate T_1N_3 plate; apply gentle pressure to ensure contact with each colony. Allow labeled filter to sit at RT for 1-30 min. Transfer each filter with colony-side up to a plastic or glass petri dish lid containing 1 ml of lysis solution. Alternatively, to avoid overheating, a Whatman #3 filter pad can be saturated with 4ml of lysis buffer in the bottom of the petri dish onto which the #541 filter is transferred.

2.5.3. Microwave filters in petri dishes (full power) for 15-20 sec/filter depending on wattage of microwave; rotate dishes with filters and repeat microwaving. Filters should be hot and almost completely dry but not brown.

2.5.4. Transfer filters to a plastic wash container (up to 30 filters can be combined in one container) and neutralize with ammonium acetate (4 ml/filter) for 5 min on shaker at RT.

2.5.5. Decant ammonium acetate and rinse filters 2 times with 1X SSC buffer (10 ml/filter), for 1-2 min each time. (Filters can be air dried and stored at this point.)

2.6. Proteinase K (proK) treatment

2.6.1. Prepare proK solution (this is made by adding 10 ml/filter of 1X SSC and 20 µl/filter of proK stock solution) for the appropriate number of filters. Place filters (up to 30) in plastic wash container of proK solution. Incubate for 30 min in a 42°C water bath with shaking (50 rpm) to destroy naturally occurring alkaline-phosphatase and digest bacterial protein.

2.6.2. Decant proK solution. Rinse filter 3 times in 1X SSC (10 ml/filter) for 10 min at RT with shaking at 50 rpm. (Filters can be air dried by placing on paper towels and stored when completely dry.)

2.7. Hybridization

2.7.1. Place up to 5 proK-treated filters (either dried or straight from treatment) in a Whirl-Pak bag. Add 10 ml of pre-warmed hybridization buffer and close bag to exclude air. Avoid trapping air bubbles. Incubate filters for 30 min at 54°C in a shaking (50 rpm) water bath.

2.7.2. Pour off buffer from bag and add 10 ml fresh pre-warmed buffer/bag. Add probe (final conc. is 0.5 pmol/ml) to bag with filters. Reseal bag, excluding air, and incubate 1 h in a 54°C water bath with shaking. The temperature is critical for hybridization and washing steps.

2.7.3. Remove filters from hybridization bags and place in plastic wash container(s).

2.7.4. Add 10 ml/filter 1X SSC/1% SDS . Incubate in a 54°C water bath with shaking for 10 min. Repeat wash a second time.

2.7.5. Rinse filter 5 times for 5 min each in 1X SSC (10ml/ filter) at RT on an orbital shaker, 100 rpm.

2.8. Color development

2.8.1. In petri dish, add 20 ml of NBT/BCIP solution. Add filters (5 or fewer) to dish and incubate with gentle shaking; cover to omit light. Incubation temperatures from room temperature up to 40˚C can be used; color development will be quicker at higher temperatures. Check development of positive control every hour.

2.8.2. Transfer filters to a plastic wash container and add tap water (10 ml/filter). Rinse filters at RT with shaking for 10 min. Repeat rinse 2 additional times to stop color development. Do not expose filters to light as they will continue to develop. Consider purple or brown spots positive.

Proposal No. **15-114**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Executive Board
Affiliation	Interstate Shellfish Sanitation Conference (ISSC)
Address Line 1	209 Dawson Road
Address Line 2	Suite 1
City, State, Zip	Columbia, SC 29223-1740
Phone	803-788-7559
Fax	803-788-7576
Email	issc@issc.org
Proposal Subject	Pre-Proposal for Male-Specific Coliphage Enumeration in Wastewater by Direct Double-Agar Overlay Method
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas .11 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	The submitter of the pre-proposal requests approval to submit a full proposal to the ISSC for approval of the analytical method for use in the NSSP. Submitted by the developer Kevin Calci (FDA Gulf Coast Seafood Laboratory) Proposed Use of the Method: This method is applicable for the enumeration of MSC wastewater influent, effluent and sewage contaminated surface waters. The method will directly determine the quantity of MSC in wastewater to provide information of the viral reduction efficiencies of wastewater treatment plants. Method is also applicable for the analysis of surface source waters as part of a shoreline survey. Description of Method: This method employs E. coli HS (pFamp) RR as a male-specific coliphage host in a direct double agar overlay for the quantification of plaque forming units. All sample volumes are plated in triplicate. Briefly, 2.5ml of sample is mixed with 2.5ml of soft agar and 0.2ml of Famp host and then poured onto bottom agar petri plate. One ml of the sample is serially diluted down to 1:10 and 1:100. Those two dilutions are then plated by placing 2.5ml of sample is mixed with 2.5ml of soft agar and 0.2ml of Famp host and then poured onto bottom agar petri plate. The plates are incubated at 35-37°C for 16-20 h. Under indirect light the plaque forming units are counted. The working range of the 9 plate method would be 14pfu/1OOml to 1.0 x 106 pfu/1 OOml.
Public Health Significance	Scientific consensus at the MSC informational meeting supported the use of MSC to evaluated wastewater treatment plant viral reduction efficiency to better inform the SSCA's conditional management plans impacted by wastewater treatment plant operations. This method would identify a consistent and accurate measure of MSC load in wastewater influent, effluent and surface waters.
Cost Information	
Action by 2015 Laboratory Methods Review Committee	Recommended referral of Proposal 15-114 to an appropriate committee as determined by the Conference Chair to await SLV data.
Action by 2015 Task Force I	Recommended adoption of 2015 Laboratory Methods Review Committee recommendation on Proposal 15-114.
Action by 2015	Adopted recommendation of Task Force I on Proposal 15-114.

General Assembly	
Action by FDA January 11, 2016	Concurred with Conference action on Proposal 15-114.
Action by 2017 Laboratory Committee	Recommended referral of Proposal 15-114 to an appropriate committee as determined by the Conference Chair.
Action by 2017 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 15-114.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 15-114.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 15-114.

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 ISSC LMR Committee for acceptance will require at a minimum 6 months for review from the date of submission.

Name of the New Method	Male-specific Coliphage for Wastewater
Name of the Method Developer	USFDA Gulf Coast Seafood Laboratory
Developer Contact Information	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36 kevin.calci@fda.hhs.gov

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
Clearly define the need for which the method has been developed.	Y	
What is the intended purpose of the method?	Y	
Is there an acknowledged need for this method in the NSSP?	Y	
What type of method? i.e. chemical, molecular, culture, etc.	Y	Culture method for Male-specific Coliphage in Wastewater Influent, Mid-process Samples, and Effluent

B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	
Method Scope	Y	
References	Y	
Principle	Y	
Any proprietary aspects	N	
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)	Y	
4. Recovery	Y	
5. Specificity	NA	
6. Working and Linear ranges	Y	
7. Limit of detection	Y	
8. Limit of quantitation / Sensitivity	Y	
9. Ruggedness	Y	
10. Matrix effects	N	
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	NA	

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	Date:
Submission of validation data and draft method to committee	Date:
Reviewing members:	
Accepted	Date:
Recommendations for further work	Date:

Comments:

Single Laboratory Validation (SLV) Protocol For Submission to the ISSC For Method Approval

Name of the New Method -	A Culture Method/Double Agar Overlay Method for the Determination of Male-specific Coliphage (MSC) for Wastewater
Name of Method Developer -	Kevin Calci, USFDA-GCSL
Developer Contact Information -	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36 kevin.calci@fda.hhs.gov
Date of Interim Submission -	May 15, 2017

Section A. Need for the New Method

FDA has long been using Male-Specific Coliphage (MSC) to evaluate the potential viral contamination of shellfish growing water by wastewater treatement plant (WTP) outfalls. Methods using MSC as an indicator of viral contamination have been successful in evaluation of viral persistence in molluscan shellfish impacted by WTP outfalls (Daskin et al, 2008)(ISSC MSC Workshop). Studies continue to show a significant inverse relationship between decreasing MSC levels in shellfish and increasing wastewater dilution, which is in turn strongly associated with increasing distance from the WTP disharges (Goblic et al, 2011). The relationship between the level of viral contamination in shellfish and dilution of treated wastewater is really contingent on the viral reduction efficientcy of the WTP impacting the area.

The purpose of this method is to assess the $\log_{10}$ reduction of MSC, as a process indicator for enteric viruses,namely Human Norovirus, in wastewater samples including raw influent, pre-disinfected effluent and final effluent. By comparing $\log_{10}$ values of these results, the viral reduction performance of a WTP can be assessed under different environmental and operational conditions (Amarasiri et al, 2017) (Pouillot et al, 2015). Understanding the viral reduction performance at different stages in a wastewater treatment process is a valuable assessment tool to determine growing area classsification and management options for shellfish growing areas adjacent to and downstream from the WTP outfall. This newly configured FDA method for the determination of MSC in wastewater samples has been adapted from previous methods so that it may be more readily implimented at NSSP Laboratories.

The recognized need for an alternative viral indicator is addressed in detail in the newly accepted 2015 Revision of the NSSP Guide for the Control of Molluscan Shellfish, Section IV Guidance Documents, Chapter II, @ .19, Determining Appropriately Sized Prohibited Areas Associated with Wastewater Treatment Plants, page 292. The need and utility for this method was likewise address at the MSC Informational Meeting of the Growing Area Committee (MSC Summit) in

Charlotte, NC in August 2014. A pre-proposal was reveiwed at the 2015 ISSC Meeting and given the Proposal Number of 15-114. The LMRC agreed that the pre-proposal was sufficient and that there is a need for the method. The LMRC recommended to Task Force I that Proposal 15-114 be referred to an appropriate committee as determined by the Conference Chair and await the SLV data.

Section B. Method Documentation

Modified Double Agar Overlay Method for Determination of Male-specific Coliphage in Wastewater

May 2017 Revision

This method for determining levels of male-specific coliphage in wastewater is based on the method described by Cabelli in work on the Narragansett Bay Project. (Cabelli, 1998) The development of an *Escherichia coli* host cell that consitutively expresses the F plasmid and is resistent to 95% of the somatic phage in wastewater was described in a subsequent paper. (DeBartolomeis and Cabelli,1991) FDA refined the method for oyster and hard clam meats as described in the workshop instructions, *Male-specific Bacteriophage (MSB) Workshop*, conducted in Gloucester, Massachusetts on March 9-12, 2004. (US Food and Drug Administration, 2004) This original FDA (2004) method was submitted as ISSC Proposal 05-114. Spinney Creek Shellfish, Inc. (SCS) further refined these procedures for soft-shelled clams and American Oysters in work funded by the Maine Technology Institute in 2006 with the assistance of Mercuria Cumbo of the Maine Department of Marine Resources. This method was approved for limited use by the 2009 ISSC in Manchester, NH. In work funded by UNH Sea Grant, SLV work continued for species extension to quahogs, which was approved for limited use by the 2013 ISSC in San Antonio, Texas. Method development and preliminary SLV trials were conducted in 2015 by Kevin Calci and Ashley Cooper at USFDA-GCSL. Additional SLV trials were conducted at the Spinney Creek Shellfish Laboratory in collaboration with Kevin Calci, the method developer in work supported by UNH Sea Grant.

A. Apparatus and Materials.

Equipment and Materials for Collection and Transport of Wastewater Samples:
250 or 500 ml Sterile Sample Containers
Sealable Bio-hazard Bags (used when shipping)
Labels
Cooler
Gel Packs
Sampling Device
10% Sodium Thiosulfate Solution (for effluent samples)

Laboratory Equipment:
Water bath, 50-52°C
Air Incubator, 35-37°C
Balance
Stir plate and magnetic stirring bars, sterile
Mini vortexer
Autoclave, 119°C - 121°C
Refrigerator, 0–4° C
Freezer, -20°C

pH meter
Erlenmeyer flasks, 2L and 4L
Graduated cylinders, 1000 ml
500 ml jars, autoclavable with caps
Inoculating loops (3 mm in diameter or 10 □L volume)
Bacti-cinerator or flame
Sterile swabs
Sterile, disposable filters, 0.22 or 0.45µm pore size
Syringes, sterile disposable; 5ml
Serological Pipets- 1 ml, 2 ml, 5 ml, 10 ml
Pipet-aid, or
(Micropipette option; 100 µL and 1000 µL (marked with red tape for positive controls), 200 µL (for aliquots of host cells), 2500 µL (for sample aliquots),
Micropipette tips, sterile 100 µL, 1000 µL, 2500 µL
Micropipette Stand)
Petri dishes, sterile disposable 100 x 15 mm
Petri dish racks
Test tubes 16 x 100 mm (for soft agar)
Dilution tubes, 16 x 150 mm, sterile with screw caps
50ml conical tubes, sterile with screw caps
Test tube racks--sizes to accommodate tubes
Freezer vials, sterile 30 ml with screw caps
Baskets with tops to hold freezer vials
Parafilm tape
Aluminum foil
Counter-pen, digital

Reagents:
Reagent water
Glycerol- sterile
Ethanol, 70% or laboratory disinfectant
Calcium chloride, 1M
Mineral oil
Sodium Thiosulfate (for effluent sample bottles to eliminate chlorine residual)

Antibiotic stocks:
Ampicillin sodium salt (Sigma A9518)
Streptomycin sulfate (Sigma S6501)
Streptomycin and Ampicillin stock solutions (50 µg/ml each).
Note: Antibiotics must always be added to liquids and media after these have been autoclaved and cooled.

Media Components:
Agar, Granulated
Dextrose
NaCL

$CaCl_2$
DI water

Media:
Bottom Agar
DS Soft Agar
Growth Broth

Bacterial Host Strain:
E.coli F_{amp} □ *E. coli* HS(pF_{amp})RR (ATCC # 700891).

MSC (Coliphage) Stock:
Type Strain - MS2, ATCC # 15597

B. Media Composition.

Bottom Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
Agar	15.0 g
DI water	990 ml
Final pH	6.7 ± 0.2 at 25°C

1. With gentle mixing, add all the components, except antibiotics, to 990ml of dH2O in a 1000ml flask (increase flask size to make larger volumes). Dissolve, heat until clear, bringing to a boil.

2. Sterilize at 121°C ± 2°C for 15 minutes.

3. Temper to 50°C in the water bath.

4. Add 5 ml of Streptomycin sulfate/Ampicillin solution, aseptically to the flask (50 µg/ml each in final) and mix. Transfer to 2 – 500ml sterile jars (easier to pour plates from jars).

5. Pour 15-17 ml aliquots aseptically into sterile 100 x 15 mm Petri dishes and allow the agar to harden. Tip Petri dish lids off slightly to reduce condensation.

6. Store bottom agar plates inverted at 4°C and warm to room temperature for 1 hour before use.

7. Plates stored sealed at 4°C can be used up to 3 months.

Streptomycin sulfate/Ampicillin Solution:

1. Dissolve 0.5g of streptomycin sulfate and 0.5g of ampicillin in 50 ml of dH_2O with a sterile 100 ml graduated cylinder in sterile 600 ml beaker with sterile stir bar.

2. Stir for 2 to 3 minutes, no heat.

3. Filter by injecting through a sterile 0.22 µm filter.

4. Store in 5 ml aliquots in sterile 30 ml capped freezer vials at -20°C for up to one year. Label and date.

5. Allow to come to room temperature before adding and mixing in tempered bottom agar at 50°C.

DS Soft Agar:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
1M $CaCl_2$	0.5 ml
Agar	7.0 g
DI water	500 ml
Final pH	6.7 ± 0.2

1. With gentle mixing, add all the components to 500 ml of dH_2O in a 1000 ml flask.

2. Bring flask contents to a boil.

3. Dispense in 2.5 ml aliquots into 16 x 100 ml tubes, cover and freeze (-20°C)

4. Sterilize prior to use at 121°C ± 2°C for 15 minutes, then temper to 50-52°C in a water bath set to 50 ºC ± 2 ºC for no longer than 2 hours.

5. Store up to 3 months at -20 °C.

1M $CaCl_2$ Solution:

1. Add 11.1 g of $CaCl_2$ anhydrous (FW 111.0, Dihydrate FW 147) to 100 ml dH_2O in a screw top bottle and dissolve or use prepared from VWR.

2. Sterilize by autoclaving at 121°C for 15 minutes.

3. Store up to three months at 4°C.

Growth Broth:

Tryptone	10.0 g
Dextrose	1.0 g
NaCl	5.0 g
DI water	1000 ml

1. With gentle mixing, add all the components to 1000 ml of dH_2O water in a 2000 ml flask.

2. Dissolve and dispense into sterile screw top containers.

3. Sterilize at 121°C ± 2°C for 15 minutes.

4. Store for up to three months at 4°C.

C. Storage and Propagation of Host Strain, E. coli F_{amp}:

Storage:

1. Lab stock culture – Frozen at – 80°C indefinitely (most desirable method) in broth culture containing 10% glycerol under no selective pressure. Selective pressure is reapplied when the culture is retrieved, by streaking onto Bottom Agar plates containing the two antibiotics.

2. Long-term working stock culture – Grown tryptic soy agar slant with sterile mineral oil overlay under no selective pressure and stored at room temperature in the dark for up to 2 years.

3 Long-term working stock – 6-hour grown tryptic soy agar slant and deep stab with sterile mineral oil overlay containing the two antibiotics, Ampicillin and Streptomycin (least desirable method).

4. Short-term working stock culture - Grown Bottom Agar streak plate stored at 4°C up to 3 weeks.

Glycerol Solution, 10%:

1. Add 9 ml of distilled water to 1 ml of undiluted glycerol.

2. Autoclave resulting 10% glycerol solution at 121°C for 15 minutes and use at room temperature.

3. For storage, add 1/5th volume of 10% glycerol solution, let stand for 30 minutes, dispense 1 ml aliquots in 2 ml cryo-vials and store at -70 to –80°C (best) or at –20°C.

D. Control Plates.

1. Negative Control - Add 2.5 ml of Growth Broth and 0.2 ml host to the 2.5 ml DS Soft Agar tube.

2. Positive Control - Make serial dilutions using growth broth of the concentrated MS2 control (to grow approximately 50-100 PFU per 2.5 ml), and add 2.5 ml of appropriate MS2 dilution and 0.2 ml of host to 2.5 ml DS Soft agar.

E. MSC Density Determinations in wastewater Treatment Plant (WWTP) Samples.

Sample Requirements:

1. Sterile 250 or 500 ml Nalgene bottles (or comparable bottle) with a permanent fill mark at the approximate 200 or 400 ml level are recommended for wastewater samples including influent, pre-treated effluent, and effluent.

2. Sample collection bottles must be properly labeled with sample number, location, sample type, date and time.

3. Sample bottles are filled to the 200 or 400 ml line. Effluent sample bottles must contain 1.0 ml of 10% sodium thiosulfate solution for 200 ml or 2.0 ml of 10% sodium thiosulfate solution to for 400 ml to inactivate any residual chlorine.

4. Wastewater samples are held under refrigerated conditions at 1-4ºC.

Note - A sealed bio-hazard bag is recommended for the samples bottles containing sewage samples going into an insulated shipping box when using overnight carriers.

Propagation of Host Cells:

1. Allow grown Bottom Agar streak plate and Growth Broth to temper to room temperature.

2. Vortex to aerate 20 ml of Growth Broth in a 16 x 150 mm tube, with screw cap.

3. Transfer host strain to Growth Broth using sterile swab to collect material from three colonies off grown Bottom Agar streak plate.

4. Gently shake to mix, then incubate at 35–37°C for 4-6 hours.

5. Once turbidity is observed, use of the host strain broth culture (log-phased growth) may commence.

Note - Following initial inoculation and mixing, do not shake or mix the host strain broth culture (to avoid mixing of cell debris at bottom with log-phase E. coli with pili)

Preparation of Wastewater samples for Analysis:

1. Analyst must wear gloves during handling of stir bars and sample bottle.

2. Water samples are removed from 1 - 4º C.

3. Sample bottle is shaken vigorously for 20 seconds (ensure cap is tightened), and a sterile magnetic stir bar is aseptically transferred to bottle.

4. Sample bottle is placed on stir plate set to medium for five minutes prior to analysis.

5. For the **high range** of this method a 10^{-2} decimal dilution is prepared by transferring 1ml of sample with a sterile 2 ml pipette (using a pipette aid) to a sterile 16x150mm screw cap tube containing 9 ml of growth broth. Sample tube is then vortexed for 10 seconds. For the second decimal dilution, 2ml are transferred from the first tube to a sterile 50ml conical tube with cap containing 18ml of growth broth using a second sterile 2ml pipette. The appropriately labeled 50 ml conical tube is then vortexed for 10 seconds.

6. For the **low range** of this method, 30ml of sample is transferred to a sterile 50 mm conical tube with cap using a sterile 10 ml pipette. The appropriately labeled 50 ml conical tube is vortexed for 10 seconds.

7. Prepped samples in labeled 50ml conical tube are stored in a test tube rack which can be stored short term at 0-4°C.

8. Return sample bottles to refrigeration and clean the work surface with disinfectant.

Note: The samples bottles containing wastewater samples should be autoclaved prior to disposal. Sample bottles must be washed and sterilized for re-use.

Direct Analytical Technique for WWTP samples:

This MSC method for wastewater has both a **high range** and a **low range** routine. Combined, the working range is from 5 to 1,200,000 PFU/100ml. The **high range** routine is adequate for enumeration of MSC in WWTP influent and has a working range from 1,000 to 1,200,000 PFU/100ml. The **low range** routine is generally adequate for enumeration of MSC in final effluent and has a working range from 5 to 12,000 PFU/100ml. When testing for pre-treatment effluent (before disinfection) or at times when the effluent is questionable, both high and low ranges routines should be used together.

1. In the morning, propagate host cells as described above.

2. Tubes may be inoculated on a staggered time schedule:

Tubes in incubator at 7:00am	Ready at 11:00am
Tubes in incubator at 8:00am	Ready at 12:00pm
Tubes in incubator at 10:00am	Ready at 2:00pm
Tubes in incubator at 11:00am	Ready at 3:00pm

3. Before experimentation, prepare the wastewater samples for analysis as described above.

4. One hour before experimentation (at 3 hours of host growth), autoclave required number of soft agar tubes at 121ºC for 15min. Temper soft agar tubes in water bath set to 50-52ºC.

High Range Routine:

For each high range (influent) sample, four (4) Bottom Agar plates and four (4) 2.5 ml DS Soft Agar tubes are prepared. Always begin the day's analyses with a negative control (blank) plate and finish analyses with a positive control plate and a negative control plate.

1. Allow prepared samples (50ml conical tubes, racked and labeled) to warm to room temperature immediately before analysis (20-30 minutes)

2. Remove tubes containing appropriate timed host cells from incubator immediately before analysis.

3. Vortex sample in 50ml conical tube for 10 seconds.

4. Moving quickly and smoothly, gently pipette 200µL of host cells into each of 4 soft agar tubes using a 1 ml serological pipet or 200µL micropipette with sterile tip.

5. Immediately thereafter, pipette 2500µL aliquot of sample into each of the 4 soft tubes using a 10 ml serological pipet and pipet aid or 2500µL micropipette with sterile tip.

6. Mix sample, host and soft agar by gently rolling test tube between hands.

Note: *Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.*

7. Then overlay the contents of the soft agar tubes onto a Bottom Agar plates. Drag the mixture into a clear area and gently swirl the plates to spread sample and agar

mixture.

8. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

9. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 – 20 hours of incubation are counted as plaques. PFUs on each plate are counted and totaled using a digital counter pen and adequate light

10. Calculations of **High Range** Routine Results;

N = Total number of PFUs counted on 4 the plates,

The maximum readable limit on PFUs count is 1000 on the four plate,

PFU count exceeding 1,000/4 plate is considered TNTC or >1,000,000 PFU/100gm

$$\text{Result} = \frac{(\text{N PFUs}) * 100}{.1 \text{ ml}} = \text{N} * 1{,}000 \text{ PFU/100ml}$$

Example: High range version plate counts - 13, 23, 12, and 16 PFUs

Result = (64)(1000) = 64,000 PFU/100ml*

Low Range Routine:

For each low range (effluent) sample, eight (8) Bottom Agar plates and eight (8) 2.5 ml DS Soft Agar tubes are prepared. Always begin the day's analyses with a negative control (blank) plate and finish analyses with a positive control plate and a negative control plate.

1. Allow prepared samples (50ml conical tubes, racked and labeled) to warm to room temperature immediately before analysis (20-30 minutes)

2. Remove tubes containing appropriate timed host cells from incubator immediately before analysis.

3. Vortex sample in 50ml conical tube for 10 seconds.

4. Moving quickly and smoothly, gently pipette 200µL of host cells into each of 8 soft agar tubes using a 1 ml serological pipet or 200µL micropipette with sterile tip.

5. Immediately thereafter, pipette 2500µL aliquot of sample into each of the 8 soft tubes using a 10 ml serological pipet and pipet aid or 2500µL micropipette with sterile tip.

6. Mix sample, host and soft agar by gently rolling test tube between hands.

Note: *Once E. coli F_{amp} is added to the mixture do not shake, only gently mix contents by rolling the tube between palms.*

7. Then overlay the contents of the soft agar tubes onto a Bottom Agar plates. Drag the mixture into a clear area and gently swirl the plates to spread sample and agar mixture.

8. Allow plates to set then inverted and incubated for 16 - 20 hours at 35- 37°C.

9. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16 – 20 hours of incubation are counted as plaques. PFUs on each plate are counted and totaled using a digital counter pen and adequate light

10. Calculations of **Low Range** Routine Results;

N = Total number of Plaque forming units (PFUs) counted on 8 the plates,

The maximum readable limit on PFUs count is 2000 on the eight plate,

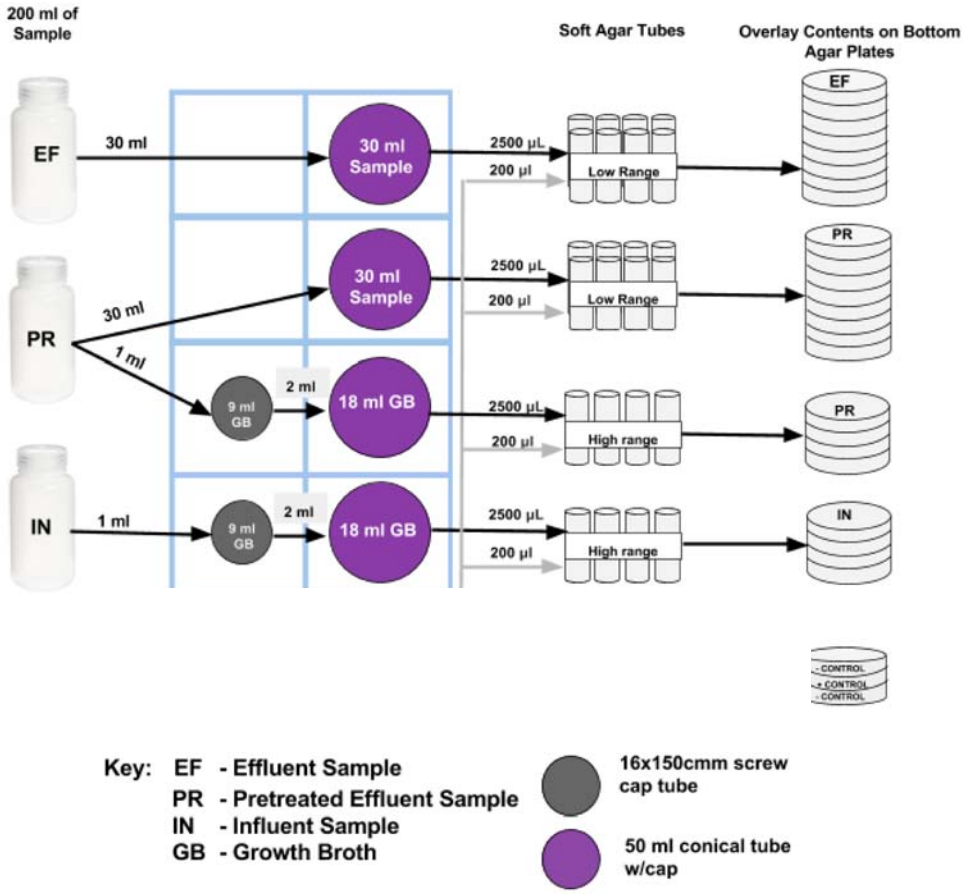
PFU count exceeding 2,000/8 plates is considered TNTC or >10,000 PFU/100gm

$$\text{Result} = \frac{(\text{N PFUs}) * 100}{20 \text{ ml}} = \text{N} * 5 \text{ PFU/100ml}$$

Example: High range version plate counts - 21, 17, 20, 19, 13, 23, 12, and 16 PFUs

Result = (141)(5) = 702 PFU/100ml. Rounding off result to nearest 10s... Result = 700PFU/100gm*

MSC Method for Wastewater Schematic:



Samples Bench Sheet:

Sample Bench Sheet
For use with the NSSP Method for Determination of MSC in Wastewater
2016 Edition

Initiation Analysts: Jerry Jone Date: 10/10/16 Time: 1:00PM
Completing Analysts: Bill Bellicheck Date: 10/11/16 Time: 8:00AM

Low Range Routine

Sample#:1EF	WWTP: Hopetown, NE				Type: Effluent					Date/Time:	10/9/16
PFU Counts	3	2	0	1	0	2	0	1	=	9	45 PFU/100ml

Sample#:3Pre	WWTP: Hopetown, NE				Type: Pre-treatment					Date/Time:	10/9/16
PFU Counts	199	215	203	170	233	210	206	188	=	1,624	8,120 PFU/100ml

Sample#:	WWTP:				Type:					Date/Time:	
PFU Counts									=		

Hign Range Routine

Sample#:2In	WWTP: Hopetown, NE				Type: Influent		Date/Time:	10/9/16
PFU Counts	171	193	201	177	= 742			177,000 PFU/100ml

Sample#:	WWTP: Hopetown, NE				Type: Pre-treatment		Date/Time:	10/9/16
PFU Counts	3	1	2	0	= 6			6,000 PFU/100ml

Sample#:	WWTP:				Type:		Date/Time:	
PFU Counts					=			

For unknown or mid-range sample, run both rountines above on the sample

Controls:	Start Neg Contol:	count	End Pos Control:	count
		0	43	
			End Neg Control:	0

F. Sample Collection and Storage.

1. Record all pertinent information on the collection form.

2. During transportation store samples in a cooler at 0 to 10°C

3. At laboratory, store samples in a refrigerator at 0 to 4 °C.

4. Maximum holding times for wastewater samples is up to 72 hours.

G. Quality Assurance.

1. Positive and negative control plates are run with MSC analyses each day.

2. Media sterility checks are made per batch and records are maintained.

3. Media log book is maintained (pH, volume, weights of each components, lot numbers, etc.).

4. An intra- and inter-laboratory performance program is developed.

5. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 16- 20 hours of incubation are counted as plaques. (Count the number of plaques on each plate.)

6. MSC determinations are reported as plaque forming unit (PFU) per 100 grams.

7. The desired range for counting is 0 to 300 PFU per plate. If the count exceeds the upper range or if the plaques are not discrete, results should be recorded as "too numerous to count" (TNTC) or >12,000 PFU/100ml for **Low Range** Routine and >1,200,000 PFU/100ml for **High Range** Routine.

8. Temperatures incubators are checked twice daily (at least 4 hours apart) to ensure operation within the stated limits of the method, and results are recorded in a logbook.

9. Check thermometers at least annually against a NIST-certified thermometer.

10. Calibrate the balance monthly using ASTM-certified Class 1 or 2 or NIST Class S reference weights.

11. Laboratory analysts adhere to all applicable quality control requirements set forth in the most recent version of FDA's *Shellfish Laboratory Evaluation Checklist*.

12. Calibration of micropipettes needs to be checked quarterly and records kept. Micropipettes used for handling MSC control and transferring host cells need to have a barrier tip or be dedicated to the specific use to prevent contamination

H. Safety.

Samples, reference materials, and equipment known or suspected to have sewage, coliphage and/or *E.coli* attached or contained must be sterilized prior to disposal.

I. Technical Terms.

°C	-	degrees Celsius
µL	-	microliter
g	-	gram
L	-	liter
M	-	molar
ml	-	milliliter
Ave.	-	average
MSC	-	Male-specific Coliphage, Male-specific Bacteriophage, F+ Bacteriophage
NIST	-	National Institute of Standards and Technology
PFU	-	plaque forming units
RT	-	room temperature
TNTC	-	too numerous to count

Host Strain:	*E.coli* F_{amp} bacteria (*E.coli* HS(pFamp)RR)
Male-specific Coliphage:	Viruses that infect coliform bacteria only via the F-pili.
Plaque :	Clear circular zones (typically 1 to 10 mm in diameter) in lawn of host cells after incubation.

References:

Amarasiri, M., M. Kitajima, T.H. Nguyen, S. Okabe, and D. Sano. Bacteriophage removal efficiency as a validation and operational monitoring tool for virus reduction in wastewater raclamation: Review. Water Research 121 (2017) 258-269.

Cabelli, V.J. 1988. Microbial indicator levels in shellfish, water, and sediments from the upper Narragansett Bay conditional shellfish-growing area. Report to the Narragansett Bay Project, Providence, RI.

Daskin, J. H., K.R. Calci, W. Burkhardt III, and R.H. Carmichael. Use of N stable isotope and microbial analyses to define wastewater influence in Mobile Bay, AL. Marine Pollution Bullentin 56 (2008) 860-868.

DeBartolomeis, J. and V.J. Cabelli. 1991. Evaluation of an *Escherichia coli* host strain for enumeration of F male-specific Coliphages. Appl. Environ. Microbiol. 57(4):1201-1205.

Goblic, G.N., J.M. Anbarcian, J. Woods, W. Burkhardt III, and K.R. Calci. Evaluating the dilution of wastewater treatment plant effluent and viral impacts on shellfish growing areas in Mobile Bay, Alabama. Journal of Shellfish Research. 30:3, 1-9, 2011.

Pouillot, R., J.M. van Doren, , J. Woods, , D. Plante, M. Smith, G. Goblick, C. Roberts, A. Locas, W. Hajen, J. Stobo, J. White, J. Holtzman, E. Buenaventura, W. Burkhardt III, A. Catford, R. Edwards, A. DePaola, and K.R. Calci, 2015. Meta-analysis of the reduction of norovirus and male-specific coliphage concentrations in wastewater treatment plants. Appl. Environ. Microbiol. 81, 4669-4681.

U.S. Food and Drug Administration. 2004. Male-specific Coliphage (MSC) Workshop, conducted in Gloucester, Massachusetts on March 9-12, 2004.

Other Information:

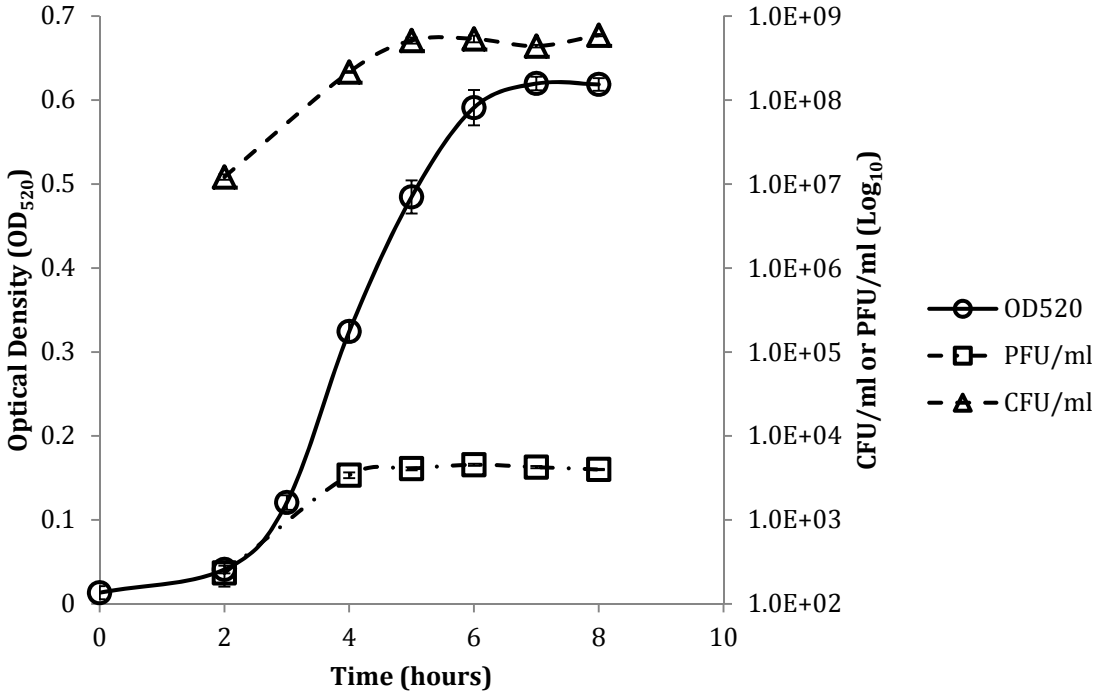
This method for the enumeration of male-specific coliphage in wastewater samples is inexpensive, easy to perform, and rapid, providing results within 24 hours. The cost of laboratory glassware, plastic-ware, agars, and reagents is approximately $18 per series of samples (an influent, a pre-disinfection effluent, and a final effluent sample). In a well set-up laboratory, the method requires 6 hours of time from initiating host to pouring plates. Hands on technician time to perform this test is significantly less on the order of 1-4 hours per test depending upon how many tests are done per day. There are no special skill sets required beyond those required to operate a state-approved shellfish laboratory under the NSSP.

To standardize these assessments, an index of viral performance for use in the NSSP to determine classification options adjacent to WWTP outfall can be estimated. Subtracting the log value of final effluent score from the log value of the corresponding raw influent score will yield an index of viral performance ranging from 0 to 5. A viral index of 4 to 5 indicates 99.99% to 99.999% reduction of enteric viruses and would be consider high performance. A viral index of 1 to 2 indicates 90% to 95% reduction of enteric viruses through the treatment process and would be considered poor performance. A viral index <1 would be considered ineffective and should lead the SSCA to consider 10,000:1 rather than 1000:1 for the determination of the size of the prohibited zone adjacent to the outfall.

C. Validation Criteria

Determination of Optimal Optical Density of Famp Host (OD)

Procedures for enumeration of double-agar overlay method for male-specific coliphage provide different ranges for OD of host growth. Effort was taken to determine the working range of the host *E. coli* at 520nm, which is the current EPA measurement. Graph 1 shows that at approximately 4 to 6 hours growth the OD520 of host is in the range of 0.35 to 0.6, during which time the MS2 plaquing efficiency of the host *E. coli* is optimal and consistent. Therefore, we conclude that a host OD520 of 0.35 to 0.7, or approximately 4 to 6 hours of growth, is ideal for MSC enumeration.



Graph 1. Optical Density (OD520) of *E. coli* HS(pFamp)RR in tryptone broth compared to plaque forming units (PFU) of MS2 coliphage. 10 ml of tryptone broth was inoculated with *E. coli* and incubated at 35ºC. OD was measured every hour starting at t = 2 hours. At these intervals 100 µL of host was serially diluted and 100 µL of pre-determined dilutions were plated to determine CFU/mL. 200 µL of the same host sample was used to determine PFU/ml of stock MS2 controls.

The Determination of LOD, LOQ, and Linear Range using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

The SOP for the determination of LOQ, LOD, and the Linear Range is the most robust of the SOPs and yields a database from which subsets of data can be use to generate other validation criteria. For this database ten trials were run using clean effluent samples. Great effort was taken to find and verify clean effluent. The Dover, NH WWTP and the Hampton, NH WWTP were identified as high performing plants of different design capable of consistently producing clean effluent. Table 1 list the metadata for the effluent samples collected for these trials

Table 1. Effluent Samples used for the Determination of LOD, LOQ, Linear Range

Trial #	Date Sampled	WWTP	Treatment Process
1	4/11/17	Dover	Tertiary, UV Dis-infection
2	4/11/17	Hampton	Secondary, Chlorine
3	4/18/17	Dover	Tertiary, UV Dis-infection
4	4/18/17	Hampton	Secondary, Chlorine
5	4/24/17	Dover	Tertiary, UV Dis-infection
6	4/24/17	Hampton	Secondary, Chlorine
7	5/1/17	Dover	Tertiary, UV Dis-infection
8	5/1/17	Hampton	Secondary, Chlorine
9	5/8/17	Dover	Tertiary, UV Dis-infection
10	5/8/17	Hampton	Secondary, Chlorine

For each of the 10 validation trials, 150 ml of clean effluent sample was aseptically transferred into 5-200ml sterile dilution bottles. A master spike solution was prepared in growth broth and was varied in concentration during the trials. The master spike solution was on the order of 10^3 MSC/ml. Four subsequent serial dilutions were made for each trial from the master spike at a 3:1 dilutions. This represented different spike concentrations over the working range of the method. The 5 dilution bottles were asceptically spiked with 5 ml of spike concentration 1 through 5, shaken vigourously and then 4 aliquots of 30 ml were transferred into 4-50 ml sterile conical tubes for each spike concentration (3 conical tubes for the replicates and a 4th tube for spike determination). In this way, 3 true replicates were generated at each of the 5 spike concentrations. This methodology was consistently applied throughout the ten trials. The 5 sets of 3 aliquots were processed and plated according to the method description above. Clean effluent was used instead of growth broth for the spike determination. Spike determinations using growth broth were underestimating the sample results. This problem was solved by using the same clean effluent similiarly spiked. This strategy was employed as there is no standard method available for a truly independant spike determinations.

Table 2 below shows the Spiking Study Database for MSC Method in Wastewater SLV. Spike Concentrations and MSC replicate plate count results are in units of PFU of MSC/100ml.

Table 2. Spiking Study Database for MSC Method in Wastewater SLV

WW Spiking Database 8 plate -

Date	X Value Measured Spike (PFU/100ml)	Repicate Plates (PFU/100ml)	Log of plates	Y Value RSD	Log RSD	X Value log of spike
	3400	4105	3.613	0.0025	-2.603	3.531
		4140	3.617			
		3980	3.600			
	1050	1025	3.011	0.0134	-1.874	3.021
		1225	3.088			
		1180	3.072			
Trial 1						
Dover	315	245	2.389	0.0352	-1.453	2.498
4/11/17		330	2.519			
		360	2.556			
	60	75	1.875	0.0629	-1.202	1.778
		60	1.778			
		45	1.653			
	10	30	1.477	0.2090	-0.680	1.000
		10	1.000			
		30	1.477			

	5950	5490	3.740	0.0046	-2.340	3.775
		5110	3.708			
		5155	3.712			
	1515	1355	3.132	0.0051	-2.289	3.180
		1365	3.135			
		1450	3.161			
Trial 2						
Hampton	410	225	2.352	0.0304	-1.517	2.613
4/11/17		225	2.352			
		170	2.230			
	70	65	1.813	0.0204	-1.690	1.845
		55	1.740			
		60	1.778			
	25	25	1.398	0.0321	-1.494	1.398
		30	1.477			
		25	1.398			

	5135	4315	3.635	0.0063	-2.199	3.711
		4800	3.681			
		4550	3.658			
	980	1085	3.035	0.0139	-1.856	2.991
		1005	3.002			
Trial 3		1220	3.086			
Dover						
4/18/17	405	385	2.585	0.0241	-1.618	2.607
		315	2.498			
		415	2.618			
	75	65	1.813	0.0379	-1.422	1.875
		90	1.954			
		80	1.903			
	25	20	1.301	0.1072	-0.970	1.398
		25	1.398			
		40	1.602			

	5175	4925	3.692	0.0065	-2.189	3.714
		5300	3.724			
		5490	3.740			
	1130	1280	3.107	0.0103	-1.986	3.053
		1160	3.064			
Trial 4		1340	3.127			
Hampton						
4/18/17	355	280	2.447	0.0317	-1.499	2.550
		335	2.525			
		405	2.607			
	40	60	1.778	0.0590	-1.229	1.602
		100	2.000			
		75	1.875			
	20	25	1.398	0.0634	-1.198	1.653
		30	1.477			
		20	1.301			

	11575	10655	4.028	0.0128	-1.891	4.064
		12800	4.107			
		10220	4.009			
	2080	2025	3.306	0.0212	-1.674	3.318
		2650	3.423			
		2735	3.437			
Trial 5						
Dover	525	680	2.833	0.0360	-1.444	2.720
4/24/17		705	2.848			
		465	2.667			
	190	205	2.312	0.0179	-1.746	2.279
		185	2.267			
		170	2.230			
	20	45	1.653	0.2069	-0.684	1.301
		60	1.778			
		15	1.176			

	12210	11140	4.047	0.0076	-2.121	4.087
		12165	4.085			
		10580	4.024			
	2555	2720	3.435	0.0058	-2.239	3.407
		2510	3.400			
		2520	3.401			
Trial 6						
Hampton	495	555	2.744	0.0395	-1.403	2.695
4/24/17		350	2.544			
		395	2.597			
	85	90	1.954	0.1082	-0.966	1.929
		110	2.041			
		45	1.653			
	20	20	1.301	0.1396	-0.855	1.301
		35	1.544			
		15	1.176			

	4430	3530	3.548	0.0131	-1.882	3.646
		4370	3.640			
		4075	3.610			
	1035	1100	3.041	0.0256	-1.592	3.015
		780	2.892			
		880	2.944			
Trial 7	240	275	2.439	0.0188	-1.727	2.380
Dover		230	2.362			
5/1/17		230	2.362			
	65	80	1.903	0.1126	-0.949	1.813
		75	1.875			
		35	1.544			
	10	20	1.301	0.1305	-0.884	1.000
		15	1.176			
		10	1.000			

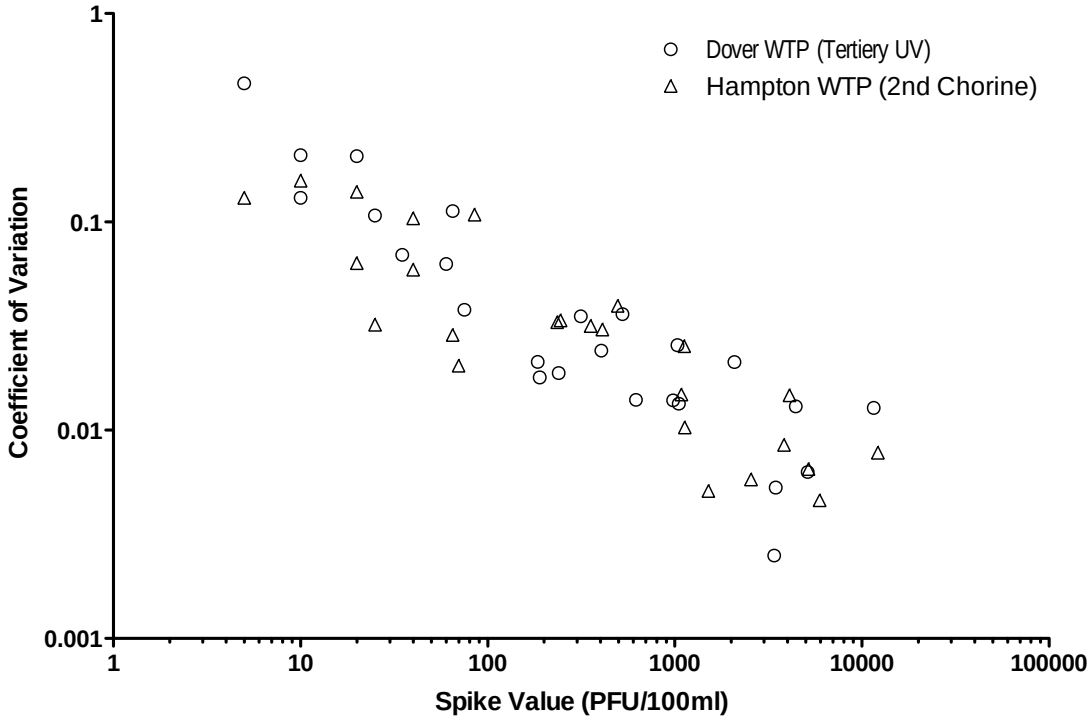
	4110	4415	3.645	0.0147	-1.833	3.614
		5630	3.751			
		5260	3.721			
	1125	955	2.980	0.0253	-1.596	3.051
		1060	3.025			
		1350	3.130			
Trial 8	245	315	2.498	0.0336	-1.474	2.389
Hampton		450	2.653			
5/1/17		325	2.512			
	40	35	1.544	0.1040	-0.983	1.602
		55	1.740			
		80	1.903			
	10	10	1.000	0.1580	-0.801	1.000
		10	1.000			
		20	1.301			

	3460	2765	3.442	0.0053	-2.273	3.539
		2940	3.468			
		3000	3.477			
	620	605	2.782	0.0140	-1.853	2.792
		725	2.860			
		650	2.813			
Trial 9						
Dover	185	210	2.322	0.0213	-1.672	2.267
5/8/17		175	2.243			
		215	2.332			
	35	35	1.544	0.0694	-1.158	1.544
		25	1.398			
		40	1.602			
	5	10	1.000	0.3618	-0.442	0.699
		5	0.699			
		3	0.477			

	3840	3490	3.543	0.0085	-2.073	3.584
		3675	3.565			
		4005	3.603			
	1085	825	2.916	0.0148	-1.831	3.035
		710	2.851			
Trial 10		855	2.932			
Hampton						
5/8/17	235	175	2.243	0.0330	-1.482	2.371
		135	2.130			
		185	2.267			
	65	60	1.778	0.0386	-1.413	1.813
		55	1.740			
		75	1.875			
	5	10	1.000	0.1305	-0.884	0.699
		15	1.176			
		20	1.301			

The replicate plate count results were log transformed and the relative standard deviation (RSD) were calculated. The RSD or coefficient of variation was plotted against the spike concentration and appears in Graph 2 below.

Graph 2. Coefficient of Variation verses Spike Concentration for Clean Effluent



To accurately determine the LOD and LOQ graphically, it was necessary to take the Coefficient of Variation and the Spike Determinations and to re-plot these as log values. Graph 3 below show the linear regression of the log transformed replicate and spiking data. Graphically, the LOQ/sensitivity of the method may be found at the point of intersection of the log spike concentration and the log coefficient of variation of –1.0 (or its antilog, 10%). The LOD may be found at the point of intersection of the log spike concentration and the log coefficient of variation of –0.477 (or its antilog of, 33%). Taking the antilog of the spike concentrations at these points of intersection gives the LOQ and LOD, respectively. Graph 3 indicates the LOQ and LOD for clean effluent to be 5.8 PFU/100gm and 0.9 PFU/100ml, respectively. The biostatistics program Prism 5.0 for Mac OS was used linear regression analysis and plots. The statistical summary of the linear regression from the log coefficient of variation verses log spike for the clean effluent data is presented in Table 3.

Graph 3. The LOD and LOQ/Sensitivity for Clean Effluent Samples

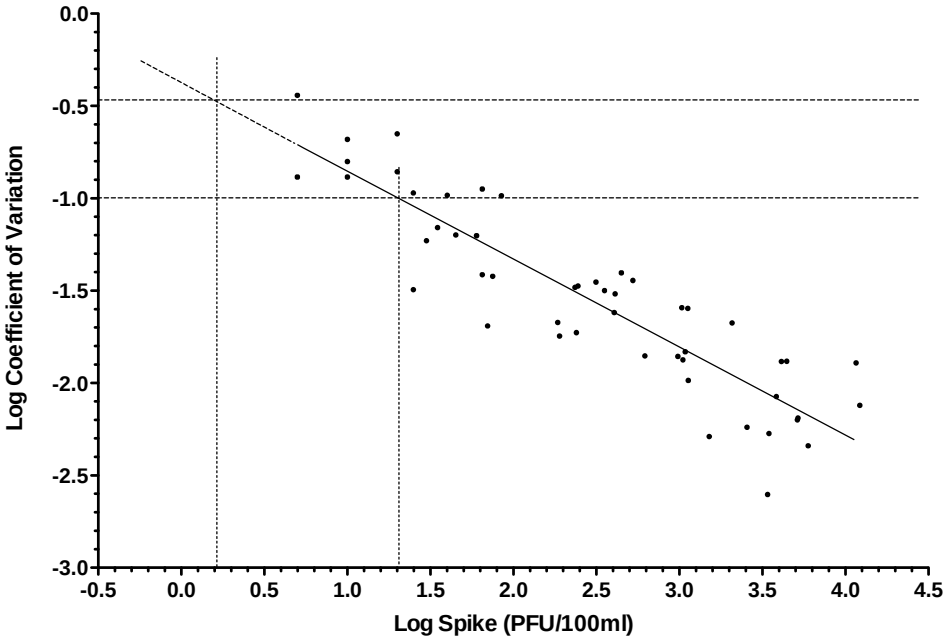


Table 3. Linear Regression Statistics for the Effluent Spiking Trials

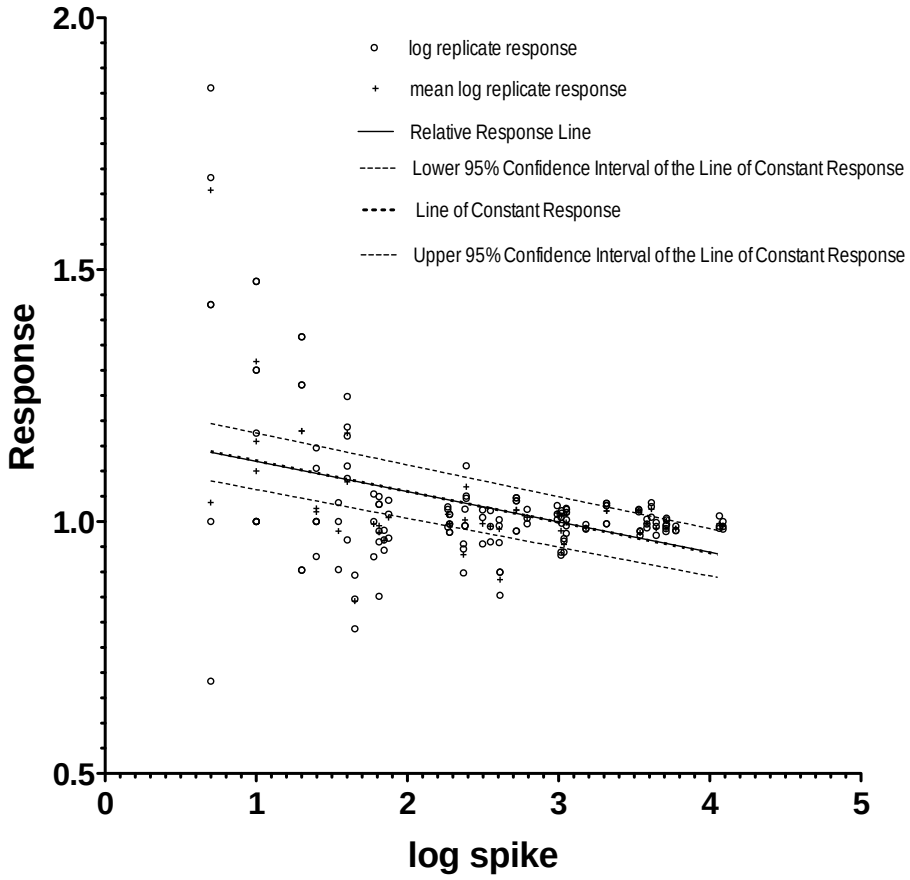
Best-fit values	
Slope	-0.4767 ± 0.03387
Y-intercept when X=0.0	-0.3746 ± 0.08890
X-intercept when Y=0.0	-0.7859
1/slope	-2.098
95% Confidence Intervals	
Slope	-0.5449 to -0.4085
Y-intercept when X=0.0	-0.5536 to -0.1957
X-intercept when Y=0.0	-1.345 to -0.3618
Goodness of Fit	
R square	0.8049
Sy.x	0.2244
Is slope significantly non-zero?	
F	198.1
DFn, DFd	1.000, 48.00
P value	< 0.0001
Deviation from zero?	Significant
Data	
Number of X values	50
Maximum number of Y replicates	1
Total number of values	50
Number of missing values	0

LOQ = Antilog [-2.098 (-1.0 + 0.375)] = 20.42 PFU/100ml
LOD = Antilog [-2.098 (-0.477 + 0.375)] = 1.63 PFU/100ml

The correlation coefficient (R square value) of this linear regression is 0.8049 which is above the threshold level of 0.64 and indicates a good fit. The LOD and LOQ as determined by the spiking trials shows LOD and LOQ of 1.63 PFU/100ml and 20.42 PFU/100ml, respectively. Rounding up, the LOD and LOQ are 2 PFU/100ml and 21 PFU/100ml, respectively.

To determine the **Linear Range**, data from Table 2 was manipulated to construct the relative response line, the line of constant response and the upper and lower 95% confidence interval bracketing the line of constant response as instructed in the SOP. Graph 4 below show that the upper (1.05) and the lower (.95) 95% confidence interval estimates are essentially parallel to the Relative Response line. This suggests that the method is linear through the working range of 5 PFU/100ml to 12,000 PFU/100ml.

Graph 4. Linear Range Determination



Data Summary:

Linear range of the method as implemented 5 to 12,000 PFU/100ml
The limit of detection of the method as implemented 2 PFU/100ml
The limit of quantitation/sensitivity of the method as implemented 21 PFU/100ml
Indeterminates <2 PFU/100ml to >12,000 PFU/100ml

The Determination of Accuracy/Trueness and Measurement Uncertainty is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust databases acquired from the determination of the LOQ/LOD/Linear Range. The Accuracy/Trueness is calculated by dividing the log average of the plates by the log average of the spike concentrations, then multiplying the result by 100 to get a percent value. Table 4 shows the results for the Accuracy/Trueness of the method.

Table 4. Calculation of the Accuracy/Trueness of the MSC Method for Wastewater (Low Range Routine).

Average log of plates (2.473 PFU/100ml)/Average log of spike (2.455 PFU/100ml)
= Accuracy/Trueness of 100.7 %.

The Measurement Uncertainty is determined by subtracting the log mean replicate plate values from the reference or log spike values, then calculating the 95% confidence limits of the mean difference. Table 5 show the results of statistical analyis for Method Uncertainty.

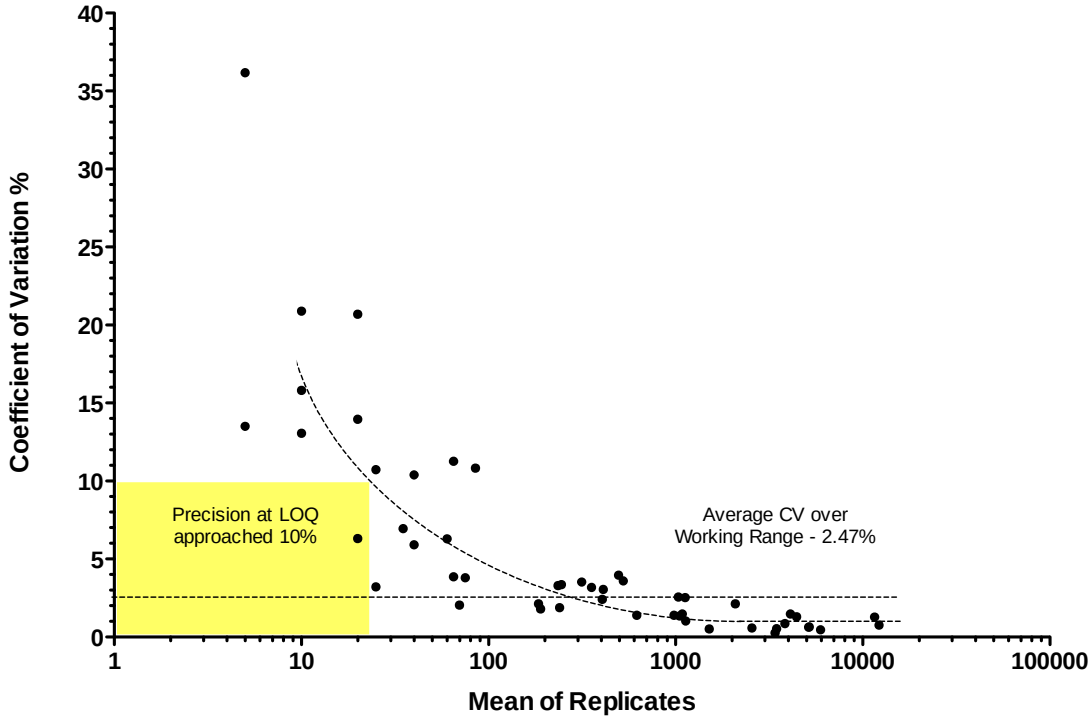
Table 5 – Measurement Uncertainty in wastewater using low range routine.

		Antilog
Number of values	150	
Mean	-0.01787	**0.960**
Std. Deviation	0.1566	
Std. Error	0.01278	
Lower 95% CI of mean	-0.04314	**0.905**
Upper 95% CI of mean	0.00739	**1.017**

Data Summary: Wastewater using Low Range Routine
Calculated % accuracy/trueness 100.7 %
Calculated measurement uncertainty 0.905 to 1.017

The Determination of the Precision and Recovery is based upon the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods using the more robust data set acquired from the determination of the LOQ/LOD/Linear Range. To examine the precision over the working range of the method, a simple graphical approach was followed. The coefficients of variation were determined from the log transformed replicate data (50 sets of three true replicates) and were plotted verses the mean of the triplicate results (non log transformed data). The results are shown in Graph 5 for effluent using the low range routine.

Graph 5 - Coefficient of Variability (%) of Replicate verses Mean of Replicate for Effluent Samples using the Low Range Routine of the Method.



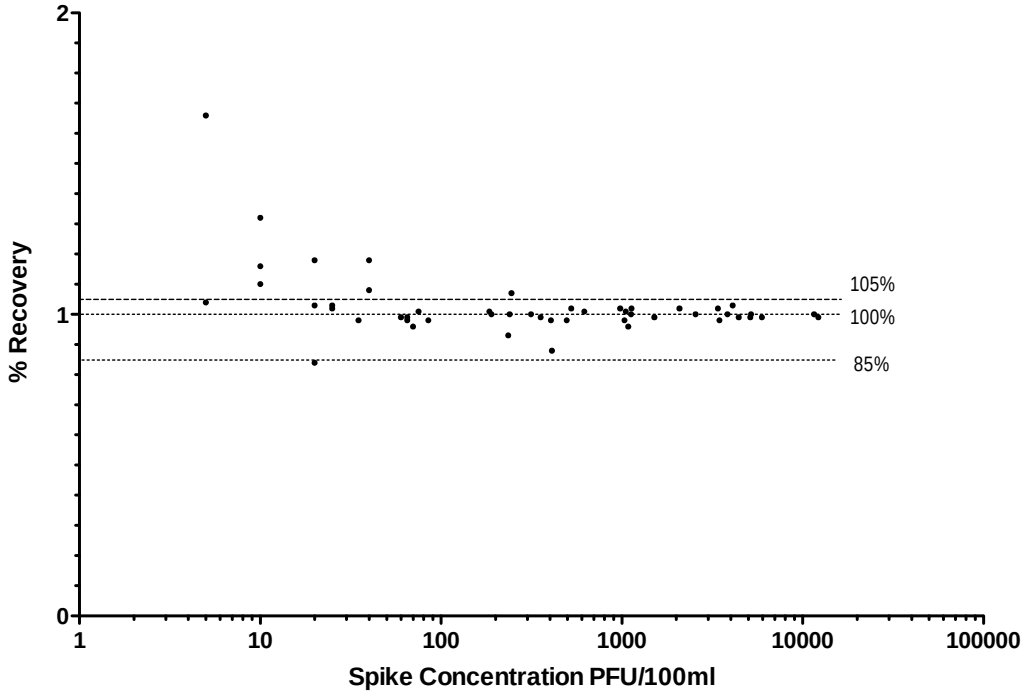
In Graph 4 above, the precision decreases as the LOQ and LOD are approached. The mean, minimum, and maximum coefficient of variations as determined over the working range for effluent samples appear in Table 6 below.

Table 6 – Mean, Minimum, and Maximum Coefficient of Variation over the Working Ranges.

Average Coefficient of Variation = 2.47%
Minimum Coefficient of Variation = 0.25%
Maximum Coefficient of Variation = 36.2%

To examine the **Recovery** over the working range of the method, a simple graphical approach was followed. The data from the LOD/LOQ/Linear Range was used for this determination. The mean of replicates was divided by the spike concentration. The percent recovery was then plotted against the spike concentrations. Graph 6 show these recovery plots with the recoveries bracketed at 85% and 105% for clean wastewater samples using the low range method routine. Recovery by the method is highly variable due to the problems associated with spike determinations. However, recovery for the method over all is high at 98.8%. (see Table 7)

Graph 6 - Percent Recovery verse the Spike Concentration for effluent using the low range method routine



As indicated above, the percent recovery of the method as implemented by this laboratory was calculated by dividing the log average of the replicates by the log spike concentration and multiplying by 100 to get a percent. Table 6 below shows this calculation from effluent trials for the low range method routine

Table 7 – Method Recovery from effluent trials.

Average Log of Spike	Average Log Replicates	% Recovery
2.455	2.473	100.7%

Data Summary:

- Is the precision of the method under study consistent through the working range? **N, It varies as expected as the method approaches the LOD**
- The coefficient of variation of the test method as implemented is **2.5%**
- Is the recovery of the method under study consistent through the working range? **N, It varies as expected as the method approaches the LOD**
- What is the overall percent recovery of the method under study? **100.7%**

Ruggedness was determined using the NSSP SOP for the Single Laboratory Validation of Marine Biotoxin and Non-MPN Based Microbiological Methods.

Two batches of bottom plates, soft agar tubes, and growth browths were prepare using two different lots of granulate agar (Media A and Media B), well in advance of the trials. Ten samples of clean effluent from the Dover and Hampton WTPs were similarly spiked and plated using media A and media B batches. The spike level was varied throughout the experiment. Table 8 show the data, data analysis, and the results of the paired t-test for effluent

Table 8 - Determination of the Method Ruggedness for Effluent

Media A PFU/100gm	Media B PFU/100gm	Log Media A	Log Media B
3000	3575	3.48	3.55
5130	5055	3.71	3.70
5335	5465	3.73	3.74
14055	14980	4.15	4.18
13370	15955	4.13	4.20
12275	12200	4.09	4.09
5545	4845	3.74	3.69
5340	4495	3.73	3.65
210	235	2.32	2.37
190	175	2.28	2.24
	Skew	-1.35	-1.29
	Variance	0.47	0.48
	Ratio of Larger Var to Lower Var	0.01	

skew between -2 and 2 indicates symmetry
Ratio of Varieances < 2 indicates homogeneity of variance

Paired t-test (Media A verses Media B)

P value	0.0.7648
P value summary	ns
Are means signif. different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=0.3084 df=9
Number of pairs	10

Data Summary:

Value for the test of symmetry of the distribution of Media A data -1.35
Value for the test of symmetry of the distribution of Media B data -1.29
Variance of Media A data .0.47
Variance of Media B data .0.48
Ratio of the larger to the smaller of the variances of Media A and Media B 0.01
Is there a significant difference between Media A and Media N

Proposal No. **17-100**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	J. Michael Hickey
Affiliation	Massachusetts Division of Marine Fisheries
Address Line 1	1213 Purchase Street
Address Line 2	
City, State, Zip	New Bedford, MA 02740
Phone	508-965-2273
Fax	508-990-0449
Email	Michael.hickey@state.ma.us
Proposal Subject	Marina Definition
Specific NSSP Guide Reference	Section I Purposes and Definitions B. Definition of Terms (71) Marina
Text of Proposal/ Requested Action	**(71) Marina** means any water area with a structure (docks, basin, floating docks, etc.) which is: (a) Used for docking or otherwise mooring vessels to a dock or pier; and (b) Constructed to provide temporary or permanent docking space for more than ten boats.
Public Health Significance	There has been ever increasing pressure to include mooring areas which are not defined in the Model Ordinance into the Marina Proper; Section II- Chapter IV **@ .05 Marinas**. When the criteria were developed to deal with the classification of Marinas as defined, and the determination of a buffer zone in adjacent waters; mooring areas were purposely not included. It was left to the discretion of the SSCA to determine, classification criteria that could be different from the marina calculations depending on local circumstances and local knowledge. FDA is now interpreting anchors, chains and mooring blocks as "structures "and as such is requiring that mooring areas be treated as Marinas. Structure in the Marina definition means "(docks, basin, floating docks, etc.)" not anchors and chains. There are many different kinds of marinas, some essentially parking lots with no overnight occupancy and others that are destination mooring areas. Some states have outstanding boat pump out programs and large areas, if not the entire state, that are federal No Discharge Areas, in addition to local well enforced no discharge and occupancy regulations or by-laws. SSCAs should be allowed to assess the pollution impact of mooring areas based on actual circumstances and data not just an assumed risk.
Cost Information	NONE, Possible savings to SSCAs.
Action By 2017 Task Force I	Recommended referral of Proposal 17-100 to an appropriate committee as determined by the Conference Chair.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-100.
Action by FDA February 7, 2018	Concurred with Conference action on proposal 17-100 with comments. (See February 7, 2018 FDA response to ISSC Summary of Actions)

Proposal No. **17-103**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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
Fax	301-436-2601
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 in 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.

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:

Whitney Stutts, Ph.D. and Jonathan Deeds, Ph.D.

Developer Contact Information:

FDA Center for Food Safety and Applied Nutrition
5100 Paint Branch Parkway
College Park, MD 20740

Phone: 240-402-1474 (Deeds) or 240-402-1895 (Stutts)

whitney.stutts@fda.hhs.gov; jonathan.deeds@fda.hhs.gov

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.

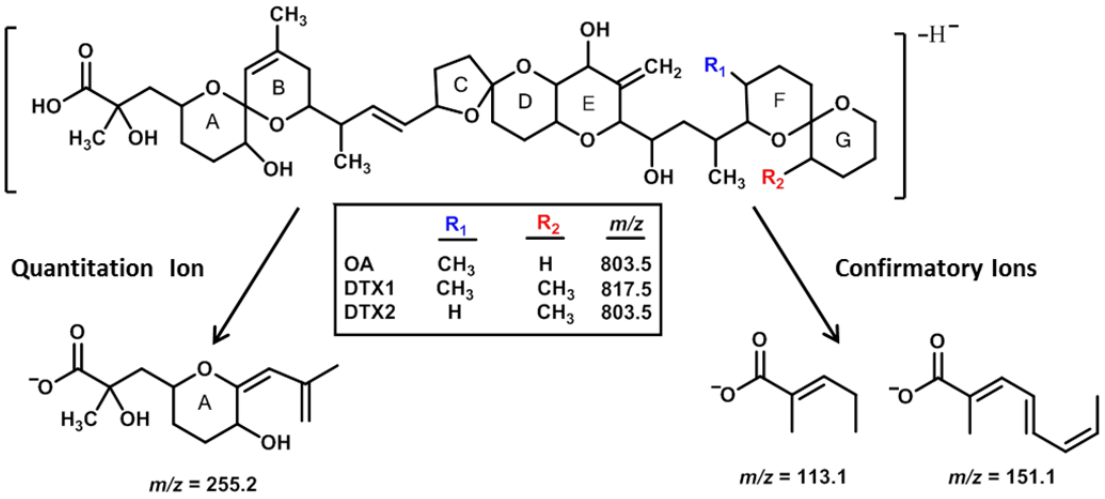


Figure 1. Structure of precursor ions and proposed product ion structures for DSTs.

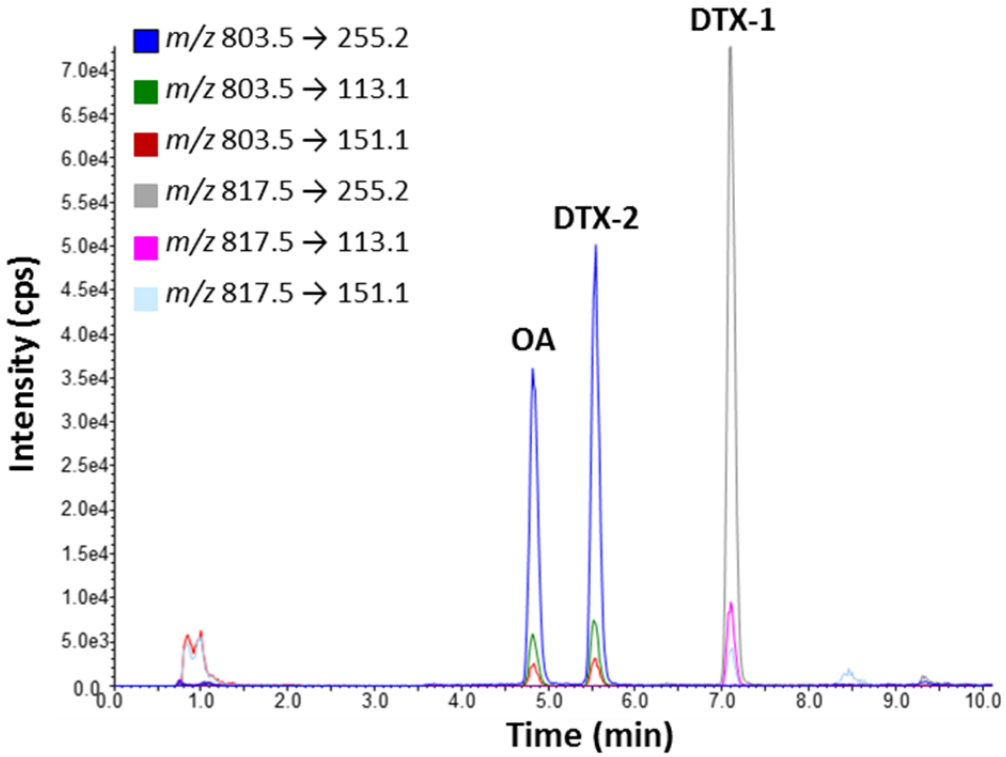


Figure 2. MRM chromatogram for the 12.5 ng/mL (regulatory level = 12.8 ng/mL) spike of OA, DTX1, and DTX2 into blank clam matrix.

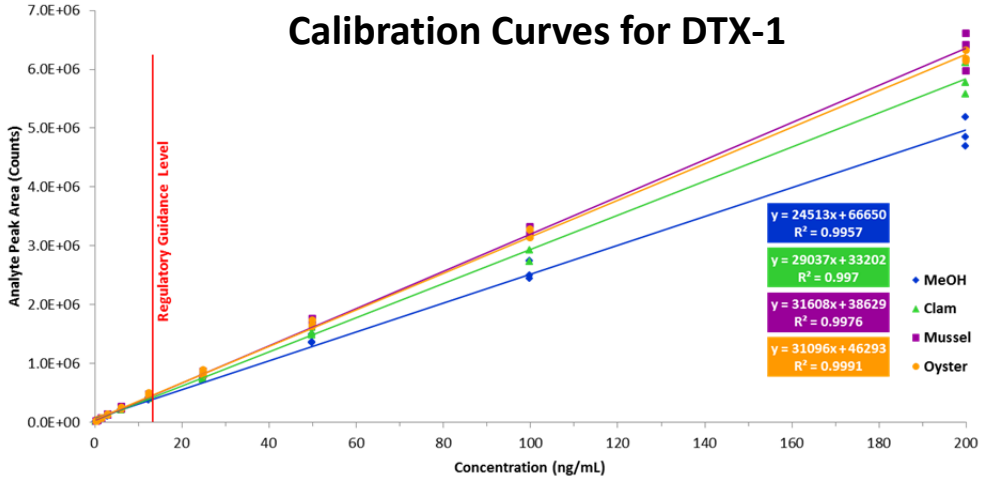
Pre-Validation Study Results (Testing for potential shellfish matrix effects for clam, mussel, and oyster, and ruggedness testing for acidic versus basic chromatography)

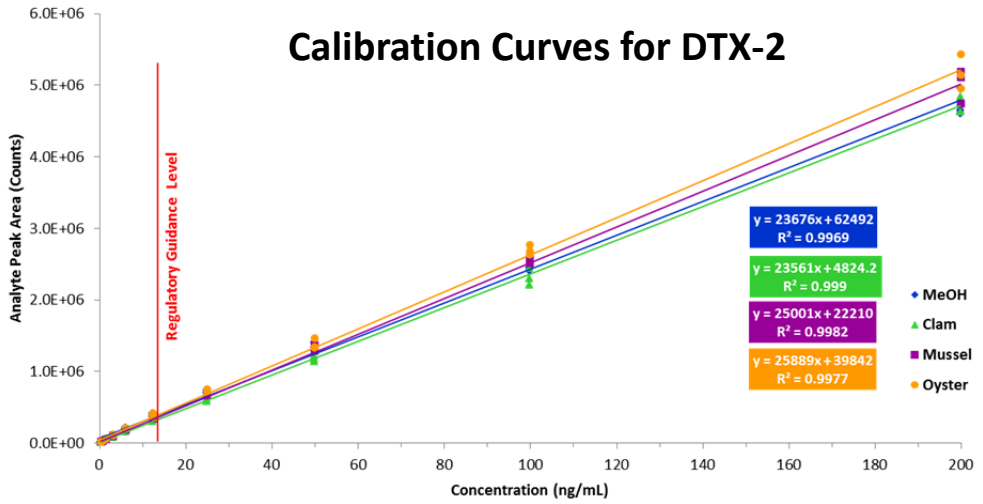
Initial pre-validation testing investigated the impact of mobile phase pH on method performance, and potential matrix effects (i.e. MS signal suppression or enhancement) for clam (*Mercinaria mercinaria*), mussel (*Mytilus edulus*), and oyster (*Crassostrea virginica*) in neat versus matrix matched spiked 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.





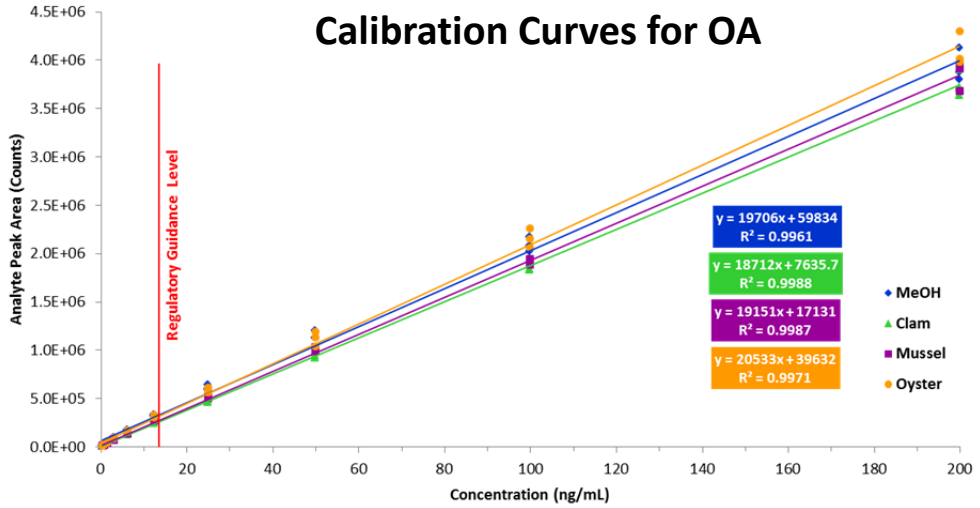
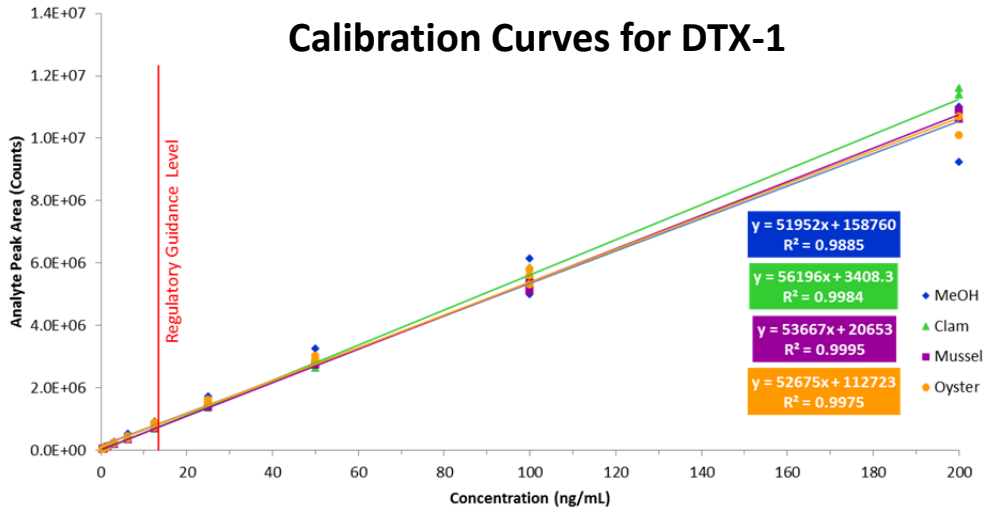


Figure 3. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under acidic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.



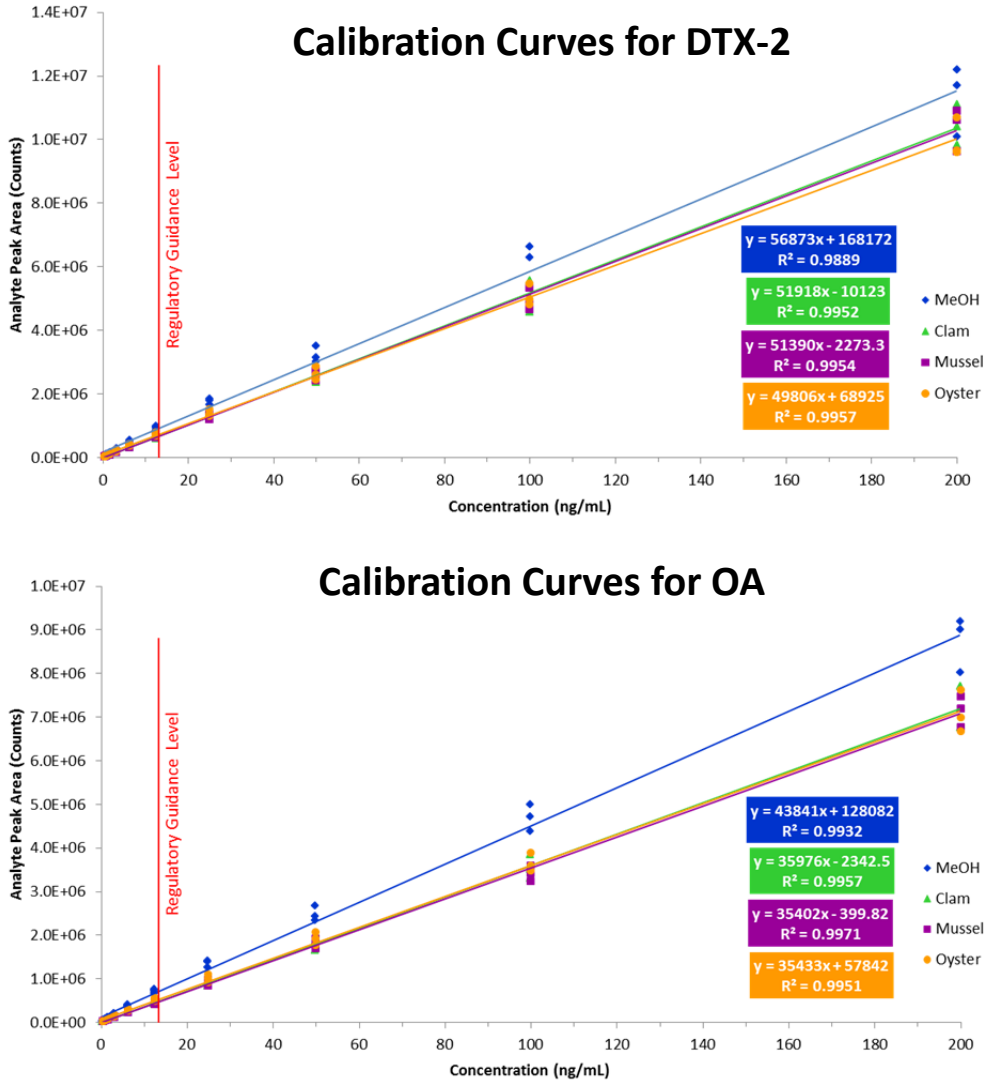


Figure 4. Calibration curves from neat standard solutions and spiked methanolic extracts of clam, mussel, and oyster analyzed under basic chromatographic conditions. A working range of 0.39–200 ng/mL (n=10) was used.

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 No. **17-106**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Pacific Rim Shellfish Sanitation Association
Affiliation	Sitka Tribe of Alaska
Address Line 1	456 Katlian St
Address Line 2	
City, State, Zip	Sitka, AK 99835
Phone	907-747-7356
Fax	907-747-4915
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	This 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 the 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.

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).

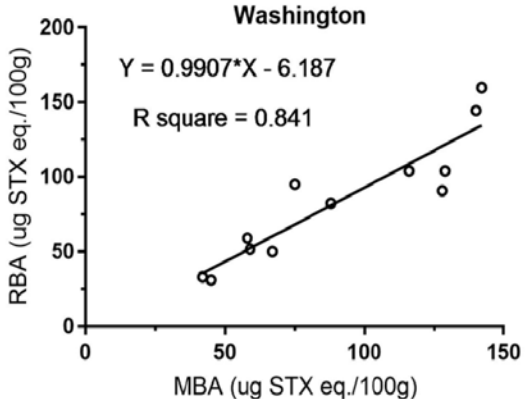
10. Matrix Effects

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)

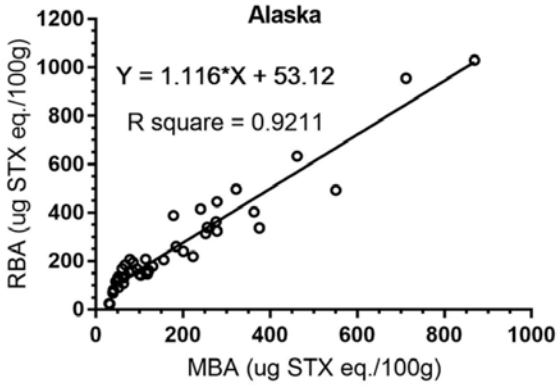
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)

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

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.

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

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

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.

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

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).]

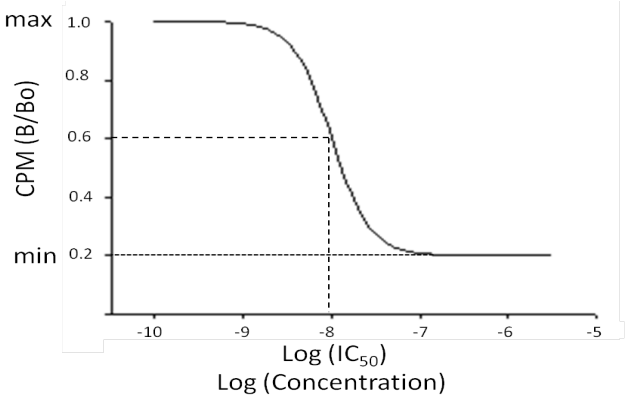


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC50.

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)

Appendix 2

FOOD CHEMICAL CONTAMINANTS

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

FRANCES M. VAN DOLAH, TOD A. LEIGHFIELD, and GREGORY J. DOUCETTE
NOAA National Ocean Service, Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412
LAURIE BEAN
Maine Department of Natural Resources, McKown Point, W. Boothbay Harbor, ME 04575
BARBARA NIEDZWIADEK and DOROTHEA F.K. RAWN
Health Canada, Food Research Division, Sir Frederick Banting Research Centre, Ottawa, ON, Canada K1A 0L2

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.

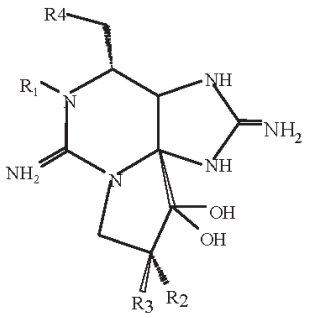
Received March 9, 2009. Accepted by AP May 10, 2009.
Corresponding author's e-mail: fran.vandolah@noaa.gov

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 Duropore 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

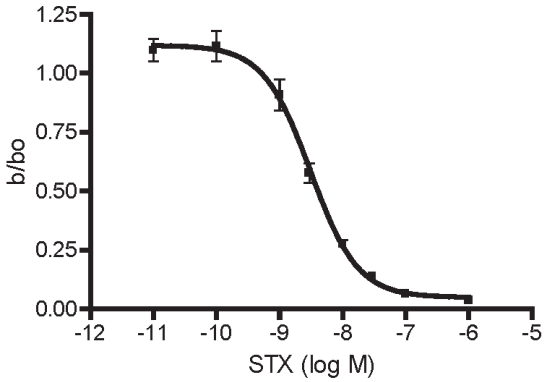


Figure 3. Average of five calibration curves obtained by one analyst in five independent assays on separate days. IC_{50} = 2.23 ± 0.23 nM, slope = 0.96 ± 0.06, error bars are ± SD.

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

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:

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

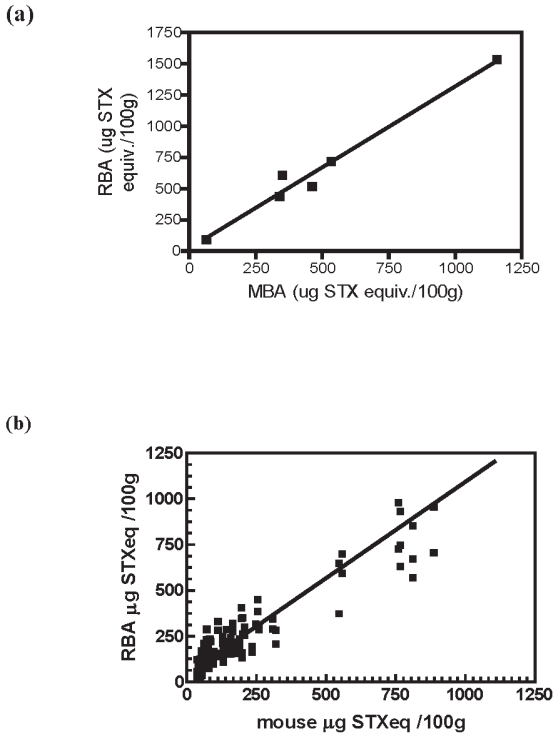


Figure 4. Linear correlation analysis between the RBA and mouse bioassay. (a) Average values of six naturally contaminated samples analyzed on five independent RBA assay days (r^2 = 0.98, slope = 1.29). (b) A separate study of 110 shellfish extracts analyzed by RBA and MBA yielded an r^2 of 0.88 with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

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

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

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.

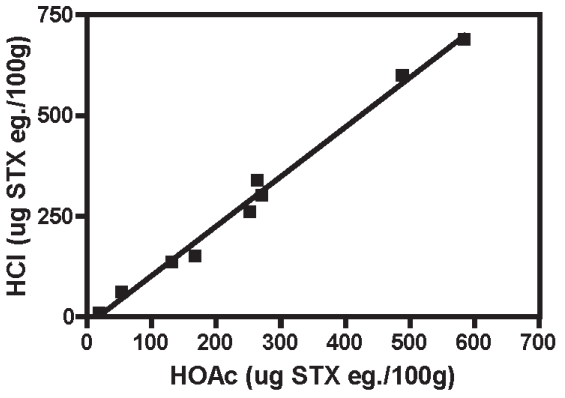


Figure 5. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by RBA. Results are average values of nine naturally contaminated samples obtained from four independent assays (r^2 = 0.99, slope = 1.23).

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.

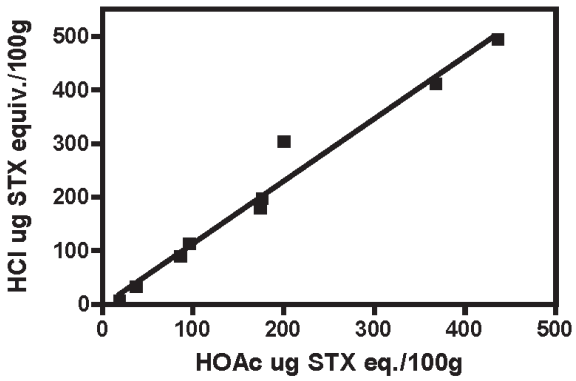


Figure 6. Linear correlation between HCl and acetic acid (HOAc) extracts analyzed by HPLC (slope = 1.16, r^2 = 0.97).

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.

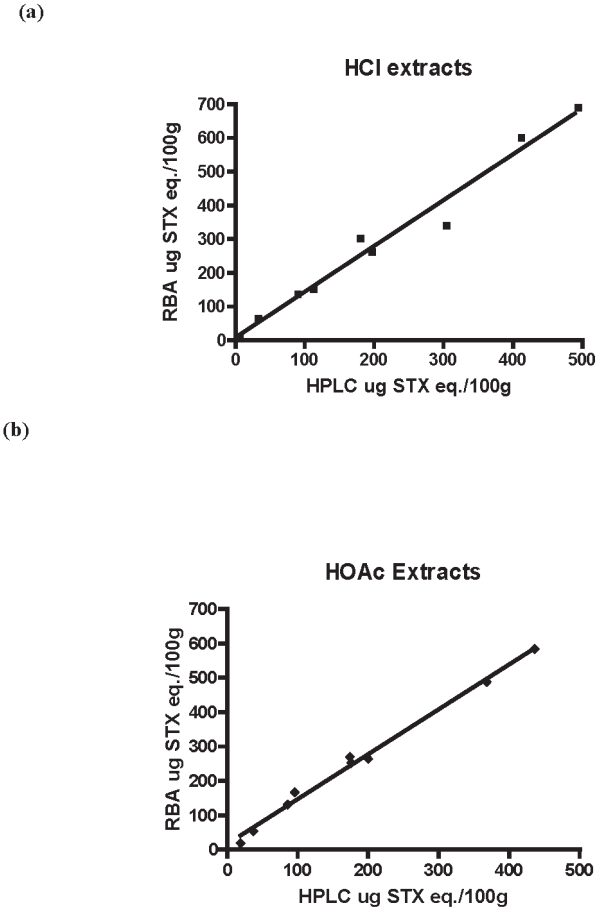


Figure 7. Linear correlation between RBA and HPLC for samples extracted (a) by the HCl method (r^2 = 0.98, slope = 1.39) and (b) by the acetic acid method (r^2 = 0.99, slope = 1.32).

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.

Acknowledgments

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

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2006) *J. AOAC Int.* **88**, 1714–1732

(3) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(4) Turner, A.D., Norton, D.M., Hatfield, R.G., Morris, S., Reese, A.R., Algoet, M., & Lees, D.N. (2009) *J. AOAC Int.* **92**, 190–207

(5) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425

(6) Hall, S., Strichartz, G., Moczydlowshi, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origins, Structure, and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(7) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636

(8) Van Dolah, F.M., Finley, E.L., Haynes, B.L., Doucette, G.J., Moeller, P.D., & Ramsdell, J.S. (1994) *Nat. Toxins* **2**, 189–196

(9) Powell, C.L., & Doucette, G.J. (1999) *Nat. Toxins* **7**, 393–400

(10) Suarez-Isla, B.A., & Valez, P. (2000) in *Seafood and Freshwater Toxins,* L.M. Botana (Ed.), Marcel Dekker Inc., New York, NY, pp 187–202

(11) Llewellyn, L.E., Doyle, J., Jellett, J., Barrett, R., Alison, C., Bentz, C., & Quilliam, M. (2001) *Food Addit. Contam.* **18**, 970–980

(12) Ruberu, S.R., Liu, Y.-G., Wong, C.T., & Perera, S.K. (2003) *J. AOAC Int.* **86**, 1–9

(13) Usup, G., Leaw, C.-P., Cheah, M.-Y., Ahmad, A., & Ng, B.-K. (2004) *Toxicon* **44**, 37–43

(14) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667

(15) Oshima, Y. (1995) *J. AOAC Int.* **78**, 528–532

Appendix 3

FOOD CHEMICAL CONTAMINANTS

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

FRANCES M. VAN DOLAH, SPENCER E. FIRE, TOD A. LEIGHFIELD, CHRISTINA M. MIKULSKI, and GREGORY J. DOUCETTE
Center for Coastal Environmental Health and Biomolecular Research, NOAA Marine Biotoxins Program, 219 Fort Johnson Rd, Charleston, SC 29412

Collaborators: Å. Andersson; L. Bean; D. Couture; S. DeGrasse; A. DeLeon; V. Dell'Ovo; L. Flewelling; P. Holland; G. Langlois; R. Lewis; M. Masuda; P. McNabb; C. Mikulski; B. Niedzwiadek; B. Porntepkasemsan; D. Rawn; E. Sombrito; K. Srisuksawad; B. Suarez; S. Subsinserm; A. Tubaro

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.

Submitted for publication December 3, 2011.
The recommendation was approved by the Method Committee on Paralytic Shellfish Toxins as First Action. *See* "Methods News," (2011) *Inside Laboratory Management*, January/February issue.

Corresponding author's e-mail: Fran.vandolah@noaa.gov
DOI: 10.5740/jaoacint.CS2011_27

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%.

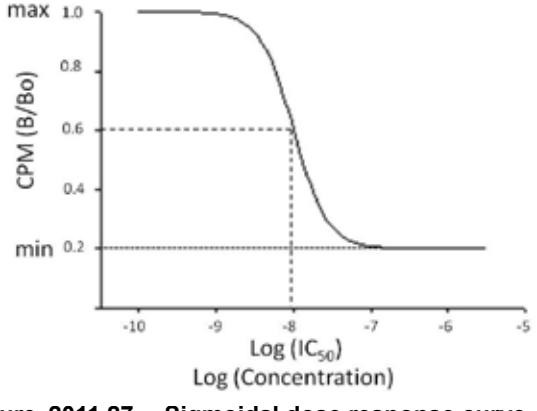


Figure 2011.27. Sigmoidal dose response curve. Dashed lines indicate log IC_{50}.

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

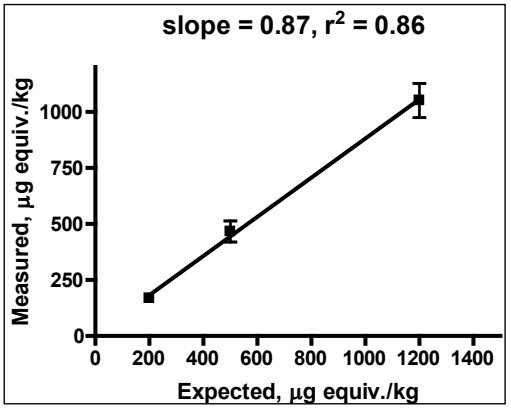


Figure 2. Recovery of spiked STX diHCl in homogenates of blue mussel. Values are in µg STX diHCl equiv./kg.

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

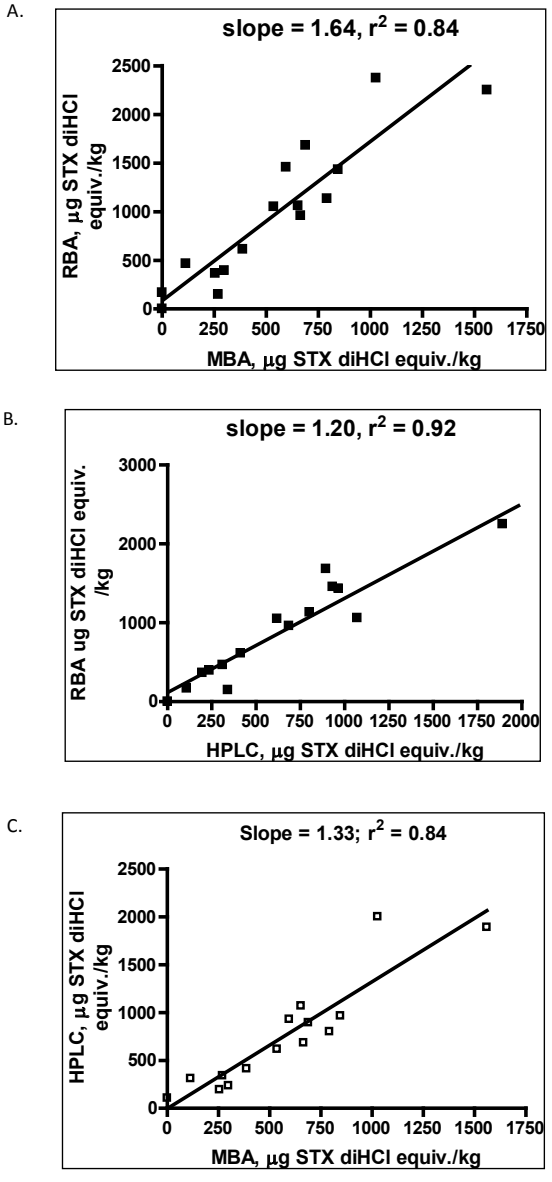


Figure 3. Correlation of the RBA results on PSP toxins in shellfish homogenates with mouse bioassay (A) and HPLC (B). Correlation between the current AOAC *Official Methods*, mouse bioassay, and HPLC (C).

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.

Acknowledgments

We would like to thank Laurie Bean (Maine Department of Marine Resources), Stacey DeGrasse (U.S. Food and Drug Administration), Patrick Holland (Cawthron Institute), Gregg Langlois (California Department of Health Services), Bob Lona (Washington State Department of Health), and Benjamin Suarez (University of Chile) for providing the shellfish material used in the study. We are grateful to Barbara Niedzwiadek and Dorothea Rawn (Health Canada) for HPLC analyses and to Laurie Bean and Darcie Couture (Maine Department of Marine Resources), Benjamin Saurez, and Supanoi Subsinserm (Thailand Department of Fisheries) for performing mouse bioassays. We are indebted to the following collaborators for their dedication and perseverance in carrying out the RBAs for this study:

Leanne Flewelling, Florida Wildlife and Marine Research Institute, St. Petersburg, FL

Stacey DeGrasse, U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition, College Park, MD

Patrick Holland and Paul McNabb, Cawthron Institute, Nelson, New Zealand

Gregg Langlois and Melisa Masuda, California Department of Public Health, Richmond, CA

Richard Lewis and Åsa Andersson, University of Queensland, Queensland, Australia

Christina Mikulski, NOAA Marine Biotoxins Program, Charleston, SC

Elvira Sombrito and Aileen DeLeon (Philippine Nuclear Research Institute, Manila, the Philippines

Kanitha Srisuksawad and Boonsom Porntepkasemsan, Thailand Nuclear Research Institute, Bangkok, Thailand

Aurelia Tubaro and Valeria Dell'Ovo, Dipartimento dei Materiali e Risorse Naturali, Trieste, Italy

This project was funded by the International Atomic Energy Agency Interregional Project INT7017 9001 and NOAA Marine Biotoxins Program Project 02E0017. FDA saxitoxin diHCl used for tritiation by IICH and STX diHCl reference standard (NIST RM 8642) were provided by Sherwood Hall, FDA Office of Seafoods.

References

(1) *Official Methods of Analysis* (1999) 17th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **959.08**

(2) LeDoux, M., & Hall, S. (2000) *J. AOAC Int.* **83**, 305–310

(3) Lawrence, J.F., Niedzwiadek, B., & Menard, C. (2005) *J. AOAC Int.* **88**, 1714–1732

(4) *Official Methods of Analysis* (2005) 18th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **2005.06**

(5) *Official Methods of Analysis* (2011) AOAC INTERNATIONAL, Gaithersburg, MD, Method **2011.02**

(6) Jellett, J.F., Robert, R.L., Laycock, M.V., Quilliam, M.A., & Barrett, R.E. (2002) *Toxicon* **40**, 1407–1425. http://dx.doi.org/10.1016/S0041-0101(02)00153-8

(7) Hall, S., Strichartz, G., Moczydlowski, E., Ravindran, A., & Reichardt, P.B. (1990) in *Marine Toxins: Origin, Structure and Molecular Pharmacology*, S. Hall & G. Strichartz (Eds), ACS Symposium Series No. 418, American Chemical Society, Washington, DC, pp 29–65

(8) Llewellyn, L.E. (2006) *Chem. Res. Toxicol.* **19**, 661–667. http://dx.doi.org/10.1021/tx050277i

(9) Doucette, G.J., Logan, M.L., Ramsdell, J.S., & Van Dolah, F.M. (1997) *Toxicon* **35**, 625–636. http://dx.doi.org/10.1016/S0041-0101(96)00189-4

(10) Van Dolah, F.M., Leighfield, T.A., Doucette, G.J., Bean, L., Niedzwiadek, B., & Rawn, D.F.K. (2009) *J. AOAC Int.* **92,** 1705–1713

(11) McFarran, E.F. (1959) *J. AOAC Int.* **42**, 263–271

(12) EU Reference Laboratory for Marine Biotoxins (2010) *Report*

on the EURLMB 2010 proficiency testing on saxitoxin group (PSP) toxins determination, Vigo, Spain

(13) Eurachem Guide (1998) *The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics,* http://www.eurachem.org/index.php/publications/guides/mv

(14) Kleivdahl, H., Kristiansen, S.-I., Nilson, M.A., Goksoyr, A., Briggs, L., Holland, P., & McNabb, P. (2007) *J. AOAC Int.* **90**, 1011–1027

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.

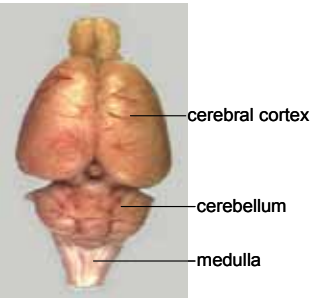


Figure 1. Rat brain.

(*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.

Proposal No. 17-106
Taylor & Francis
Taylor & Francis Group

Food Additives & Contaminants: Part A
2012, 1–10, iFirst

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

S.R. Ruberu*, G.W. Langlois, M. Masuda and S. Kusum Perera

Drinking Water and Radiation Laboratory Branch and Environmental Management Branch, California Department of Public Health, 850 Marina Bay Parkway, Richmond, CA 94804, USA

(*Received 13 April 2012; final version received 9 July 2012*)

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

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

*Corresponding author. Email: Shiyamalie.Ruberu@cdph.ca.gov

ISSN 1944–0049 print/ISSN 1944–0057 online

http://dx.doi.org/10.1080/19440049.2012.712063
http://www.tandfonline.com

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).

- 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

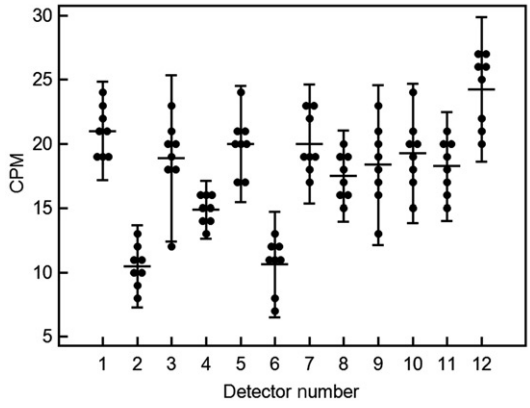


Figure 2. Plot of the instrument background plate second count cycle depicting the randomness of CPMs in the 96 wells. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

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.

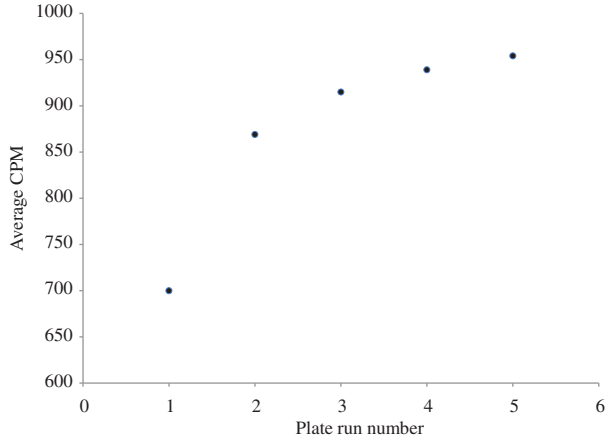


Figure 3. Graph of the blank plate five sequential readings showing a gradual increase in average CPM.

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

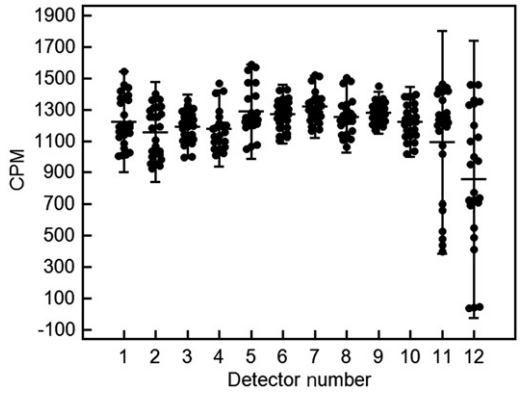


Figure 4. Scatter plot of the reference plate, first reading showing detector variability. Each datum point represents the CPM of a well read by the respective detector. Also shown for each detector are the mean and error bars at 2 SDs for the group of data.

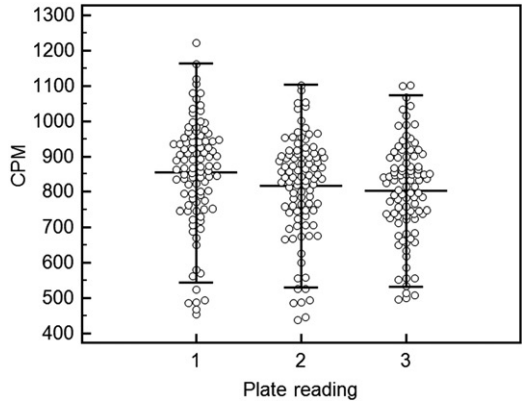


Figure 5. Results of the QC plate showing the variability of CPM in the three successive readings. For each run the mean and error bars at 2 SDs per plate are shown.

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.

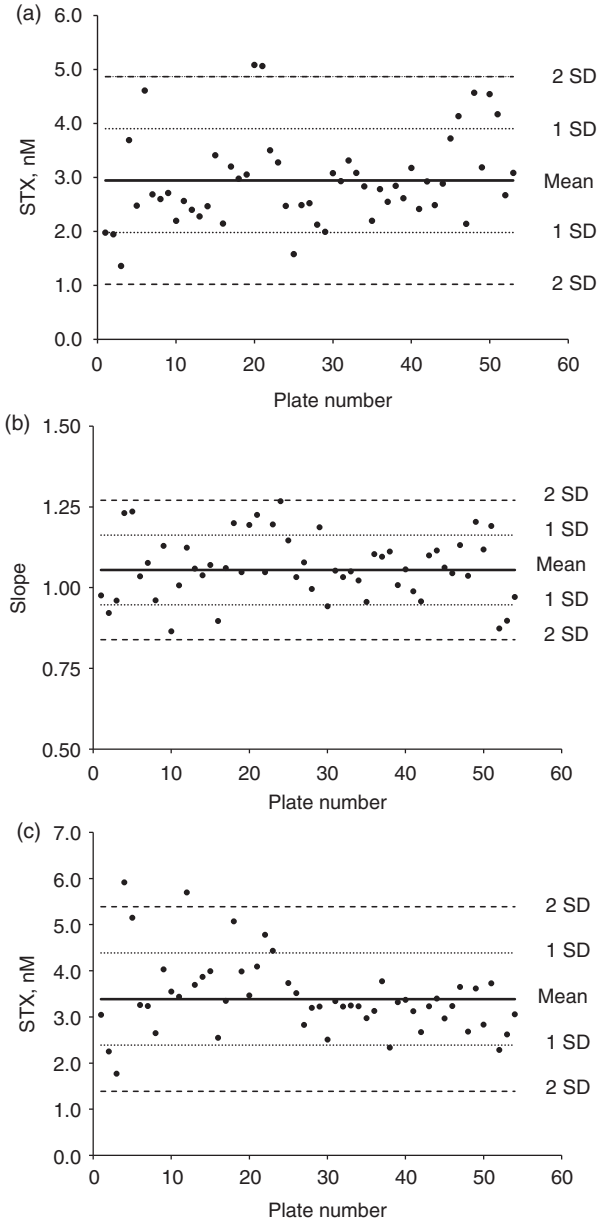


Figure 6. Control charts for (a) averaged daily QC samples on a plate, (b) slope of the binding curve and (c) EC_{50} per plate.

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.

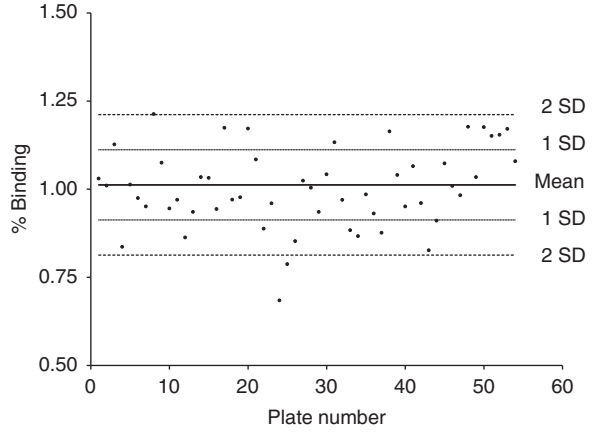


Figure 7. Control chart for the top plateau, maximum binding (B_{max}), of the calibration curve.

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

This study was supported under NOAA grant NA04NOS4780239 from the Monitoring and Event Response for Harmful Algal Bloom (MERHAB) programme. This is MERHAB Publication Number 152. The authors would like to thank Roger Ho for helping with the Graphpad Prism data analysis; Clive Kittredge and Vanessa Zubkousky for the preparation and analysis of plates; and American Radiolabeled Chemicals Inc. for providing ^{3}H-STX reagents. Special thanks to Sherwood Hall of the US Food and Drug Administration's Office of Regulatory Science for FDA STX reference standards and helpful discussions on this study. The authors would also like to thank Fran van Dolah and Greg Doucette for their guidance in the RBA method.

References

American Public Health Association (APHA). 1970. Recommended procedures for the examination of sea water and shellfish. 4th ed. New York (NY): American Public Health Association. p. 57–61.

Doucette GJ, Logan MM, Ramsdell JS, van Dolah FM. 1997. Development and preliminary validation of a microtiter plate-based receptor-binding assay for paralytic shellfish poisoning toxins. Toxicon. 35(5):625–636.

Grubbs FE. 1969. Procedures for detecting outlying observations in samples. Technometrics. 11(1):1–21.

Lawrence JF, 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. J AOAC Int. 88:1714–1732.

Powell CL, Doucette GJ. 1999. A receptor-binding assay for paralytic shellfish poisoning toxins: recent advances and applications. Natural Toxin. 7:393–400.

Ruberu SR, Liu YG, Wong CT, Perera KS, Langlois GW, Doucette GJ, Powell CL. 2003. Receptor-binding assay for paralytic shellfish poisoning toxins: optimization and inter-laboratory comparison. J AOAC Int. 86(4):737–745.

Sokal RR, Rolf FJ. 1981. Biometry. 2nd ed. New York (NY): W. H. Freeman. p. 229–231.

van de Riet JM, Gibbs RS, Chou FW, Muggah PM, Rourke WA, Burns G. 2009. Liquid chromatographic post-column oxidation method for analysis of paralytic shellfish toxins in mussels, clams, scallops, and oysters: single-laboratory validation. J AOAC Int. 92(6): 1690–1704.

van Dolah FM, Fire SA, Leighfield TA, Mikulski CM, Doucette GJ. 2012. Determination of paralytic shellfish poisoning toxins in shellfish by receptor-binding assay: a collaborative study. J AOAC Int. 95(3):795–812.

van Dolah FM, Leighfield TA, Doucette GJ, Bean L, Niedzwiadek B, Rawn DFK. 2009. Single-laboratory validation of the microplate receptor-binding assay for paralytic shellfish toxins in shellfish. J AOAC Int. 92(6):1705–1713.

Proposal No. **17-108**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	Titan Fan, Ph.D
Affiliation	Beacon Analytical Systems, Inc.
Address Line 1	82 Industrial Park Road
Address Line 2	
City, State, Zip	Saco, Maine 04072
Phone	(207) 571-4302
Fax	(207)602-6502
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.

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.
Dr. Titan Fan, President
Contact Person: Holly Lawton, Director of New Product Development

Beacon Analytical Systems, Inc
82 Industrial Park Rd.
Saco, ME 04072
Phone: 207-571-4302
email: holly@beaconkits.com
Cell: 207-289-6390

Date of submission: June 30, 2017
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.

Beacon Analytical Systems Inc.®

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#).

Beacon Analytical Systems Inc.®

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.

Beacon Analytical Systems Inc.®

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 %

Beacon Analytical Systems Inc.®

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

Beacon Analytical Systems Inc.®

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

Beacon Analytical Systems Inc.®

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

Beacon Analytical Systems Inc.®

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).

Beacon Analytical Systems Inc.®

Figure 1: Linearity of Beacon DA ELISA

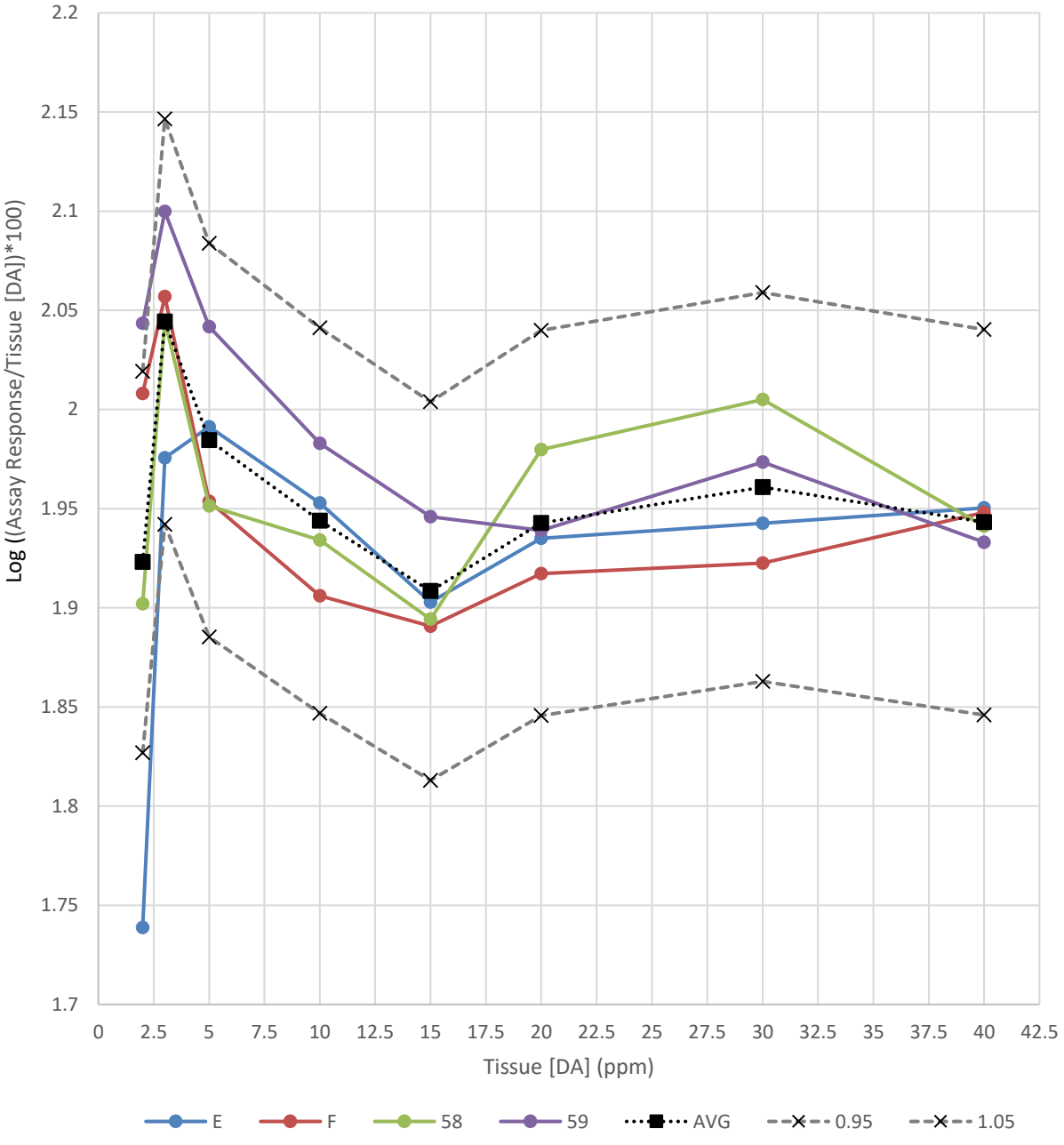
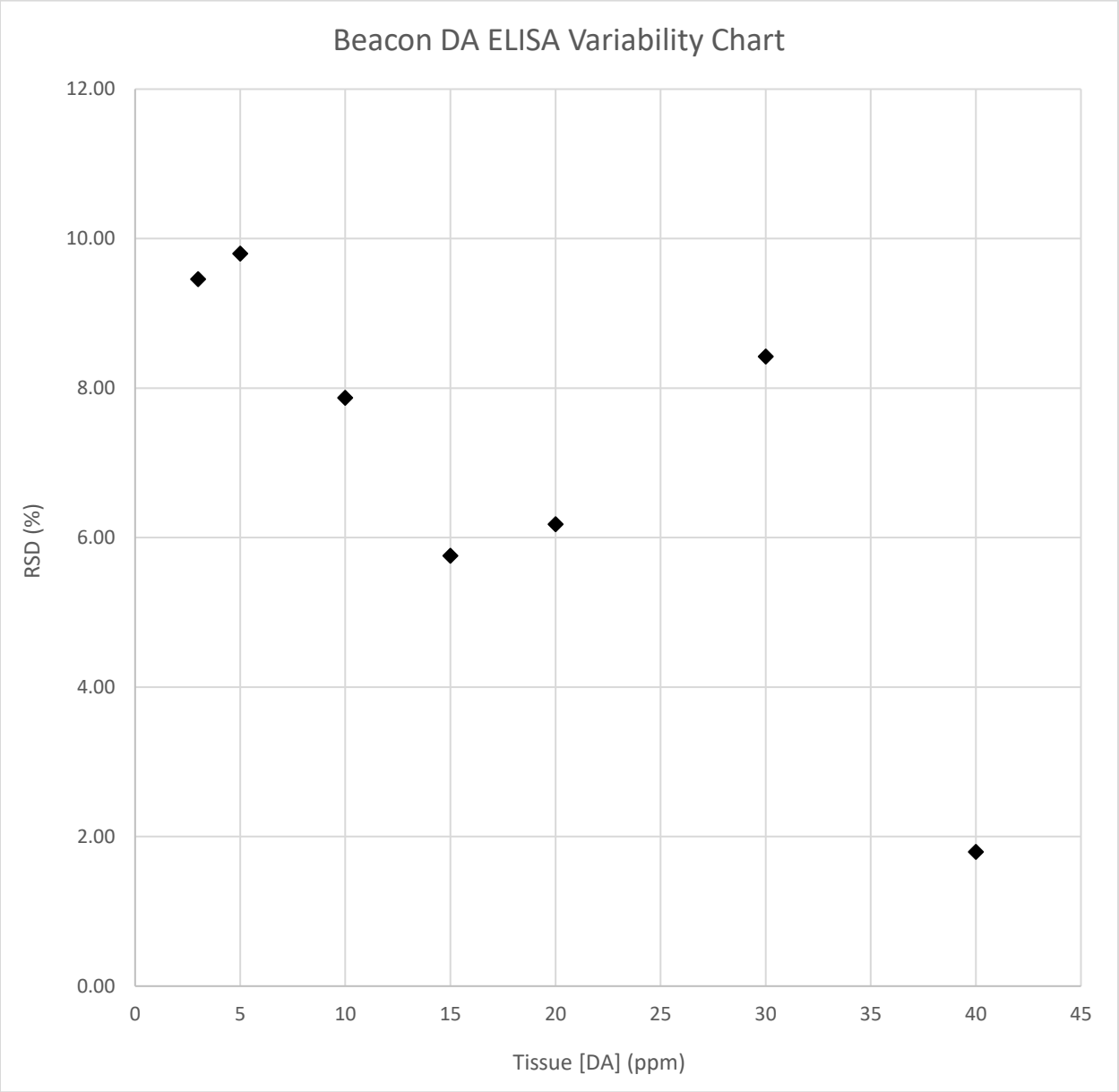


Figure 2: Limit of Detection & Limit of Quantitation/Sensitivity



Beacon Analytical Systems Inc.®

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.

Proposal No. 17-108

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.

BEACON ANALYTICAL SYSTEMS, INC.®

82 Industrial Park Road

Saco, ME 04072

Tel. (207) 571-4302 Fax (207) 602-6502

www.beaconkits.com

Proposal No. **17-110**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration (FDA)
Affiliation	FDA
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Alkaline Phosphatase Probe Method for *Vibrio vulnificus* and *Vibrio parahaemolyticus* Detection in Oysters - 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 Checklists
Text of Proposal/ Requested Action	The requested action is to adopt the text of the attached checklist for the probe method for detecting *Vibrio vulnificus* (Vv) and *Vibrio parahaemolyticus* (Vp) in oysters 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	Currently, there is no checklist adopted by the ISSC for the probe method for detecting Vv and Vp in oysters. The attached checklist provides the quality assurance and method requirements that laboratory evaluation officers will use to evaluate laboratories implementing this method in support of 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	NA
Action By 2017 Laboratory Committee	Recommended Proposal 17-110 be referred to an appropriate committee as determined by the Conference Chair.
Action By 2017 Task Force I	Recommended adoption of Laboratory Committee recommendation on Proposal 17-110.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-110.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 17-110.

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

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

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]

PART I – QUALITY ASSURANCE			
			ITEM
Code	REF		
			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 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 All working thermometers are checked annually against the standards thermometer at the temperature(s) of use. Results for 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	**6, 8**	☐	**1.6.3 The autoclave provides a sterilizing temperature of 121 ± 2 ºC as determined for each load using a calibrated maximum registering working 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	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 heterotropic 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 at 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:** **a. *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.** **b. *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.**
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.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. U.S. Department of Commerce. 1976. *NBS Monograph 150*. U.S. Department of Commerce, Gaithersburg, Maryland.
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.
8. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
9. Fisher, J. 1985. Measurement of pH. American Laboratory 16:54-60.
10. Association of Official Analytical Chemists (AOAC). 1999. AOAC *Methods Validation and Technical Programs – Criteria for Laboratories Performing Food Testing*. AOAC, Arlington, Va.
11. American Public Health Association. 2001. *Compendium of Methods for the Microbiological Examination of Foods*. APHA, Washington, D.C..
12. U.S. Food and Drug Administration (FDA). 1995. *Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
13. Angelo DePaola, Jessica L. Jones, Jacquelina Woods, et al. 2010. Bacterial and Viral Pathogens in Live Oysters: 2007 United States Market Survey. *Applied and Environmental Microbiology.* 76(9): 2754-2768.
14. ISSC email communication.
15. David W. Cook, Angelo DePaola, and Susan A. McCarthy. DIRECT PLATING PROCEDURE FOR THE ENUMERATION OF TOTAL AND PATHOGENIC Vibrio parahaemolyticus IN OYSTER MEATS. FDA / Office of Seafood Gulf Coast Seafood Laboratory Dauphin Island, AL 36528-0158.
16. Section IV Guidance Documents, Naturally Occurring Pathogens, NSSP *Guide for the Control of Molluscan Shellfish*, 2009 Revision.
17. Consult pH electrode product literature.
18. U.S. Department of Commerce. 1986. *NBS Monograph 174*, Gaithersburg, Maryland.

LABORATORY:			**DATE OF EVALUATION:**
SHELLFISH LABORATORY EVALUATION CHECKLIST **SUMMARY OF NONCONFORMITIES**			
Page	**Item**	**Observation**	**Documentation Required**

LABORATORY:

Page	Item	Observation

Page ___ of ___

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	

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, Key and Other (O) Nonconformities in Parts I-III	_____

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:_____

Proposal No. 17-115

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	J. Michael Hickey Margaret Barette David Fyfe
3. Affiliation	Massachusetts Division of Marine Fisheries Pacific Coast Shellfish Growers Association NWIFC Treaty Tribes
4. Address Line 1	1213 Purchase Street 120 State Avenue NE, #142 19472 Powder Hill Place NE, Suite 210
5. Address Line 2	
6. City, State, Zip	New Bedford, MA 02740 Olympia, WA 98501 Poulsbo, WA 98370
7. Phone	508-965-2273 360-754-2744 360-397-6502
8. Fax	508-990-0449 360-754-2743
9. Email	Michael.hickey@state.ma.us margaretbarrette@pcsga.org dfyfe@nwifc.org
10. Proposal Subject	Reconditioning of Recalled Shellfish Implicated in a Norovirus Outbreak
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter II. Risk Assessment & Risk Management @.01 Outbreaks of Shellfish Related Illness.
12. Text of Proposal/ Requested Action	J. 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 PHPs or placing 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 State Shellfish Control Authority (SSCA). 2. Product associated with a Norovirus outbreak may be reconditioned by returning the product, within three (3) days of the recall, to the growing area from which it was harvested for an appropriate period of time. The period of time shall not be less than twenty-one (21) days. The Authority shall ensure appropriate controls and provide documentation of the activity.
13. Public Health Significance	A twenty-one (21) day submergence period is consistent with the amount of time required at Section II. Chapter IV. A. (5) (b) (ii) and C. (2) (c) (iii), Shellstock Growing Areas.
14. Cost Information	No substantial increased cost to SSCAs and to the shellfish industry. would

	constitute a cost saving
Action By 2017 Task Force I	Recommends referral of Proposal 17-115 to an appropriate committee as determined by the Conference Chair.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-114.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 17-114.

Proposal No. **17-116**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

Submitter	U.S. Food and Drug Administration (FDA)
Affiliation	U.S. Food and Drug Administration (FDA)
Address Line 1	5001 Campus Drive
Address Line 2	HFS-325
City, State, Zip	College Park, MD 20740
Phone	240-402-1401
Fax	301-436-2601
Email	Melissa.abbott@fda.hhs.gov
Proposal Subject	Sanitary Control of Molluscan Shellfish Harvested From Federal Waters
Specific NSSP Guide Reference	Section I Purposes & Definitions Section II Model Ordinance Chapter IV Shellstock Growing Areas Section II Model Ordinance Chapter VI Shellfish Aquaculture
Text of Proposal/ Requested Action	Insert the following definition for Federal Waters in Section I Purposes & Definitions as follows: **Federal Waters** means the waters that fall outside of State and local jurisdiction but within U.S. sovereignty (typically 3-200 nautical miles offshore). Federal waters include the territorial sea and exclusive economic zone. Insert the language below for Section II Model Ordinance 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, and marine biotoxin hazards) and involve commercial shellfish resources . Insert the language below for Section II Model Ordinance Chapter VI Shellfish Aquaculture just after the text in @.03and prior to Shellfish Gardening @.04 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. @.~~04~~05 Shellfish Gardening Insert the language below for Section II Model Ordinance Chapter VI Shellfish Aquaculture just after .07 .08 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 @.04. 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(a)(5) 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 obtain 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.
Public Health Significance	Currently, the NSSP Guide does not explicitly cover requirements for the sanitary control of molluscan shellfish harvested from U.S. Federal waters. The lack of standards for this activity has impeded the harvest of shellfish, notably aquaculture, from Federal waters to date. FDA's policy on the classification of growing areas in offshore Federal waters as described in Verber 1977 was followed in drafting the Proposal. Adding specific language to the Model Ordinance on the appropriate requirements for this activity will facilitate safe and sanitary access to additional shellfish resources.
Cost Information	N/A
Action By 2017 Task Force I	Recommended adoption of Proposal 17-116 on an interim basis with a sunset date of November 1, 2021 and that during this period a committee be appointed to evaluate aquaculture activities in federal waters.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-116.

Action by FDA February 7, 2018	Concurred with Conference action on Proposal 17-116.

Proposal No. **17-121**

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	☒ Growing Area ☐ Harvesting/Handling/Distribution ☐ 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
Fax	301-436-2601
Email	Melissa.Abbott@fda.hhs.gov
Proposal Subject	Disposal of Human Sewage and Bodily Fluids
Specific NSSP Guide Reference	Section II. Model Ordinance Chapter VIII. Control of Shellfish Harvesting Requirements for Harvesters .02 Shellstock Harvesting and Handling. Section II. Model Ordinance Chapter IX. Transportation Requirements for Harvesters .01 Conveyances Used to Transport Shellstock to the Original Dealer and .02 Conveyances Used to Transport Shellstock from Dealer to Dealer
Text of Proposal/ Requested Action	Chapter VIII. .02 Shellstock Harvesting and Handling D. Disposal of Human Sewage and Bodily Fluids~~from Vessels~~. (1) Human sewage and bodily fluids shall not be discharged overboard from ~~a~~any vehicle or vessel used in the harvesting of shellstock, or from vehicles or vessels which buy shellstock while the vehicles or vessels are in growing areas. (2) As required by the Authority, in consultation with FDA, an approved marine sanitation device (MSD), portable toilet or other sewage disposal receptacle shall be provided on the vehicle or vessel to contain human sewage and bodily fluids. (3) Portable toilets shall: (a) Be used only for the purpose intended; (b) Be secured while on board and located to prevent contamination of shellstock by spillage or leakage; (c) Be emptied only into a sewage disposal system; (d) Be cleaned before being returned to the vehicle or vessel~~boat~~; and (e) Not be cleaned in equipment used for washing or processing food. (4) Use of other receptacles for sewage disposal may be approved by the Authority if the receptacles are: (a) Constructed of impervious, cleanable materials and have tight fitting lids; (b) Indelibly labeled “Human Waste” in contrasting letters at least three (3) inches in height; and (c) Meet the requirements in Section D. (3). Chapter IX. .01 Conveyances Used to Transport Shellstock to the Original Dealer G. Disposal of Human Sewage and Bodily Fluids (1) Human sewage and bodily fluids shall not be discharged overboard from any vehicle or vessel used in the harvesting of shellstock, or from vehicles or vessels which buy shellstock while the vehicles or vessels are in growing areas. (2) As required by the Authority, in consultation with FDA, an approved marine sanitation device (MSD), portable toilet or other sewage disposal receptacle

	shall be provided on the vehicle or vessel to contain human sewage and bodily fluids. Portable toilets shall meet the requirements of VIII. .02. D. (3). Chapter IX. 02 Conveyances Used to Transport Shellstock from Dealer to Dealer C. Disposal of Human Sewage and Bodily Fluids (1) Human sewage and bodily fluids shall not be discharged overboard from any vehicle or vessel used in the harvesting of shellstock, or from vehicles or vessels which buy shellstock while the vehicles or vessels are in growing areas. (2) As required by the Authority, in consultation with FDA, an approved marine sanitation device (MSD), portable toilet or other sewage disposal receptacle shall be provided on the vehicle or vessel to contain human sewage and bodily fluids. Portable toilets shall meet the requirements of VIII. .02. D. (3).
Public Health Significance	During evaluations, harvesters and certified dealers buying trucks are observed within harvesting areas and aquaculture lease site areas. The vehicles are often there for hours while harvesting, husbandry, and purchasing activities are taking place. In many areas, there are no nearby toilet facilities to accommodate emergency (or non-emergency) needs for toilet facilities to accept human digestive waste or vomit, putting the area at risk of foodborne illness, e.g. norovirus, hepatitis A, etc. The requirement for marine sanitation devices should not only pertain to vessels in order to protect the public health.
Cost Information	~$5.00 for a five (5) gallon bucket with a lid.
Action By 2017 Task Force I	Recommended referral of Proposal 17-121 to an appropriate committee as determined by the Conference Chair.
Action by 2017 General Assembly	Adopted the recommendation of Task Force I on Proposal 17-121.
Action by FDA February 7, 2018	Concurred with Conference action on Proposal 17-121.

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Determining Emergency Conditions
11. Specific NSSP Guide Reference	Section I. Purposes and Definitions Section II. Model Ordinance Chapter IV @.03 A.(1)
12. Text of Proposal/ Requested Action	Section I. Purposes and Definitions New Definition: B.(39) **Emergency Conditions** means potential or actual pollution conditions which were not specifically represented in the sanitary survey information used to establish the classification and support the status of a shellfish growing area. Emergency conditions include, but are not limited to, tropical storms, hurricanes, sewage spills, oil spills, poisonous or deleterious substance spills, excessive rainfall, and flooding events. Chapter IV @.03 A.(1): (1) Emergency Conditions. A growing area shall be placed in the closed status under Section @.03A. (5) when ~~pollution conditions exist which were not included in the database used to classify the area~~ emergency conditions exist. The Authority shall: (a) Develop a written emergency conditions protocol defining the thresholds and criteria used to determine if emergency conditions exist, including defining what conditions would trigger a growing area closure, and how to reopen a growing area once the emergency conditions no longer exist. The thresholds and criteria used to determine if emergency conditions exist, shall be based on the potential or actual pollution conditions which were not specifically represented in the sanitary survey information or database used to establish the classification and support the status of a shellfish growing area. These potential or actual pollution conditions may include, but are not limited to, tropical storms, hurricanes, sewage spills, oil spills, poisonous or deleterious substance spills, excessive rainfall, and flooding events; (b) Make a determination within 24 hours of a potential emergency condition event as to whether conditions exceed the established thresholds and criteria defined in the emergency conditions protocol and maintain a written record of the determination assessment; (c) Notify FDA and ISSC of the determination within 24 hours; (d) Once it is determined that an emergency condition exists, ~~If it is~~

	~~determined that an emergency condition or situation exists, then the growing area will be~~ immediately (within 24 hours) ~~placed in the closed status.~~ place the growing area in the closed status; (e) If a determination cannot be made within 24 hours, notify FDA and ISSC and immediately place the growing area in the closed status; (f) If the growing area is closed due to a precautionary closure and a determination is later made that the growing area did not experience emergency conditions based on the established protocol, the area may be immediately re-opened. The determination shall be documented in a written report and included in the sanitary survey for the area; and ~~(e)~~(g) If the growing area is closed due to emergency conditions, prior to re-opening, conduct an assessment of the growing area based on the established protocol and field observations and document the results in a written report to be included in the sanitary survey. Field observations include, but are not limited to, observations of actual or potential pollution sources made via shoreline survey, boat survey, sample collection, and/or analysis of sample results. The assessment shall include documentation of any new pollution sources and their effect on the growing area.
13. Public Health Significance	Current Model Ordinance language in Chapter IV states "If it is determined that an emergency condition or situation exists…", but does not specify the circumstances under which a determination must be made by the Authority. It will not be clear to a state Authority that pollution conditions exist which were not included in the data used to classify a growing area unless the Authority decides to check the data within the sanitary survey and perform an assessment in a situation which has the potential to meet emergency conditions. Not all Authorities do this in all situations that have the potential to meet "Emergency Conditions" under NSSP MO @.03 A.(1), such as excessive rainfall events with higher rainfall totals that what's recorded in the Authority's database. Additionally, the current language for "Emergency Conditions" does not clearly define "pollution conditions" or "the database used to classify the area". The "database" could be referring to the most recent 12 year sanitary survey or to all of the data ever collected for a growing area or to the most recent 30 water quality samples – it is not clear. In some instances, this has led to disagreements between FDA and state Authorities as to when a growing area needs to be closed due to emergency conditions, such as in the event of a tropical storm with rainfall levels or river stage levels which may or may not exceed the levels in the state's database. Since emergency conditions have the potential to significantly impact the water quality of a growing area and could lead to human fecal contamination, petroleum contamination, or poisonous or deleterious substance contamination in the area and possible shellfish-borne illnesses, it is important to clarify the definition of "Emergency Conditions".
14. Cost Information	Minimal Cost

Proposal No. 19-101

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 ISSC Accepted SLV Method; or (b) Complete a FDA Shellfish LEO or FDA certified State Shellfish LEO approved Method Verification based on ISSC SLV protocols; 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	A technical Laboratory evaluation, as outlined in MO Chapter 1.@.03B.1.b.ii, is

Significance	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

Proposal No. 19-102

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Updating epidemiological investigation reference.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter II. Risk Assessment and Risk Management @.01 Outbreaks of Shellfish-Related Illness A NOTE.
12. Text of Proposal/ Requested Action	NOTE: For additional guidance refer to the International Association for Food Protection ~~of Milk, Food, and Environmental Sanitarians'~~ *Procedures to Investigate Food Borne Illness*.
13. Public Health Significance	The name of the organization producing the referenced publication has changed.
14. Cost Information	No cost.

Proposal No. 19-103

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Bill Dewey
3. Affiliation	Taylor Shellfish Farms
4. Address Line 1	130 SE Lynch Rd
5. Address Line 2	
6. City, State, Zip	Shelton, WA 98584
7. Phone	360-790-2330
8. Fax	360-432-3344
9. Email	billd@taylorshellfish.com
10. Proposal Subject	Alternative for allowing harvest for raw consumption from a growing area closed due to *V.p.*
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter II. Risk Assessment and Risk Management @.02 Shellfish Related Illnesses Associated with *Vibrio parahaemolyticus* (*V.p.*), Section A. (6)
12. Text of Proposal/ Requested Action	(6) 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) Implementing a process that has been validated to achieve <100 mpn/gram total *V.p.*; ~~(b)~~(c) 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)~~(d) 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.
13. Public Health Significance	The Center for Disease control estimates 45,000 people get ill each year in the United States from *V.p.*. In an effort to reduce *V.p.* illnesses SSCAs have developed and implemented vibrio control plans and industry has diligently implemented strict temperature controls and harvest practices. Despite these efforts *V.p.* illnesses persist. There are several possible explanations for this. It could be the result of more oysters being produced for raw consumption and therefore greater exposure or because the adopted controls are ineffective or because of improper handling during retail distribution and sale at facilities beyond the authority of ISSC to control or because of increased reporting of illnesses because of improved awareness or changes in reporting procedures. Regardless of the reason, the fact is consumers continue to get ill from eating raw shellfish contaminated with *V.p.* bacteria and it is incumbent on the ISSC to consider all options for reducing *V,p.* illnesses. With this proposal we hope to enlighten ISSC participants to the apparent efficacy of utilizing a < 100 MPN/gram tlh standard to reduce V.p. illnesses and establish the standard as an option for states to use.

While based in Washington State, Taylor Shellfish Farms has farms, a processing facility and oyster bar in British Columbia. Because of this we are familiar with Canadian *V.p.* regulations. Following a *V.p.* outbreak in 2015 Canada implemented a requirement for processors to reduce total V.p. (tlh) levels below 100 MPN/gram prior to sale or distribution. This new regulation appears to have been effective at reducing *V.p.* illnesses while adjacent Washington State continues to see significant *V.p.* illnesses despite a vibrio control plan updated in 2015 with stringent harvest controls and time to documented temperature reduction.

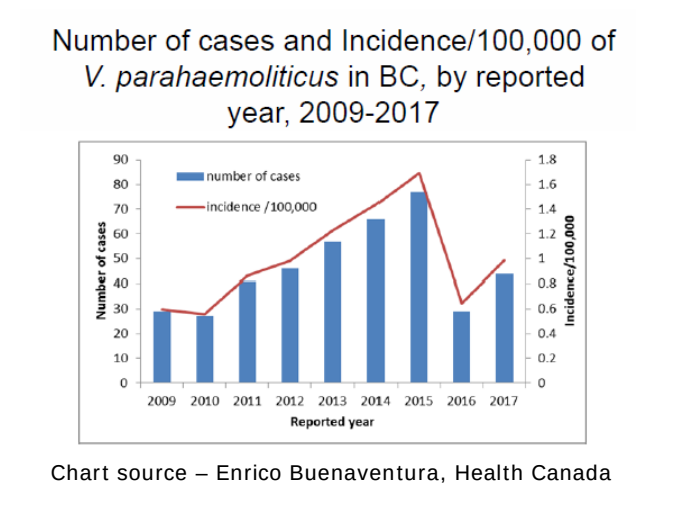


Chart source – Enrico Buenaventura, Health Canada

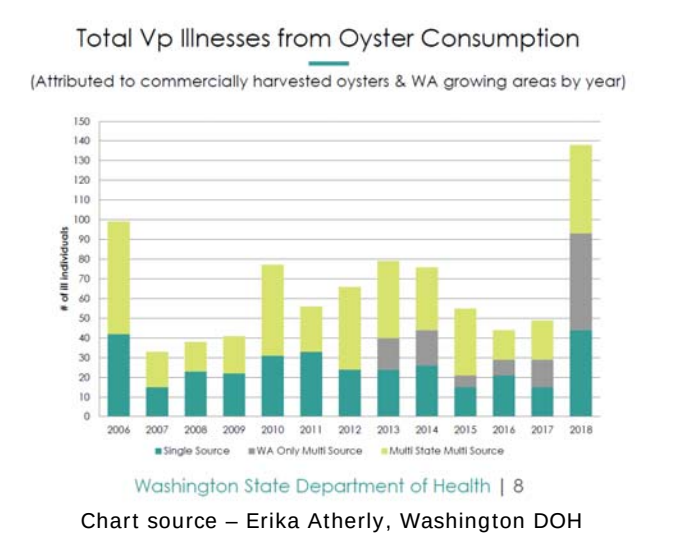


Chart source – Erika Atherly, Washington DOH

On Taylor Shellfish farms in British Columbia (d.b.a. Fanny Bay Oyster) we can predictably achieve the < 100 MPN/gram Canadian standard by holding oysters in culture trays at growing densities in 12-15 C water for 5 to 7 days. In Washington, we are achieving similar results after holding shellfish in a chilled recirculating wet storage system at 15 C for 3 days.

The current Chapter II. Risk Assessment and Risk Management @.02 Shellfish

	Related Illnesses Associated with *Vibrio parahaemolyticus* (*V.p.*), Section A. (6)(c) allows for harvest from areas closed due to *V.p.* with "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". This could provide the opportunity for a SSCA to allow the use of the < 100 MPN/gram to permit harvest. We are submitting this proposal to draw attention to the effectiveness of the < 100 MPN/gram tlh standard and clearly state that it is an option for inclusion in state vibrio control plans. As proposed, it is our understanding and intent that this would be an option and not mandatory. If adopted it would provide companies with an option to continue harvesting and distribution of a reduced risk product during V.p. closures. The International Commission on Microbiological Standards for Foods (ICMSF) advises that < 100 MPN/gram would be of acceptable quality in live bivalve Mollusca. Other countries, including Japan for fresh/frozen fish and shellfish and Hong Kong, Australia, New Zealand in Ready to Eat (RTE) foods and Russia (for imported shellfish) have adopted the 100 MPN/gram standard. U.S. companies exporting live shellfish to countries that have adopted this standard already have to demonstrate their product achieves the standard. This is yet another reason we feel it makes sense for the U.S. to consider including it as an option in the Model Ordinance. As a major seafood and shellfish consumer Japan has had a history of large numbers of *V.p.* illnesses. Their response warrants review as it appears to have been very effective at reducing illnesses. Following a peak in 1998 with 839 outbreaks and 12,318 cases, Japan's Ministry of Health, Labor and Welfare (MHLW) instituted a series of regulations from production through consumption including adoption of a ≤ 100 MPN/gram standard. Subsequently, the number of cases and out- breaks of *V. parahaemolyticus* infections decreased by an unprecedented 99- and 93-fold, respectively, from 1998 to 2012. The 2014 paper: Impact of seafood regulations for *Vibrio parahaemolyticus* infection and verification by analyses of seafood contamination and infection by Kara-Kudo and Kumagai reviews Japan's response including an explanation of how they arrived at the ≤ 100 MPN/gram tlh standard while considering various serotypes and pathogenic thermostable direct haemolysin (TDH) and/or TDH-related haemolysin (TRH)-positive strains. Further, according to Kara-Kudo and Kumagai's review article total V. parahaemolyticus levels in seafood associated with 11 outbreaks from 1998 were analyzed. The contamination levels in 8 out of 11 outbreaks were >100 V. parahaemolyticus MPN/g food, suggesting that the regulatory level of ≤100 V. parahaemolyticus MPN/g is effective for food control. Taylor Shellfish Farms is confident based on recommendations from the International Commission on Microbiological Standards for Foods (ICMSF), that results seen in BC and documented in Japan that the < 100 MPN/gram tlh standard provides considerable *V.p.* illness risk reduction. So much so that we have begun construction of a 90,000 gallon chilled live holding system at our Shelton, Washington processing facility with the goal of ensuring all our shellfish destined for raw consumption meets this standard.

14. Cost Information	If adopted as intended, it would be optional for states to include it in their vibrio control plans and for companies to pursue validation of a process to achieve the standard. It is anticipated that the tests associated with the validation process and periodic verification would be at the expense of the participating company. The costs would only be incurred if a company opted to pursue validation of their process. It is anticipated that states would recoup the cost of the validation tests if they were performed at a state operated laboratory. Presumably SSCAs could also impose fees to cover cost associated with overseeing validation of a company's process and periodic verification. Costs incurred by companies would theoretically be recouped by having the advantage of continued sales when growing areas might otherwise be closed due to *V.p.*.

Proposal No. 19-104

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Centers for Disease Control and Prevention (CDC)
3. Affiliation	CDC
4. Address Line 1	1600 Clifton Road
5. Address Line 2	MS H24-9
6. City, State, Zip	Atlanta, GA 30329
7. Phone	404-718-1175
8. Fax	404-235-1735
9. Email	Estokes@cdc.gov
10. Proposal Subject	*Vibrio vulnificus* risk evaluation
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter II. Risk Assessment and Risk Management @.06 *Vibrio vulnificus* Control Plan Section III. Public Health Reasons and Explanations Chapter IV. Shellstock Growing Areas @.01 Sanitary Survey ISSC Constitution, Bylaws & Procedures Procedure XVI. Procedure for *Vibrio vulnificus (V.v.)* Illness Review Committee Procedures
12. Text of Proposal/ Requested Action	**Section II. Model Ordinance Chapter II. Risk Assessment and Risk Management @.06 *Vibrio vulnificus* Control Plan** C. All States not currently implementing a *V.v.* Control Plan shall develop and implement a *V.v.* Control Plan ~~should~~ if the risk evaluation indicates two (2) or more etiologically confirmed, and epidemiologically linked *V.v.* ~~septicemia~~ illnesses from ~~the~~ consumption of commercially harvested ~~raw or undercooked~~ oysters that originated from the growing waters of that State within the previous ten (10) years **Section III. Public Health Reasons and Explanations Chapter IV. Shellstock Growing Areas @.01 Sanitary Survey** **A.** General. 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. Feces deposited on land surfaces can release pathogens into surface waters via runoff. Most freshwater streams eventually empty into an estuary where fecal bacteria and viruses may accumulate in sediment and subsequently can be re-suspended. Shellfish pump large quantities of water through their bodies during the normal feeding process. During this process the shellfish also concentrate microorganisms, which may include pathogenic microorganisms. Epidemiological investigations of shellfish-caused disease outbreaks have found difficulty in establishing a direct numerical correlation between the

bacteriological quality of water and the degree of hazard to health. 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 portions of water examined were positive for coliforms (an MPN of approximately seventy [70] per 100 ml), provided the areas were not subject to direct contamination with small amounts of fresh sewage which would not be revealed by bacteriological examination.

Following the oyster-borne typhoid outbreaks during the winter of 1924-25 in the United States, the NSSP was initiated by the States, the Public Health Service, and the shellfish industry. Water quality criteria were then 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 dilution of the growing area water. Once the standards were adopted in the United States in 1925, reliance on this three-part standard for evaluating the safety of shellfish harvesting areas has generally proven effective in preventing major outbreaks of disease transmitted by the fecal-oral route. Similar water quality criteria have been used in other countries with favorable results.

Nevertheless, some indicators and pathogens are capable of persisting in terrestrial soil, fresh and marine waters, and aquatic sediment for many days while others are even capable of growth external to a host. A small number of shellfish-borne illnesses have also been associated with bacteria of the genus Vibrio. The *Vibrio spp.* are free-living aquatic microorganisms, generally inhabiting marine and estuarine waters.
Among the marine *Vibrio spp.* classified as pathogenic are strains of non-01 *Vibrio cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. All three (3) species have been recovered from coastal waters in the United States and other parts of the world. These and other *Vibrio spp.* have been detected in some environmental samples recovered from areas free of overt sewage contamination and coliform.

In general, shellfish-borne Vibrio infections have tended to occur in coastal areas in the summer and fall when the water was warmer and *Vibrio* spp. counts were higher. *V. parahaemolyticus* and non~~-01~~O1 *V. cholerae* are commonly reported as causing diarrhea illness associated with the consumption of seafood including shellfish. In contrast, *V. vulnificus* has been related to~~ two (2) distinct syndromes:~~ wound infections, invasive disease usually characterized by bacteremia, and less commonly diarrheal illness associated with the consumption of seafood. ~~often with tissue necrosis and bacteremia, and primary septicemia characterized by fulminant illness in individuals with severe chronic illnesses such as liver disease, hemochromatosis, thalassemia major, alcoholism or malignancy. Increasing e~~Evidence shows that individuals with such chronic diseases such as liver disease, hemochromatosis, thalassemia major, alcoholism or malignancy are susceptible to ~~septicemia~~ severe illness and death from raw seafood, especially raw oysters. Shellfish-borne Vibrio infections can be prevented by cooking seafood thoroughly, keeping them from cross contamination after cooking, and

eating them promptly or storing them at hot (60 °C or higher) or cold (4 °C or lower) temperatures. If oysters and other seafood are to be eaten raw, consumers are probably at lower risk to Vibrio infection during months when seawater is cold than when it is warm.

In addition to pathogenic microorganisms, poisonous or deleterious substances may enter shellfish growing areas via industrial or domestic waste discharges, seepage from waste disposal sites, agricultural land or geochemical reactions. The potential public health hazard posed by these substances must also be considered in assessing the safety of shellfish growing areas.

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 the growing area to one (1) of five (5) classifications. The survey information must be updated periodically to ensure that it remains current and must be readily accessible to both the Authority and the harvester. Experience has shown that the minimum sanitary survey components required in this chapter are necessary for a reliable sanitary survey. A more detailed explanation is provided in the NSSP Model Ordinance Guidance Documents: *Sanitary Survey and the Classification of Growing Waters* (ISSC/FDA, 2017).

ISSC Constitution, Bylaws & Procedures Procedure XVI. Procedure for *Vibrio vulnificus (V.v.)* Illness Review Committee Procedures

Section 1. Committee Charge

The *V.v.* Illness Review Committee will annually review all *V.v.* cases involving the consumption of shellfish which are reported to FDA regional specialists and the Center for Disease Control (CDC). The Committee will determine which cases meet the case definition of a National Shellfish Sanitation Program (NSSP) *V.v.* case as outlined in Model Ordinance Section II. Chapter II. @.05. All cases meeting the NSSP definition will be included in an annual report which will be presented to the Interstate Shellfish Sanitation Conference (ISSC) Executive Board and the Vibrio Management Committee. Following ISSC Executive Board approval the report will be made available to the ISSC membership and posted on the ISSC website. This data is expected to be used by USFDA, State Authorities, and the ISSC for the following purposes:

Subdivision a. Conducting annual *V.v.* Risk Evaluations;
Subdivision b. Risk per serving determinations;
Subdivision c. *V.v.* Control Plan Evaluations;
Subdivision d. *V.v.* Contingency Plan Evaluations; and
Subdivision e. Reviewing illness trends.

Section 2. Procedures.

Subdivision a. The Committee will only consider cases that are

reported on a CDC and Prevention Cholera Vibrio Illness Surveillance Report (COVIS) Form CDC 52.79 or other means.

Subdivision b. FDA will coordinate the collection of cases and COVIS forms, and other information and after redacting identifying information will make this information available to the Committee.

Subdivision c. The information from the COVIS forms will be shared with the *V.v.* Illness Review Committee for review.

Subdivision d. The *V.v.* Illness Review Committee will review the cases and incorporate the appropriate information into a chart which will serve as the Committee report.

Subdivision e. The report will be presented to the ISSC Executive Board for approval and then forwarded to the Vibrio Management Committee.

Subdivision f. The availability of the report will be announced to the ISSC membership.

A copy of the report will be posted on the ISSC website.

Section 3. Criteria and Guidelines.

The Committee will use the following criteria and guidelines in reviewing reported cases:

Subdivision a. Was the illness etiologically confirmed? In this context "etiologically confirmed "shall mean laboratory confirmation by wound, stool or blood culture. Confirmation may be by a laboratory otherthan a State laboratory."

Subdivision b. Was the illness epidemiologically linked to shellfish? Epidemiologically linked will mean "associated with" the consumption of oysters. Consumption means ingested; eaten within 7 days of onset of symptoms. Date of onset may be before hospitalization. Further information may be warranted; discretion may be exercised.

Subdivision c. Were the shellfish consumed?

Subdivision d~~e~~. Were the shellfish commercially harvested? Commercially harvested shall mean the shellfish were intended for sale or distribution in commerce. Commercial harvest will include those cases involving a foreign state.

~~Subdivision d.~~ ~~Were the shellfish raw or undercooked? If the victim developed *V.v.* septicemia after consumption the shellfish are considered to have been raw or undercooked.~~

Subdivision e. From what State was the shellfish harvested?

Subdivision f. ~~Did the case involve septicemia from consumption:~~

~~The following guidance will be used in~~

		~~determining if the case is a septicemia or a gastroenteritis case. Clinical signs and symptoms *V.v.* septicemia include:~~ A case of severe *V.v.* is defined as illness in a person who had V. vulnificus infection confirmed by bacterial culture and either of the following:	
		Subdivision i.	*V. vulnificus* was isolated from blood or a site that likely indicates invasive disease (see specimen source table).~~*V.v.* bacteria isolated from blood~~.
		Subdivision ii.	Any of the following were indicated on the COVIS case report form: 1. Fever 2. Septic Shock 3. Death Any of the following sequelae: necrosis; or invasive procedure, such as surgery, amputation, skin graft, wound debridement, fasciotomy, or incision and drainage~~Fever measured as above 100 degree Fahrenheit~~.
		~~Subdivision iii.~~	~~Death as outcome (septicemia has a mortality rate of over 50% – 70%).~~
		~~Subdivision iv.~~	~~Bullae (blood filled blisters) but this also can occur after a wound infection which becomes septic.~~
		~~Subdivision v.~~	~~Shock because of the sepsis (again this can happen also because of a wound infection).~~
	~~Subdivision g.~~	~~Indications case may not be *V.v.* septicemia from consumption:~~	
		~~Subdivision i.~~	~~Bacteria are only isolated from wound fluid or stool and no clinical evidence of septicemia.~~
		~~Subdivision ii.~~	~~Cellulitis. Since cellulitis is a localized or diffuse inflammation of connective tissue with severe inflammation of dermal and subcutaneous layers of the skin (bacteria entering~~

~~bodies through the skin, there might be a visible wound or just a small scratch), therefore more likely a wound infection.~~

~~Subdivision iii.~~ ~~History of pre-existing and sustained wound infection (If both wound and oyster/seafood consumption is documented and happened within the incubation period, there is no way to differentiate why the patient is septic.)~~

~~Subdivision iv.~~ ~~Septicemia has a much shorter incubation period compared to gastroenteritis, according to CDC data. *V.v.* septicemia has an incubation period between 12-72 hours, although we have seen cases with shorter incubation periods.~~

Section 4. Challenges to Committee Findings.

Persons wishing to challenge the information included in the report must notify the ISSC Executive Director within sixty (60) days of the posting of the report on the ISSC website. The ISSC Executive Board will review all challenges at the next scheduled Executive Board meeting.

Section 5. *V.v.* Case Appeal Procedure

Subdivision a. Appropriate V.v. information will be provided to the reporting and source States at least 60 days prior to committee review. The States will be given 30 days from the date of receipt to respond.

Subdivision b. Following V.v. Illness Review Committee review, each source State with a countable case will be notified.

Subdivision c. Should a source State disagree with the Committee determination on a specific case, the source State will be provided thirty (30) days to file an appeal.

Subdivision d. Should the Committee, based on the information provided by the appellant, conclude that the original determination should be reversed, the appellant will be notified.

Subdivision e. Should the Committee, based on the information provided by the appellant, conclude that the original determination was appropriate; the Committee will provide the appellant an

opportunity to state their position. This opportunity will be either by telephone conference call or in person. The choice of venue will be determined by the Committee and will not exceed fifteen (15) minutes.

Subdivision f. The Committee will consider information presented by the appellant in the oral presentation. The appellant will be notified of the final decision of the Committee.

Subdivision g. The appellant will receive a final decision from the Committee no more than 30 days after the date the appeal is submitted; if a decision can NOT be made after 30 days, then an appeal extension must be granted by the committee, or the appeal will be considered denied.

Table: Specimen sources that likely reflect invasive disease

Blood: Includes plasma and blood components
Vascular: Includes heart, heart valves, aorta, blood vessels
Lymphatic: Includes lymph, lymph nodes, thymus
Spleen: Includes spleen, splenic abscesses
Bone: Includes bone, bone marrow
Placenta and products of conception: Includes fetus, cord blood
Nervous system Cerebrospinal fluid (CSF) Other nervous tissue; includes brain abscess
Pleural fluid
Peritoneal fluid
Joint: includes synovial/joint fluid
Hepatobiliary: Gallbladder, bile, liver (includes abscesses)
Pancreas: Includes pancreas, pancreatic cysts, and abscesses
Reproductive: Ovary, fallopian tube, uterus (includes cysts and abscesses in these sites), pelvic abscesses, amniotic fluid
Kidney: Includes renal and perinephric abscess

ISSC *Vibrio vulnificus* Illness Review Criteria Table

Review Date: _____

Case Identifier/Number:	Criteria Status		
Criteria	Yes	No	Unknown
1. Etiologically Confirmed? ~~Blood Stool~~			
2. Epidemiologically Linked?			
3. ~~Septicemia~~ Severe Illness?			
4. Reporting State?			
5. Commercial Harvest?			

6. Were shellfish consumed?				
a. Specify shellfish consumed:		Oysters	Clams	Specify Other
b. Date of consumption: _____				
c. Is onset consistent with consumption of shellfish? Date of onset _____				
7. Trace-back Information				
a. Were shipping tags available? If other trace-back information reported, list:				
b. State of harvest, harvest area (s), and harvest date (list all reported).				

Harvest	Harvest	Harvest	Species	Comment

13. Public Health Significance	Septicemia is an outdated term no longer commonly used in medicine or public health. An alternative strategy of considering only "severe" cases to reflect the magnitude of risk from food is problematic, because 1) the severity of an illness may depend on factors other than the food, such as the patient's age, underlying health conditions, access to healthcare, bacterial load ingested, and appropriateness of medical treatment, and 2) data collection practices, state resources, and availability of data can vary by geography and over time. This makes the reporting of "severe" cases potentially inconsistent. Surveillance data on method of preparation can be limited and subjective. Any oyster that transmits illness can be considered insufficiently cooked; consumers may not realize they have eaten an undercooked food. Counting all etiologically confirmed cases associated with consumption of commercially harvested oysters is the most clear and consistent measure of *V. vulnificus* illness risk to the public.

14. Cost Information	NA

Proposal No. 19-105

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Scott Berbells
3. Affiliation	Washington State Department of Health
4. Address Line 1	P.O. Box 47824
5. Address Line 2	
6. City, State, Zip	Olympia, Washington 98504-7824
7. Phone	360.236.3324
8. Fax	360.236.2257
9. Email	Scott.Berbells@doh.wa.gov
10. Proposal Subject	Laboratory approval for sample analysis with no Model Ordinance defined method or action level
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter III. Laboratory @.01 Quality Assurance (A)
12. Text of Proposal/ Requested Action	Chapter III. @.01 A. NSSP Conformance Required~~. for all laboratories supporting the NSSP~~. All laboratory analyses for compliance with classification requirements that require a specific method, actions level, and use defined in the Model Ordinance shall be performed by a laboratory found to conform or provisionally conform by the FDA Shellfish LEO or FDA certified State Shellfish LEO in accordance with the requirements established under the NSSP.
13. Public Health Significance	This proposed amendment to Chapter III, @.01 (A) updates the requirement related to the use of data analyzed by a laboratory that has not been certified by the FDA Shellfish LEO or FDA certified State Shellfish LEO and potentially used for regulatory purposes. The amendment allows state shellfish authorities to use non FDA approved laboratories when methods and action levels have not been defined in the Model Ordinance. Washington state has developed an extensive array of partnerships aimed at evaluating pollution conditions around shellfish growing areas primarily related to microbiological conditions and remediating any impacts identified. Local and state government agencies, tribes, and wastewater treatment plant operators collect data that may be used by the Shellfish Authority to manage the status of shellfish harvesting areas. Sampling activities from sewage spills, agricultural manure discharges, failing septic systems, and treatment loss at wastewater treatment plants have resulted in temporary closures of harvest areas. In turn, data collected from partner agencies has been used to identify when the pollution issue has been resolved and when the growing area can be opened. All sample analysis is completed by laboratories inspected by state regulatory agencies but have not evaluated for conformance by the FDA Shellfish LEO or FDA certified State Shellfish LEO.

	Washington state periodically uses laboratory analysis to determine if shellfish and shellfish harvesting areas are impacted by poisonous and deleterious substances. Shellfish closures or consumption advisories may be implemented based on this data. There are currently no laboratories approved by FDA Shellfish LEO for the analysis of poisonous and deleterious substances. The proposal assures that an FDA approved laboratory is required when laboratory methods and action levels are defined in the Model Ordinance and data may be used for regulatory action (marine water quality, marine biotoxins, Male Specific Coliphage). This proposal will give state shellfish authorities the flexibility to adapt to ongoing environmental conditions and make appropriate public health decisions based on laboratory data.
14. Cost Information	

Proposal No. 19-106

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Executive Office
3. Affiliation	Interstate Shellfish Sanitation Conference
4. Address Line 1	209 Dawson Road
5. Address Line 2	Suite 1
6. City, State, Zip	Columbia, SC 29223
7. Phone	(803) 788-7559
8. Fax	(803) 788-7576
9. Email	issc@issc.org
10. Proposal Subject	Delete Notification Requirement to Pollution Control Agencies
11. Specific NSSP Guide Reference	Section II Model Ordinance Chapter IV Shellstock Growing Areas @.01
12. 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, 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 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 file shall include all data, results, and analyses from: (a) The sanitary survey; (b) The triennial reevaluation; and (c) The annual review. ~~(4) Wherever possible, the Authority shall provide the necessary information to Federal, State, or local agencies which have the responsibility to minimize or eliminate pollution sources identified in the sanitary survey.~~ ~~(5)~~(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.
13. Public Health Significance	This requirement does not have public health significance.

14. Cost Information	

Proposal No. 19-107

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Determining shoreline survey area.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas Section @.01 Sanitary Survey D.(1) and (2)(a).
12. Text of Proposal/ Requested Action	(1) In the shoreline survey for each growing area, the Authority shall: (f) Conduct an in-field assessment of pollution sources which may include: (i) A drive-through survey; (ii) Observations made during sample collection; and/or (iii) 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 ~~in-field~~ investigation ~~which identifies only the properties with the potential to impact the shellfish waters~~ that shall include, but not limited to, all properties with the potential to impact the shellstock growing area based on area topography, as well as field observations, and other sources of information;
13. Public Health Significance	The minimum requirements of the shoreline survey include an investigation and evaluation of pollution sources by trained, qualified, personnel. The investigation must be accomplished through an in-field assessment where the surveyor identifies actual and potential sources of pollution that might influence water quality. Given the technology available today, there are mutltiple options for identifing properties with the potential to impact growing areas. The Authority can define the shoreline survey area boundry by using various data resources such as geoprapohic information such as on-line maps. Using the term “only” as it is used in the existing language is confusing and, if taken literally, limiting. Example: One property two miles from the growing contains a large wastewater treatment plant that has the potential to impact shellfish waters. Another property one- and one-half miles from the growing area between that growing area and the property with the wastewater treatment plant on it has no identifiable pollution sources on it so that it does not have potential to impact shellfish waters. If the shoreline survey area is defined as a single area that includes the property with the

	wastewater treatment plant, it will also include the property with no identifiable pollution sources on it. Thus, it will not be an area that has "only" the properties with potential to impact the shellfish waters in it.
14. Cost Information	No cost.

Proposal No. 19-108

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Robert Rheault
3. Affiliation	ECSGA
4. Address Line 1	1121 Mooresfield Rd
5. Address Line 2	
6. City, State, Zip	Wakefield RI 02879
7. Phone	(401) 783-3360
8. Fax	
9. Email	bob@ECSGA.org
10. Proposal Subject	Aquaculture Seed Shellstock
11. Specific NSSP Guide Reference	Section II Model Ordinance, Chapter VI. Shellfish Aquaculture, Requirements of the Authority @.02
12. 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. D. ~~C.~~ All sources of seed produced or collected in prohibited waters shall be sanctioned by the Authority.
13. 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. C. 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)
14. Cost Information	Proposal would not impact the enforcement costs for the authority and would simplify management for growers.

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

SAMPLE COLLECTION FORM

LAB # 210357 12 AM 10:09

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

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**
		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		6, 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	DATE 12.12.01	TIME 10:15 AM	HOW STORED on ice
AGENT # PRINT NAME 164 John Mellen	PRINT NAME Theodore G. Tsikas			
SIGNED FROM	SIGNED TO			
PRINT NAME Theodore G. Tsikas	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 213115 29 MAR 9:22

SAMPLE COLLECTION FORM

4/5/02 - 4/9/02 4/12/02 PLE

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
3/28/02 Paul By ICE/Jus	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 / SOURCE
			SAMPLE #1 SM 11 SM 17 SM 18 / 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 Phan Tw	DATE 3-29-02	TIME 9.30	HOW STORED in cooler	
AGENT'S PRINT NAME 16 4 John Miller	PRINT NAME				
SIGNED FROM Phan Tw	SIGNED TO Kerry Patterson	3-29-02	10:00	in refrigulator 7	
PRINT NAME Phanca Tw	PRINT NAME Kerry PATTERSON				
SIGNED FROM Kerry Patterson	SIGNED TO Chris Ellis	4/12/02	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 13115 29 MR 922

SAMPLE COLLECTION FORM

Analyst By: Miller Miller Miller PCE

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
3/28/02 PCE/RES	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 [signature]	SIGNED TO [signature]	DATE 3-29-02	TIME 9:30	HOW STORED in cooler	
AGENT # PRINT NAME 164 John Miller	PRINT NAME				
SIGNED FROM [signature]	SIGNED TO [signature]	3-29-02	10:00	in Refrigerator	
PRINT NAME Phang Tan	PRINT NAME Kerry L Patterson				
SIGNED FROM [signature]	SIGNED TO [signature]	4/2/02	10:40	Ref	
PRINT NAME Kerry Patterson	PRINT NAME Chris Eley				

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 - Water 4/16/02 PCE

DATE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
3/28/02 Final By PCE (Tue)	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	SOURCE
			10

FOLLOW UP SAMPLE #1: SM 11 SM 13 SM 27

ANALYSIS: 24(8,13)

SHIPPER/PACKER & ADDRESS

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

1. CEREAL %	2. MEAT PROT %	3. TOT H_2O %	4. SOY PL %	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	57. TOT COLIFORM/MPN	58. CL BOT	59. CL PERF	60. COAG STAPH	61. NON-COAG STAPH	62. LISTERIA	63. CAMPYLO
<20	<20						

64. SPC	65. SALMONELLA	66. B CEREUS	67. YERSINIA	ORGANOLEPTIC
2100				

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:
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

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

SAMPLE COLLECTION FORM

LAB #

TE OF COLLECTION	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
8/4/98 Amal BX MLS	8/13/98	8/17/98	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	REASON
		Billington Cove Maine	01
		ADDRESS	SOURCE
			10
SHIPPER/PACKER & ADDRESS		FOLLOW UP SAMPLE #1	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 M. Silva	DATE 8/4/98	TIME 1:45	HOW STORED ambient
AGENT PRINT NAME 164 John Mullen	PRINT NAME Molly Ann 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. $NaNO_3$ 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:

Moonstone 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 #

SAMPLE COLLECTION FORM

202209 16 PM 1:52

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 Ellis	DATE 11/16/00	TIME 2pm	HOW STORED on ice	
AGENT# PRINT NAME 164 John Muller	PRINT NAME Chris Ellis				
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 Hesselt

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 GOLDENROD - 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 Ant Bx MWS		10/27/00	10/27/00	PCE

CONDITION	SEALED	ITEM	PRODUCT CODE/DOM	MOP	SAMPLE CODE
HOT FROZEN COLD OTHER	YES NO	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: 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				
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

SAMPLE COLLECTION FORM

LAB # 198250 13 AM 10:32

DATE OF COLLECTION	Analyzed By	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
7/13/00	MLS	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 [signature]	SIGNED TO [signature]	DATE 7/13/00	TIME 10:35	HOW STORED ambient
AGENT PRINT NAME 164 John Miller	PRINT NAME MAKEPEACE/LOA			
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

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

SAMPLE COLLECTION FORM

LAB # 198249 13 AM 10:32

DATE OF COLLECTION	Rec'd By	DATE OF ANALYSIS	DATE OF REPORT	LAB SUP
7/13/00	MS	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
		Moorstone 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 / 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 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
Rept. '99

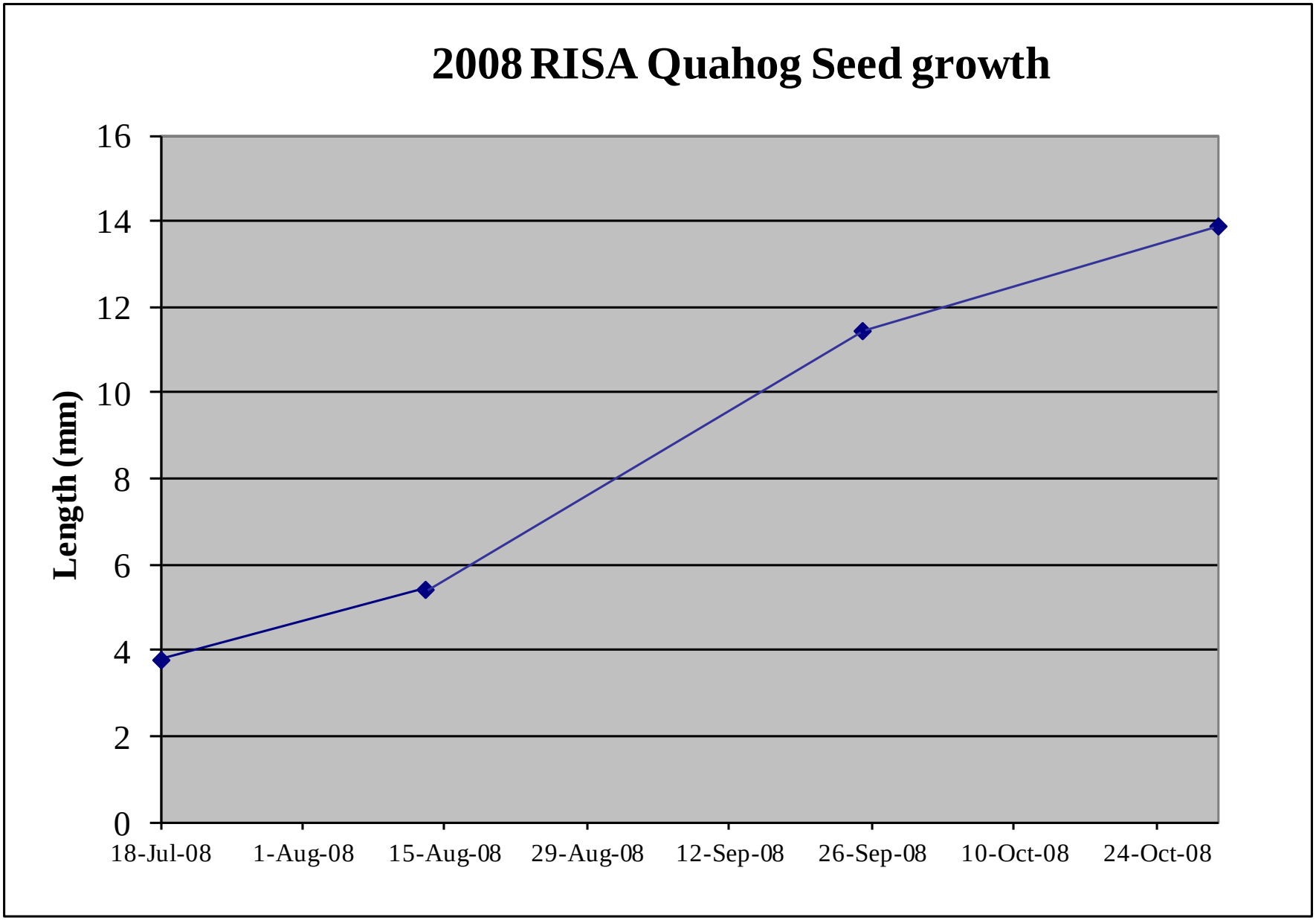
LABORATORY NOTES:
Fe: 38.3 ppm

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


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 No. 19-109

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Jill Fleiger
3. Affiliation	Department of Agriculture and Consumer Services
4. Address Line 1	600 S Calhoun Street
5. Address Line 2	Suite 217
6. City, State, Zip	Tallahassee, FL, 32399
7. Phone	850-617-7615
8. Fax	850-617-7601
9. Email	Jillian.Fleiger@freshfromflorida.com
10. Proposal Subject	Offshore State Water classification requirements
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.02
12. Text of Proposal/ Requested Action	**@.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 assure 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 where a sanitary survey shows that there are no pollution sources that will impact the microbiological quality of the water. Offshore state waters are classified as approved.
13. Public Health Significance	State waters extend 9 miles off shore of the State of Florida. If a sanitary survey can show there are no pollution impacts (ie. Rivers, WWTPs discharges) to proposed areas for aquaculture the required 30 samples to classify should not be required.
14. Cost Information	This would reduce the cost and burden to state authorities having to sample waters that are far removed from any potential pollution sources.

Proposal No. 19-110

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Point source approved standard station locations.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas Section @.02 Microbiological Standards E.(3)(c).
12. Text of Proposal/ Requested Action	(c) Sample station locations shall be adjacent to actual or potential sources of pollution and adequate in terms of number and spatial distribution to support the conclusion that the growing area is characterized by water quality meeting the approved classification bacteriological requirements.
13. Public Health Significance	Stations in waters classified as approved are frequently not adjacent to pollution sources. Stations represent a miniscule portion of points within a growing area. The stations should be located so that it is reasonable to believe that, if a station were established at any point in the area where no station currently exists, that new station would yield bacteriological data meeting the relevant bacteriological standard consistent with the classification.
14. Cost Information	No cost.

Proposal No. 19-111

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Scott Berbells
3. Affiliation	Washington State Department of Health
4. Address Line 1	P.O. Box 47824
5. Address Line 2	
6. City, State, Zip	Olympia, Washington 98504-7824
7. Phone	360.236.3324
8. Fax	360.236.2257
9. Email	Scott.Berbells@doh.wa.gov
10. Proposal Subject	Allowing the use of the SRS method in areas impacted by point sources
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.02E; Chapter IV. Shellstock Growing Areas @.02F; Chapter IV. Shellstock Growing Areas @.02F(2)(b); Chapter IV Shellstock Growing Areas @.02G; and Chapter IV. Shellstock Growing Areas @.02H
12. Text of Proposal/ Requested Action	Chapter IV, @.02 E. Standard for the Approved Classification of Growing Areas ~~Affected by Point Sources~~ when Evaluated for Adverse Pollution Conditions. Chapter IV, @.02 F. Standard for the Approved Classification of Growing Areas ~~Affected by Nonpoint Sources~~ 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~~:~~ ~~(a)~~ Impacted only by randomly occurring, intermittent events~~; and~~ ~~(b) Not impacted by discharges from sewage treatment facilities or combined sewer overflows.~~ Chapter IV, @.02 G. Standard for the Restricted Classification of Growing Areas ~~Affected by Point Sources~~ when Evaluated for Adverse Pollution Conditions and Used as a Shellstock Source for Shellstock Depuration. Chapter IV, @.02 H. Standard for the Restricted Classification of Growing Areas ~~Affected by Nonpoint Sources~~ when Evaluated for Nonpoint Sources and Used as a Shellstock Source for Shellstock Depuration
13. Public Health Significance	This proposed amendment to Chapter IV, @.02 updates the conditions under which

	the APC and SRS methods may be used. The proposal allows the use of the SRS method in areas impacted by discharges from sewage treatment facilities or combined sewage overflows where marine water stations have been placed to monitor nonpoint pollution. The intent of this proposal is to use the sampling methodology and statistical analysis most acceptable for the purpose of the marine water sampling station. If the station is placed to monitor nonpoint pollution, the SRS methodology should be used. If the station is placed to monitor adverse pollution conditions, the APC methodology should be used. In Washington state, marine water stations located in Conditionally Approved areas impacted by wastewater treatment plants are placed to monitor nonpoint pollution from the surrounding upland areas. The APC criterion is used to sample and evaluate data from these stations with the adverse condition defined as an upset at the treatment plant. Many wastewater treatment plants are high performing and upset conditions occur infrequently. The infrequency of the impact to the growing area does not allow for the intended use of the APC sampling strategy. Hydrographic studies and dilution analyses are more appropriate for the evaluation of the impact area around high performing wastewater treatment plants.
14. Cost Information	No impact

Proposal No. 19-112

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Nonpoint source approved standard station locations.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas Section @.02 Microbiological Standards F.(6)(b)(i).
12. Text of Proposal/ Requested Action	(i) Sample station locations ~~are~~ shall be adequate ~~to produce the data to effectively evaluate all nonpoint sources of pollution~~in terms of number and spatial distribution to support the conclusion that the growing area is characterized by water quality meeting the approved classification bacteriological requirements;
13. Public Health Significance	The Model Ordinance Chapter IV.@.02B indicates "The Authority shall assure that the number and location of sampling stations is adequate to effectively evaluate all pollution sources." That includes all nonpoint sources of pollution so there is no need to state that requirement within IV.@.02F. Stations represent a miniscule portion of potential points within a growing area. The stations should be located so that it is reasonable to believe that, if a station were established at any point in the area where no station currently exists, that new station would yield bacteriological data meeting the relevant bacteriological standard consistent with the classification.
14. Cost Information	No cost.

Proposal No. 19-113

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Authorizing unclassified areas and multiple classifications for single area.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas Section @.03 Growing Area Classification A.(2).
12. Text of Proposal/ Requested Action	(2) Classification of ~~All~~ Growing Areas. ~~All~~ Each growing ~~areas~~area which: (a) ~~Are~~ 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~~ (b~~e~~) ~~Are~~ 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 ~~only~~ one or more~~(1)~~ of the following: (i) Approved; (ii) Conditionally Approved; (iii) Restricted; (iv) Conditionally Restricted; and/or (v) Prohibited.
13. Public Health Significance	There is no reason to require that all growing areas be classified if the Authority is required to treat unclassified areas as prohibited areas. The current Section II. Chapter IV.@.03A.(2)(b) language is unnecessary. Requiring that each growing area be characterized by only one classification is not realistic and does not reflect common practice. There are many circumstances in which one growing area contains several classifications. Example: A 10 square mile growing area is generally classified as approved. However, there is a marina in it, so some waters associated with that marina are classified as prohibited and restricted. There is a business with a 5,000 gallon per day wastewater treatment system discharging along the shoreline so there is a prohibited zone adjacent to that point source. That circumstance literally represents violation of Chapter IV.@.03A.(2)(c) as that requirement now reads because there are multiple classifications within a single growing area.

14. Cost Information	No cost.

Proposal No. 19-114

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Emergency Conditions re-opening studies.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas Section @.03 Growing Area Classification A.(5)(c)(i).
12. Text of Proposal/ Requested Action	(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. When pathogens are of concern, ~~S~~studies establishing sufficient elapsed time shall document the interval necessary for reduction of ~~contaminant~~ coliform levels in the shellstock to pre-closure levels. ~~In addressing pathogen concerns, the~~ Such coliform studiesmay establish criteria for reopening based on coliform levels in the water. When poisonous or deleterious substances are the concern, studies 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
13. Public Health Significance	National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish, Section IV Guidance Documents, Chapter II Growing Areas, .08 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood contains target levels for many poisonous or deleterious substances. Target levels for other substances can be established through risk evaluation. The 2010 Deepwater Horizon crisis provides an example of how emergency conditions involving poisonous or deleterious substances are addressed in practice. Levels of concern were established through risk evaluation then areas were re-opened based on determining that contaminant levels were below levels of concern rather than based on comparisons between pre and post closure levels.
14. Cost Information	Cost would potentially be reduced because studies to compare post closure levels of poisonous or deleterious substances to pre closure levels would no longer be required.

Proposal No. 19-115

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Kathy Brohawn
3. Affiliation	Maryland Department of Environment
4. Address Line 1	Montgomery Park
5. Address Line 2	1800 Washington Blvd.
6. City, State, Zip	Baltimore, MD 21230
7. Phone	410 537 3608
8. Fax	410 537 3998
9. Email	Kathy.brohawn@maryland.gov
10. Proposal Subject	Emergency Conditions/closed status to reflect Chapter II use of harvest area
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.03 Growing Area Classification A. General (1) and (5)
12. Text of Proposal/ Requested Action	@.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 unpredicted pollution conditions exist ~~which were not included in the database 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. (a) If the growing area or harvest area is already closed due to resource conservation under existing fishery laws or regulation, the area is considered to be in the closed status. If the authority choses to uses this approach, an MOU detailing coordination and, communication between agencies and patrol shall be required. ~~(a)~~(b) If no harvest areas are impacted by Emergency Conditions, placement into the closed status is not required. (2)…….. (3)..... (4)…… (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 for a limited or temporary period 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) 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:
13. Public Health Significance	Closed status following an emergency situation can include an entire growing area or a harvest area within the growing area; This change is consistent with Chapter II where, if appropriate, only a harvest area is closed due to an outbreak and not necessarily the entire growing area. In addition, the text stating conditions that were not included in the data base makes no sense related to emergency conditions and actually state the obvious. Deletion of that statement clarifies this part of the MO.
14. Cost Information	There should be no need to close an area that has no shellfish resource or is already closed by existing regulation. If this proposal is accepted by the Conference, it would save money for any state that is required to post closures in the newspaper (public notice); For Maryland the cost is ~$1500, so it would represent a significant savings.

Proposal No. 19-116

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	J. Michael Hickey
3. Affiliation	Massachusetts Division of Marine Fisheries
4. Address Line 1	706 South Rodney French Blvd.
5. Address Line 2	
6. City, State, Zip	New Bedford, MA 02744
7. Phone	(508) 965-2273 (508) 742-9768
8. Fax	(508) 990-0449
9. Email	Michael.hickey@mass.gov
10. Proposal Subject	Adding a time frame to the limited or temporary period an area can be remain under a closed status prior to being reclassified.
11. Specific NSSP Guide Reference	Section II, Model Ordinance Chapter IV. Shellstock Growing Areas @.03 Growing Area Classification A. (5) (b).
12. Text of Proposal/ Requested Action	(b) Closed Status. Any classified growing area may be closed for a limited or temporary period, not to exceed more than one year prior to a reclassification because of: (i) An emergency…; (ii) The presence…; (iii) Conditions stipulated…; (iv) Failure of…; or (v) The requirements….
13. Public Health Significance	The M. O. Chapter IV @.03 A. (5) (b) states that any classified growing area may be closed for a limited or temporary period because of: (i) through (vi). The time frame "limited or temporary period "is not defined in the "Guide". The authority is required by @.03 A. (1) to place a growing area in the closed status ..." under Section @.03 A. (5) when pollution conditions exist which were not included in the database used to classify the area. If it is determined that an emergency condition or situation exists, then the growing area will be immediately (within 24 hours) placed in the closed status." Once the area is in the closed status, harvesting, attempting to harvest, possession, or sale of shellfish from the closed area is prohibited. A time limit of up to but not to exceed one year from the time the area was placed in the closed status allows the authority time with defined maximum to determine the source /cause(s) of a pollution or contamination problem before initiating a reclassification while still protecting public health by virtue of the area being in a closed status. The proposed change will not lessen public health protection.
14. Cost Information	Does not add any cost and may actually save administrative cost by averting multiple reclassifications in the process of sorting out the final correct classification.

Proposal No. 19-117

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	J. Michael Hickey
3. Affiliation	Massachusetts Division of Marine Fisheries
4. Address Line 1	706 South Rodney French Blvd.
5. Address Line 2	
6. City, State, Zip	New Bedford , MA 02744
7. Phone	(508) 965-2273 (508) 742-9768
8. Fax	(508) 990- 0449
9. Email	Michael.hickey@mass.gov
10. Proposal Subject	Shellfish cleansing studies
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.03 Growing Area Classification. C. Conditional Classifications. (2) (c) (iii)
12. Text of Proposal/ Requested Action	(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.
13. Public Health Significance	There are a number of problems related to the current M. O. language." There is no guidance or criteria in the Guide concerning what constitutes an adequate study. There are a number of study related questions: 1) How many shellfish samples of each species of shellfish and sampling stations (locations) are needed in a growing area; 2) Are studies required in every conditional area? 3) can information obtained in one growing area be applied to shellstock in another growing area? 4) The first sentence at (iii) refers "*to reducing pathogens...to acceptable levels*", what are acceptable levels of pathogens. The second sentence at (iii) refers to *reduction of coliform levels in shellstock to pre-closure levels*. Pre-closure levels in shellstock can be variable both temporally and spatially. Thus the concept of reducing coliforms to pre-closure levels is at best ambiguous. In order to obtain the required data, there is a sampling and laboratory burden. This requires time consuming shellstock sampling during open periods and again after pollution events over the year as well as increased laboratory effort to establish a data base. Shellfish samples require two lab days thus reducing lab capacity to handle water samples. In the 1980's and early 1990's Massachusetts and other states sampled shellstock one or two days after water in Conditionally Approved areas reached the criteria for an Approved classification to ensure that the shellstock was well below the then existing NSSP 230 FC market standard. Usually 150 FC or less was considered adequate to reopen because there was no actual coliform harvest standard and it made sense to only allow harvest well below the market standard. This reduction was accomplished within two days or less of the water quality returning to

	acceptable levels. This approach compared coliform levels in shellfish after water quality reached acceptable levels to an existing standard. When this policy was established, it was endorsed by the FDA Shellfish Specialist. \Shellstock can accumulate bacteria up to 100 times the level in the water. In theory shellstock in water at geometric mean of 10 FC per 100 ml could accumulate FC bacteria to a level of 1000 FC per 100 g. Thus opening an area at a level below the former 230 FC market standard would seem appropriate. Two day purging time is well established. Literature supports elimination of greater than 95% of FC bacteria from shellstock in less than 24 hours including NSSP workshop studies. Temperature is the most important factor affecting elimination of bacteria because it governs shellfish feeding activity. Naturally contaminated shellfish can eliminate fecal coliform levels in 48 hours to levels below most market standards over a range of environmental conditions (Perkins, et al, 1979). Other studies show that soft –shelled clams at MPN 10,000 FC /100 g reduced to values below 50 in 48 hours (Arcisz, et al, 1955) and oysters at MPN 39,000FC/1000g can purge to values below 50 in 48 hours.
14. Cost Information	Could produce significant savings to state shellfish classification programs.

Proposal No. 19-118

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Conditional areas not based on predicting microbiological indicator levels.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas Section @.03 Growing Area Classification C.(1).
12. Text of Proposal/ Requested Action	(1) Survey Required. The sanitary survey meets the following criteria: (a) ~~The area will be in the open status of the conditional classification for a reasonable period of time.~~ The factors determining the~~is~~ period the growing area is in open status are known and ~~, are~~ predictable~~, and are not so complex as to preclude a reasonable management approach~~; (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,~~M~~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~~is~~ data correlate~~s~~ with environmental conditions or other factors affecting the distribution and persistence of viral contaminants into the growing area.
13. Public Health Significance	Not all conditional management is based on predicted changes in microbiological water quality. Conditional management can be based, for example, on the operation of a wastewater treatment system that has never failed. In such a circumstance, demonstrating correlation with environmental conditions or other factors may play no role. The plan can be based completely on other means of predicting the impact of plant failure. Conditional management can also be based on changes in marina occupancy. Similarly, the Authority may use MSC data in some way to support conditional management without demonstrating correlation between MSC levels in shellfish tissues and environmental conditions or other factors.
14. Cost Information	No cost.

Proposal No. 19-119

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Scott Berbells
3. Affiliation	Washington State Department of Health
4. Address Line 1	P.O. Box 47824
5. Address Line 2	
6. City, State, Zip	Olympia, Washington 98504-7824
7. Phone	360.236.3324
8. Fax	360.236.2257
9. Email	Scott.Berbells@doh.wa.gov
10. Proposal Subject	Reduced marine water sampling in conditionally approved areas impacted by point sources
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.03 Growing Area Classification C3. Reevaluation of Conditional Classification(b)(ii)
12. Text of Proposal/ Requested Action	Section II Model Ordinance Chapter IV Shellstock Growing Area @.03 Growing Area Classification C3. Reevaluation of Conditional Classification (b) Water Sample Collection (ii) When the conditional management plan is based on the operation and performance of a WWSD (s); combined sewer overflows(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 performance failure; and (b) Communication requirements are documented and the WWSD operator provides immediate notification to the Shellfish Authority during a performance failure.
13. Public Health Significance	This proposed amendment to Chapter IV, @.03C3(b)(ii) updates the requirements related to the monthly sampling requirement in Conditionally Approved areas classified based on the operation and performance of a WWSD, combined sewer overflow, or other point source. The proposal allows the Shellfish Authority to reduce the number of marine water samples in the area from monthly to five or six times per year, based on the sampling methodology used, if additional studies and appropriate communication channels have been developed. Based on the high performance of many treatment plants, upset conditions occur infrequently and are not evaluated through the placement of permanent marine water sampling stations. Dye and drogue studies coupled with computer modelling are commonly used to determine the potential impact from a point source of pollution on the growing area and are used to calculate the dilution available throughout the area. In Washington state, all NPDES permits issued to wastewater treatment plants contain requirements for operators to provide immediate notification to the Shellfish Authority during upset conditions. Failure of the operator to respond in a

	timely fashion could result in a significant penalty. Upset conditions impacting Conditionally Approved shellfish growing areas in Washington State are infrequent; however, during each event the Shellfish Authority has been immediately informed. The high performance of current treatment plants, effective use of hydrographic and dilution analysis, and immediate communication during upset conditions provide more effective and efficient protection of public health in Conditionally Approved areas impacted by point sources. Upset conditions are infrequent and random which can make monthly sampling inefficient and ineffective at evaluating impacts from the point source.
14. Cost Information	The reduced sampling option would be a cost savings for the Shellfish Authority.

Proposal No. 19-120

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Tom Dameron
3. Affiliation	Surfside Foods
4. Address Line 1	2838 High St
5. Address Line 2	
6. City, State, Zip	Port Norris, NJ, 08349
7. Phone	(856) 785-2115
8. Fax	
9. Email	capttomd@gmail.com
10. Proposal Subject	Classification of Federal Waters
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter IV. Shellstock Growing Areas @.03 Growing Area Classification F.
12. Text of Proposal/ Requested Action	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.
13. Public Health Significance	The FDA has taken the position that all Federal waters are approved unless closed. Currently shellfish harvesting is being allowed in areas with known marine biotoxin hazards. To address these hazards, harvesting restrictions are being required without the designation of appropriate harvesting classification. Currently the Model Ordinance does not include any restrictions for approved areas. Shellfish harvesting areas that have been closed are considered prohibited and harvesting for human consumpltion purposes ia not allowed. If the FDA wants to continue to allow harvesting in Federal waters with restrictions, appropriate classification should be designated.
14. Cost Information	

Proposal No. 19-121

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Executive Office
3. Affiliation	Interstate Shellfish Sanitation Conference
4. Address Line 1	209 Dawson Road
5. Address Line 2	Suite 1
6. City, State, Zip	Columbia, SC 29223
7. Phone	(803) 788-7559
8. Fax	(803) 788-7576
9. Email	issc@issc.org
10. Proposal Subject	*Karenia brevis*
11. Specific NSSP Guide Reference	Section II Model Ordinance Chapter IV. Shellstock Growing Areas @.04
12. Text of Proposal/ Requested Action	Chapter IV. Shellstock Growing Areas @.04 C. Closed Status of Growing Areas. 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 - 5,000 cells/L *(Karenia brevis)* or 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)
13. Public Health Significance	The 5,000 cell count standard applies to *Karenia brevis* only
14. Cost Information	

Proposal No. 19-122

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Use of "growing area" rather than "harvest area" in Patrol requirements language.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter VIII. Control of Shellfish Harvesting @.01 Control of Shellstock Growing Areas A.(2)(d), A.(3)(b), B.(2).
12. Text of Proposal/ Requested Action	A. General. (1) The Authority shall maintain an effective program to control shellstock growing areas and to assure that shellstock are harvested only: (a) From areas in an open status; and (b) With approval from areas classified as restricted, conditionally restricted, or prohibited, or in the closed status of the approved or conditionally approved classification. (2) This program shall include: (a) The patrol of growing areas; (b) The licensing of harvesters; (c) Enforceable legal penalties sufficient to encourage compliance; and (d) Appropriate identification of growing~~harvest~~ areas and/or portions of growing areas where shellstock harvest is not allowed. (3) At the time of issuance or renewal of a harvester's license or a dealer's certification, or an annual mail out to all licensed shellfish harvesters, the Authority shall provide each harvester or dealer with: (a) Information which explains the public health risk associated with illegal harvesting shellstock in areas classified as restricted, conditionally restricted, or prohibited or in the closed status; and (b) When requested, a current, comprehensive, itemized listing of all growing~~harvest~~ areas including their geographic boundaries and their classification. B. Patrol of Growing Areas. (1) The Authority shall assure that shellstock are harvested only as provided in this Chapter. (2) The Authority shall patrol growing~~harvest~~ areas classified as restricted, conditionally restricted, or prohibited, or conditionally approved and approved when in the closed status at sufficient intervals to deter illegal harvesting...
13. Public Health	The NSSP Guide for the Control of Molluscan Shellfish contains definitions for

Significance	"Harvest Area" and "Growing Area." "Growing Area" is the more appropriate term for the indicated locations.
14. Cost Information	No cost.

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Kimberly Stryker
3. Affiliation	State of Alaska Department of Environmental Conservation
4. Address Line 1	555 Cordova Street
5. Address Line 2	
6. City, State, Zip	Anchorage, AK 99501
7. Phone	907-269-7583
8. Fax	907-269-7510
9. Email	Kimberly.stryker@alaska.gov
10. Proposal Subject	Marine Biotoxin Control - Public Health Reasons
11. Specific NSSP Guide Reference	Section III. Public Health Reasons and Explanations, Model Ordinance Chapter IV. Shellstock Growing Areas, @.04
12. Text of Proposal/ Requested Action	**. @.04 Marine Biotoxin Control** ***Marine Biotoxins*** Unlike human pathogens, marine biotoxins occur naturally in aquatic environments. Toxins are produced by certain micro-algae (also called phytoplankton), including dinoflagellates and others. Shellfish are filter feeders and may ingest and concentrate toxic phytoplankton from the water column when present in shellfish growing waters. Toxins are accumulated in the viscera and/or other tissues of shellfish and are transferred to humans when the shellfish are eaten (Gordon et al., 1973). Marine biotoxins are a public health concern for many reasons; for example, marine biotoxins: • May build up in shellfish in concentrations up to 100 times greater than in surrounding waters; • Are not normally destroyed by cooking or processing; • Cannot be detected by taste; and • Can cause illness and death if consumed in sufficient concentrations. In most cases, the toxin has no effect on the shellfish itself, and how long each shellfish vector remains toxic depends on the individual species in question. Additionally, there are non-traditional and emerging vectors of these toxins that also are potentially toxic foods. One example is that pufferfish, typically associated with tetrodotoxin, may also contain saxitoxin (e.g., puffers from coastal waters of Florida). Toxic dinoflagellates or diatoms are single-cell marine plants that are indigenous to most coastal and estuarine waters on the Atlantic, Gulf, and Pacific coasts of America, as well as in many other parts of the world. Dinoflagellates and diatoms in their vegetative stage flourish (“bloom”) seasonally when water conditions are favorable. Blooms of these organisms can occur unexpectedly and rapidly, or may follow predictable patterns. Because dinoflagellates occur naturally, their presence in the water column does not necessarily constitute a health risk. In fact, traces of their toxin in shellfish

meat does not necessarily mean they are hazardous. Toxicity depends on concentration (dose) in the shellfish.

Red tide refers to the discoloration of seawater caused by blooms of marine algae. Red tides are not always red. They occur in many colors, including amber, brown, purple, red, and pink. The relationship between red tides and biotoxin poisoning is widely misunderstood, and many people mistakenly believe that shellfish are safe to eat if no red tide is visible. While red tide can be related to harmful algae, it is helpful to remember that:

- Toxic blooms may be other colors, such as blue-green;
- Marine biotoxin poisoning can happen when there is no discoloration of the water; and
- Several marine algae that pose no public health risk to humans can turn the water red.

Diseases and Outbreaks

All humans are susceptible to shellfish poisoning. A disproportionate number of shellfish-poisoning cases occur among tourists or others who are not native to the location where the toxic shellfish are harvested, and fishermen and recreational harvesters. This may be due to disregard for either official quarantines or traditions of safe consumption.

Diagnosis of shellfish poisoning is based entirely on observed symptomatology and recent dietary history. Human ingestion of contaminated shellfish results in a wide variety of symptoms, depending on the toxin(s) present, their concentrations in the shellfish, and the amount of contaminated shellfish consumed.

Marine Biotoxin Plans – Management & Contingency

The suitability of some growing areas for shellfish harvesting is periodically influenced by the presence of marine biotoxins, such as those responsible for PSP, NSP, ASP, DSP and AZP. The occurrence of these toxins is often unpredictable, and the potential for them to occur exists along most coastlines of the United States and other countries having shellfish sanitation Memoranda of Understanding (MOU) agreements with the United States.

For this reason, even when the authority has no history or reason to expect toxin-producing phytoplankton in their growing areas, every shellfish-producing authority must have a contingency plan that defines administrative procedures, laboratory support, sample collection procedures, and patrol procedures to be implemented on an emergency basis in the event of the occurrence of shellfish toxins. For producing authorities where there is historic occurrence of toxin-producing phytoplankton and toxicity in shellfish from their growing areas, the authority must develop a management plan.

Most authorities will have a combination of management and contingency plans - management plans to address those growing areas with historic occurrence of certain toxin-producing phytoplankton, and contingency plans to address toxin-producing phytoplankton in growing areas in the event of such emergence. As an example, an authority may have statewide historical occurrence of PSP toxin-

producing phytoplankton, for which it develops a management plan; however, because of a lack of illness outbreak or historical evidence of phytoplankton that produce ASP, NSP, DSP, and AZP toxins, the authority also develops a contingency plan that addresses how the authority will manage the emergence of those particular toxins.

Guidance for the development of contingency and management plans is found at Ch IV @.04.

Shellfish Meat Analyses

Laboratory methods to detect marine biotoxins in shellfish include:

- Animal bioassay;
- Biochemical;
- Rapid test kits; and
- Chemical analytical methods.

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 appli to development of chemical analyses to replace or provide alternatives to in-vivo (liv animal) bioassays.

Marine biotoxin testing methods fall into two categories in the NSSP:

1. **Approved** (Section IV. Guidance Documents Chapter II Growing Areas .14 Table 2.)
   Approved methods are those methods that have undergone ISSC evaluation and have been adopted into the NSSP (for certain species) for regulatory decisions, including reopening a growing area after a closure.

2. **Approved Limited Use** (Section IV. Guidance Documents Chapter II Grow Areas .14 Table 4.)
   Approved limited use methods (sometimes referred to as rapid or screening methods) are testing methods that have been evaluated by the ISSC and foun fit for purpose for the NSSP, thereby providing confidence in those methods specific screening purposes. **Most limited use methods may be used for specific screening purposes, the results of which an authority may use to close a growing area; however, an approved method must be utilized to reopen an area following a closure.**

For analyses of toxins for which no method has been adopted into the NSSP, best available science is employed.

Toxin Profiles (PSP, DSP, NSP, ASP, AZP)

Paralytic Shellfish Poisoning (PSP) Toxin	
Cause	Saxitoxins are produced by the dinoflagellates of the genus *Alexandrium* (formerly *Gonyaulax*). The dinoflagellate *Pyrodinium bahamense* is also a producer of saxitoxins.
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. In addition to saxitoxin (the parent compound), monitoring laboratories typically analyze for approximately 12 other analogs that may contribute measurably to toxicity.
	Occurrences	Historically, *Alexandrium* blooms have occurred between April and October along the Pacific coasts from Alaska to California and in the Northeast from the Canadian Provinces to Long Island Sound (US Public Health Service, 1958); but these patterns may be changing. The blooms, which may or may not result in discoloration of seawater, generally last only a few weeks and most shellfish (with the exceptions of some species of clams and scallops, which retain the toxin for longer periods) clear themselves rapidly of the toxin once the bloom dissipates.
	Predictability	Toxic blooms of these dinoflagellates can occur unexpectedly or follow predictable patterns.
	Action Level	0.8 ppm (80 µg/100 g) saxitoxin equivalents. Selective species closures are allowed under the NSSP. In shellfish growing areas where low levels of PSP routinely occur, harvesting for thermal processing purposes is allowed. Thermal processing is defined by FDA regulation 21 CFR 113. Thermal processing will not entirely destroy PSP content of the shellfish; therefore, the Authority must develop and implement procedures to control harvesting and transportation of shellfish intended to be processed.
	Action Level Origin	The regulatory limit was set in the 1930s (Wekell, 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 lesser amounts of the toxin have no deleterious effects on humans.
	Monitoring	Monitoring programs for analysis of PSP toxins include: • Samples submitted by industry with a MOU. • Samples collected by shellfish authority personnel. • Sentinel species monitoring.
	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, a competition assay whereby radiolabeled saxitoxin competes with unlabeled saxitoxin for a finite number of available receptor sites as a measure of native saxitoxin concentrations in a sample, was also approved as an official AOAC method in

	2011.
Disease	Paralytic Shellfish Poisoning
Mortality	Death has been reported to occur as soon as 3 to 4 hours after consumption.
Onset	Symptoms can generally occur within 30 minutes of consuming contaminated seafood, although reports have indicated that symptoms can even ensue within a few minutes, if high enough toxin concentrations are present.
Symptoms, Illness Course	Predominantly neurologic and include tingling of the lips, mouth, and tongue; numbness of extremities; paresthesias; weakness; ataxia; floating/dissociative feelings; nausea; shortness of breath; dizziness; vomiting; headache; and respiratory paralysis. Medical treatment consists of providing respiratory support, and fluid therapy can be used to facilitate toxin excretion. For patients surviving 24 hours, with or without respiratory support, the prognosis is considered good, with no lasting side effects. In fatal cases, death is typically due to asphyxiation. In unusual cases, death may occur from cardiovascular collapse, despite respiratory support, because of the weak hypotensive action of the toxin.
General Food Associations	Mussels, clams, cockles, oysters, and scallops (excluding the scallop adductor muscle).
Outbreak Examples	In New England in 1972, shellfish suddenly became toxic in a previously unaffected portion of the coastline, which resulted in many illnesses (Schwalm, 1973). Despite widespread PSP closures, poisoning events still occur and are generally associated with recreational harvest. For example, in July 2007, a lobster fisherman harvested mussels from a floating barrel off Jonesport, Maine (an area that was currently open to shellfish harvesting), and he and his family ate them for dinner. All four consumers became ill with PSP symptoms, and three of them were admitted to the hospital. It was apparent that the barrel of mussels had originated further up the coast in an area that had been banned to commercial harvest (DeGrasse, 2014).
Diarrhetic Shellfish Poisoning (DSP) Toxin	
Cause	Certain *Dinophysis spp*. and *Prorocentrum spp*. produce okadaic acid and dinophysis toxins that cause DSP.
Analogs	A group of lipid-soluble polyether toxins that includes okadaic acid, the dinophysistoxins, and a series of fatty acid esters of okadaic acid and the dinophysistoxins (collectively known as DSTs) (Uchida, 2018).
Occurrence	DSP toxin-producing phytoplankton have been documented to occur off the coasts of Washington (Trainer et al., 2013) and Texas (Deeds et al., 2010) as well as off the coast in the northeast (e.g., Massachusetts [Tong et al., 2014], Maine, and Connecticut). Known global distribution of DSTs also

	includes Japan, Europe, Asia, Chile, Canada, Tasmania, and New Zealand (Trainer, 2013). In 2008, a large portion of the Texas Gulf Coast was closed to the harvesting of oysters due to the presence of okadaic acid in excess of the FDA guidance level. Although no illnesses were reported in 2008, these were the first closures in the U.S. due to confirmed toxins.
Predictability	Dinoflagellates are known to thrive in stratified systems and Dinophysis has particular adaptive strategies to cope with freshwater plumes (Trainer, 2013).
Action Level	0.16 ppm total okadaic acid equivalents (i.e., combined free okadaic acid, dinophysistoxins, acyl-esters of okadaic acid and dinophysistoxins)
Action Level Origin	Established by FDA in 2011 for total (esterified plus non-esterified OA + DTXs (with no guidance for PTXs and YTXs) (Trainer, 2013).
Monitoring	Production of DSTs has been confirmed in several *Dinophysis* species, including *D. fortii*, *D. acuminata*, *D. acuta*, *D. norvegica*, *D. mitra*, *D. rotundata*, *D. ovum*, *D. sacculus*, *D. caudate, and D. tripos*, and in the benthic dinoflagellates *Prorocentrum lima*, *P. concavum* (or *P. maculosum*), *P. micans*, *P. minimum*, and *P. redfieldii*. One other *Dinophysis* species, *D. hastate*, is also suspected to produce toxins (Trainer, 2013). Precautionary closures initiated based on cell abundance are not useful, but observations show promise in providing early warning to DSP events (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 DTXs in clams was approved in the NSSP. For other species, the best available science is recommended.
Disease	Diarrhetic Shellfish Poisoning
Mortality	This disease generally is not life-threatening.
Onset	Onset of the disease, depending on the dose of toxin ingested, may be as little as 30 minutes to 3 hours.
Symptoms, Illness Course	DSP is primarily observed as a generally mild gastrointestinal disorder; i.e., nausea, vomiting, diarrhea, and abdominal pain, accompanied by chills, headache, and fever. Symptoms may last as long as 2 to 3 days, with no chronic effects.
General Food Associations	Mussels, clams, cockles, oysters, and scallops (excluding the scallop adductor muscle).
Outbreak Examples	Although there have been numerous outbreaks of diarrhetic shellfish poisoning around the world, until recently there were

	no confirmed cases of DSP in the U.S. that were due to domestically harvested shellfish (Trainer, 2013). In 2011, approximately 60 illnesses occurred in British Columbia, Canada, and 3 illnesses occurred in Washington State due to consumption of DSP-contaminated mussels. Subsequent harvesting closures and product recalls were issued (Lloyd, 2013).
Neurotoxic Shellfish Poisoning (NSP) Toxin	
Cause	NSP is caused by brevetoxins produced by the dinoflagellates of the genus *Karenia* (formerly *Gymnodinium*).
Analogs	Comprised of more than 10 lipid-soluble cyclic polyethers. A number of analogs and metabolites have been identified. NSP-causing toxins in shellfish include intact algal brevetoxins and their metabolites (collectively known as NSTs). In addition to brevitoxins, numerous other *Karenia spp*. Found in the Gulf of Mexico and around the world regularly associated with blooms produce hymnodimine, karlotoxins, and other potent toxins (Watkins, 2008).
Occurrence	In Gulf coast areas, toxicity in shellfish has been associated with red tide outbreaks caused by massive blooms of the toxic dinoflagellate, *Karenia brevis* (formerly *Ptychodiscus brevis*). Naturally occurs in Gulf of Mexico, Caribbean Sea, and along New Zealand coasts; it regularly produces blooms along the coasts of Florida and Texas. Blooms may cause ocean to appear red, brown, or simply darkened and are usually accompanied by massive fish kills and mortalities in marine mammals and sea birds (Watkins, 2008). Dupuration time of brevetoxins in shellfish varies, but is typically within two to eight weeks, although reports of much longer retention (nearly one year post bloom) have been documented (Watkins, 2008).
Predictability	*Karenia* blooms show no indication of regular recurrence and shellfish generally take longer to eliminate the toxin. Blooms were once considered to be sporadic and seasonal, but historical records demonstrate these blooms have occurred in Florida almost annually in the years since the 1940s. Although more frequent in late summer and early fall, Florida blooms have been documented in almost every month of the year and may disperse in a matter of weeks, or may be present for many months at a time; in 2006, a bloom off the coast of Sarasota lasted over 12 months. Occurrence and magnitude of blooms are unpredictable.
Action Level	0.8 ppm (20 mouse units/100 g tissue or 80 µg/100 g tissue) brevetoxin-2 equivalents The cell count of members of *Karenia brevis* in the water column exceeds 5,000 cells per liter of water.
Action Level 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).
Monitoring	Water cell counts and tissue samples.
Shellfish Lab Methods	Toxicity of shellfish exposed to the dinoflagellate *Karenia brevis* has been historically assessed by mouse bioassay in the U.S.; however, mouse bioassay is not very specific for NSP toxins (Watkins, 2008). Efforts are underway to validate *in-vitro* methods for detection of brevetoxins in shellfish. 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).
Disease	Neurotoxic Shellfish Poisoning
Mortality	No fatalities have been reported, but hospitalizations occur.
Onset	Onset of this disease occurs within a few minutes to a few hours. A mean time to onset of 3-4 hours has been reported in the few documented outbreaks (Watkins, 2008).
Symptoms, Illness Course	Both gastrointestinal and neurological symptoms characterize NSP, including tingling and numbness of lips, tongue, and throat; muscular aches; dizziness; diarrhea; and vomiting. Respiratory distress has been recorded. Duration is fairly short, from a few hours to several days. Recovery is complete, with few after-effects.
General Food Associations	Oysters and clams.
Outbreak Examples	The most common public health problem associated with *Karenia* blooms is respiratory irritation; however, neurotoxic shellfish poisonings associated with *Karenia brevis* blooms have been reported in Florida (US Center for Disease Control, 1973). Until NSP toxins were implicated in more than 180 human illnesses in New Zealand in 1992/1993 due to consumption of cockles and green shell mussels, NSP was considered to be an issue only in the U.S. Outbreaks of NSP are rare where programs for monitoring *K. brevis* blooms and shellfish toxicity are implemented. An NSP outbreak involving 48 individuals occurred in North Carolina in 1987

	(Morris, 1991). A series of NSP cases occurred along the southwest coast of Florida, in 2006, after people consumed recreationally-harvested clams from waters unapproved for shellfish harvesting (Watkins, 2008).
Amnesic Shellfish Poisoning (ASP) Toxin	
Cause	ASP is caused by domoic acid that is produced by diatoms of the genus *Pseudonitzchia*.
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. Excitatory amino acid (EAA) analogues of glutamate.
Occurrence	During a 1991-1992 incident in Washington and a 2015 event on the west coast from Washington to California, high toxin levels persisted for several months (Liston, 1994; McCabe et al. 2016). There was also an extensive event in the Northeast from Maine to Rhode Island in 2016, with different regions showing varying toxicity and species dominance within the bloom. The event started in late September in eastern Maine and ended in October; however, Rhode Island experienced another bloom in February of 2017. During 1991 and 1992, there was a spread of domoic acid producing organisms throughout the world including the detection of high numbers of the diatom *Pseudonitzschia pseudodelcatissima* in Australia and *Pseudonitzschia pseudoseratia* in California. Domoic acid has also been recovered from shellfish in Washington and Oregon.
Predictability	Blooms of *Pseudonitzschia* are of varying intensity, duration and extent. Environmental factors associated with ASP in shellfish are currently unknown.
Action Level	20 ppm domoic acid
Action Level Origin	In 1987 in eastern Canada, DA poisonings sickened individuals, leading to Health Canada's establishment of the regulatory limit. (Wekell, 2004)
Monitoring	Monitoring programs for ASP toxin are designed around the shellfish species of interest.
Shellfish Lab Methods	The mouse bioassay for domoic acid is not sufficiently sensitive and does not provide a reliable estimate of potency. The NSSP approved regulatory method for detecting domoic acid in seafood is a reversed-phase HPLC method with ultraviolet (UV) detection. There is also an AOAC approved ELISA for the detection of domoic acid.
Disease	Amnesic Shellfish Poisoning
Mortality	All fatalities, to date, have involved elderly patients.
Onset	The toxicosis is characterized by onset of gastrointestinal symptoms within 24 hours; neurologic symptoms occur within 48 hours.
Symptoms, Illness	ASP is characterized by gastrointestinal disorders (vomiting, diarrhea, abdominal pain) and neurological problems

Course	(confusion, short-term memory loss, disorientation, seizure, coma). Human clinical signs of domoic acid toxicity are reported as mild gastrointestinal symptoms, from an oral dose of 0.9-2.0 mg domoic acid (DA)/kg body weight. Neurologic effects, such as seizure and disorientation, are reported from an oral dose of 1.9-4.2 mg DA/kg body weight. The toxicosis is particularly serious in elderly patients, and includes symptoms reminiscent of Alzheimer's disease.
General Food Associations	Mussels, clams, cockles, oysters, and scallops (excluding the scallop adductor muscle).
Outbreak Examples	The first human domoic acid poisoning events were reported in 1987, in Canada (Perl, 1990). While domoic acid exposure still exists, there have been no documented ASP cases since 1987, following implementation of effective seafood toxin-monitoring programs (Pulido, 2008).
Azaspiracid Shellfish Poisoning (AZP) Toxin	
Cause	*Azadinium spp*. is the producer of azaspiracids, which cause AZP.
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).
Occurrence	Coastal regions of western Europe, as well as NW Africa and eastern Canada.
Predictability	Detected between mid-summer and mid-winter from northern/western European waters, but in certain cases, the presence of AZAs in phytoplankton does correspond to the timing of shellfish contamination, yet toxin levels in bivalves can remain elevated for 8 – 12 months following initial exposure.
Action Level	160 µ/kg shellfish meat
Action Level 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.
Monitoring	Range of species in which AZAs have been detected includes mussels (*M. edulis; M. galloprovincialis*), oysters (*Crossostrea gigas*, *Ostrea edulis*), scallops (*Pecten maximus*), clams (*Tapes philipinarum*, *Ensis siliqua*, *Donax* spp.), and cockles (*Cerastroderma edule*). AZAs have also been found in crustaceans. Monitoring programs will benefit from major research efforts to identify the causative organism(s) because there is often, but not always, a correlation between the presence of potentially toxigenic phytoplankton species and the subsequent accumulation of toxins in shellfish.
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.
Disease	Azaspiracid Shellfish Poisoning
Mortality	No known fatalities to date.
Onset	Symptoms appear in humans within hours of eating AZA-contaminated shellfish.
Symptoms, Illness Course	Symptoms are predominantly gastrointestinal disturbances resembling those of diarrhetic shellfish poisoning and include nausea, vomiting, stomach cramps, and diarrhea. Illness is self-limiting, with symptoms lasting 2 or 3 days.
General Food Associations	Detected in mussels, oysters, scallops, clams, cockles, and crabs.
Outbreak Examples	The first case of AZP was detected in the Netherlands in 1995, where 8 people became ill after consuming mussels. From 1997 – 2000, approximately 80 individuals reported illnesses from mussels and scallops harvested from Ireland, Italy, France, and United Kingdom (Twiner, 2008). There have been no confirmed cases of AZP in the U.S. from domestically-harvested product. In 2008, the first recognized outbreak of AZP in the U.S. was reported, but was associated with a mussel product imported from Ireland (Klontz et al. 2009).

Resources

The 2012 version of FDA's Bad Bug Book, Foodborne Pathogenic Microorganisms and Natural Toxins, is a comprehensive resource from which a great deal of information has been used for the toxin profiles in the table above. It is accessible at https://www.fda.gov/media/83271/download

For more discussion of chemical structures and properties, methods of analysis, source organisms and habitat, occurrence and accumulation in shellfish, toxicity of toxins, prevention of intoxication, cases and outbreaks, and regulations and monitoring, see the FAO Paper 80: Marine Toxins. This may be accessed as follows:

Paralytic Shellfish Poisoning	http://www.fao.org/3/y5486e/y5486e05.htm
Diarrhetic Shellfish Poisoning	http://www.fao.org/3/y5486e/y5486e0e.htm
Neurotoxic Shellfish Poisoning	http://www.fao.org/3/y5486e/y5486e0o.htm
Amnesic Shellfish Poisoning	http://www.fao.org/3/y5486e/y5486e0n.htm
Azaspiracid Shellfish Poisoning	http://www.fao.org/3/y5486e/y5486e0p.htm
References	http://www.fao.org/3/y5486e/y5486e0t.htm

The FDA online course, Shellfish Growing Areas, introduces participants to requirements and procedures under the NSSP to ensure that shellfish are harvested from safe waters. The course contains a significant section addressing marine biotoxins. The course may be accessed at https://www.accessdata.fda.gov/ORAU/ShellfishGrowingAreas/SGA_summary.htm.

Additional information from the Centers for Disease Control and Prevention, Morbidity and Mortality Weekly Report (MMWR) contains illness reports related to these toxins. This may be accessed at https://www.cdc.gov/mmwr/index.html.

NIH/PubMed: Various Shellfish-Associated Toxins provides a list of research abstracts in the National Library of Medicine's MEDLINE database.

The specific seafood with which each toxin generally is associated is included in the profiles above to help readers link symptoms to potential sources. However, all shellfish (filter-feeding mollusks, as well as the carnivorous grazers that feed on these mollusks (such as whelk, snails, and, in some cases, even lobster and octopus), may become toxic in areas where the source algae are present.

References

Deeds, J.R., & Landsberg, J.H., Etheridge, S.M., Pitcher, G.C., Longan, S.W. (2008). Non-traditional vectors for paralytic shellfish poisoning. *Marine Drugs, 6(2)*, 308-348. Retrieved from https://doi.org/10.3390/md6020308.

Degrasse, S., & Rivera, V., Roach, J., White, K., Callahan, J., Couture, D., Simone K., Peredy, T., Poli, M. (2014). Paralytic shellfish toxins in clinical matrice extension of AOAC official method 2005.06 to human urine and serum and application to a 2007 case study in Maine. *Deep Sea Research Part II: Topical Studies in Oceanography*, 103, 368-375. Retrieved from https://doi.org/10.1016/j.dsr2.2012.08.001.

Food and Agriculture Organization of the United Nations. (2015) Codex Alimentar Standard for Live and Raw Bivalve Molluscs Codex Stan 292-2008. Retrie from http://www.fao.org/fao-who-codexalimentarius/codex-texts/all-standards/en/

Food and Agriculture Organization of the United Nations. (2004). FAO Food and Nutrition Papers, 80 - Marine Biotoxins. Retrieved from http://www.fao.org/3/y5486e/y5486e00.htm

Joint Sanitation Seminar on North Pacific Clams Juneau, A., Felsing, W. A. (Willi August)., United States. Public Health Service., Alaska. Dept. of Health an Welfare. (1966). *Proceedings of Joint Sanitation Seminar on North Pacific Clams.* Washington, D.C.: For sale by the Supt. of Docs., G.P.O.. Retrieve from https://babel.hathitrust.org/cgi/pt?id=pur1.32754081175147&view=1up&se

Klontz, K.C., & Abraham, A., Plakas, S., Dickey, R. (2009). Mussel-associated azaspiracid intoxication in the United States. *Annals of Internal Medicine*, 150(5), 361. Retrieved from https://www.researchgate.net/publication/24174858_Mussel-

Associated_Azaspiracid_Intoxication_in_the_United_States

Liston, J. (1994). Association of *Vibrionaceae*, natural toxins, and parasites with fe indicators, p. 215-216. In Hackney, C.R. and M.D. Pierson (eds.). *Environmental Indicators and Shellfish Safety*. Chapman and Hall, New Yo NY.

Lloyd, J.K., & Duchin, J., Borchert, J., Quintana, H.F., Robertson, A. (2013). Diarrh Shellfish Poisoning, Washington, USA, 2011. Emerging Infectious Diseases 19(8), 1314-1316. Retrieved from https://doi.org/10.3201/eid1908.121824.

Marsden I.D., & Contreras, A.M., MacKenzie, L., Munro, M.H.G. (2015). A comparison of the physiological responses, behaviour and biotransformatic of paralytic shellfish poisoning toxins in a surf-clam (*Paphies donacina*) a the green-lipped mussel (*Perna canaliculus*). *Marine and Freshwater Research*, 67, 1163-1174. Retrieved from http://www.publish.csiro.au/mf/MF14374

McCabe, R.M., & Hickey, B.M., Kudela, R.M., Lefebvre, K.A., Adams, N.G., Bill B.D., Gulland, F.M.D., Thomson, R.E., Cochlan, W.P., Trainer, V.L. (201 An unprecedented coastwide toxic algal bloom linked to anomalous ocean conditions. *Geophysical Research Letters*, 43(19), 10,366–10,376. Retriev from https://DOI.org/10.1002/2016GL070023.

Morris, P.D., & Campbell, D.S., Taylor, T.J., Freeman, J.I. (1991). Clinical and epidemiological features of neurotoxic shellfish poisoning in North Carolir *American Journal of Public Health*, 81(4), 471-474. Retrieved from: https://DOI.org/10.2105/ajph.81.4.471.

National Shellfish Sanitation Workshop., United States. Shellfish Sanitation Branch. (1964). *Proceedings - National Shellfish Sanitation Workshop.* [Washington]: U.S. Dept. of Health, Education, and Welfare, Public Health Service, Food and Drug Administration, Shellfish Sanitation Branch. Retrieved from https://catalog.hathitrust.org/Record/006685147

Perl, T.M., & Bedard, L., Kosatsky, T., Hockin, J.C., Todd, E.C.D., NcNutt, L.A., Remis, R.S. (1990). Amnesic shellfish poisoning: a new clinical syndrome due to domoic acid. In: Hynie, I., Todd, E.C.D., editors. Proceedings of a symposium, domoic acid toxicity. Canada Disease Weekly Report; Ottawa, Ontario. Pp. 7-8.

Prakash, A., & Medcof, J.C., Tennant, A.D. (1971). Paralytic shellfish poisoning i eastern Canada. Bulletin 177, Fisheries Research Board of Canada. Ottawa, Canada. Retrieved from http://dfo-mpo.gc.ca/library/1498.pdf.

Pulido, O.M. (2008). Domoic acid toxicologic pathology: a review. *Marine Drugs, 6(2)*, 180-219. Retrieved from https://doi.org/10.3390/md20080010.

Quayle, D.B. (1969). Paralytic shellfish poisoning in British Columbia. Bulletin 168, Fisheries Research Board of Canada. Ottawa, Canada.

Schwalm, D.J. (1973). The 1972 PSP outbreak in New England. FDA Report, Boston, MA. U.S. Food and Drug Administration, Washington, D.C.

Tong, M., & Smith, J.L., Richlen, M.L., Steidinger, K., Kulis, D., Fux, E., Anderson, D.M. (2014) Characterization and comparison of toxin-producing isolates of *Dinophysis acuminata* from New England and

Canada. *Journal of Phycology*, 51(1), 66-81. Retrieved from https://www.researchgate.net/publication/267340694_Characterization_and_comparison_of_toxin-producing_isolates_of_Dinophysis_acuminata_from_New_England_and_Canada .

Trainer, V.L., & Moore, L., Bill, B.D., Adams, N.G., Harrington, N., Borchert, J., da Silva, D.A.M., Eberhard, B.T.L. (2013). Diarrhetic shellfish toxins and other lipophilic toxins of human health concern in Washington State. *Marine Drugs*, 11, 1815–1835. Retrieved from https://doi.org/10.3390/md11061815.

Twiner, M.J., & Bottein Dechraoui, M.Y., Wang, Z., Mikulski, C.M., Henry, M.S., Pierce, R.H., Doucette, G.J. (2007). Extraction and analysis of lipophilic brevetoxins from the red tide dinoflagellate *Karenia brevis*. *Analytical Biochemistry*, 369(1), 128-135. Retrieved from https://DOI.org/10.1016/j.ab.2007.06.031.

Twiner, M.J., & Rehmann, N., Hess, P., Doucette G.J. (2008). Azaspiracid shellfish poisoning: a review on the chemistry, ecology, and toxicology with an emphasis on human health impacts. *Marine Drugs*, *6(2)*, 39-72. Retrieved from https://doi.org/10.3390/md6020039.

Uchida, H., & Watanabe, R., Matsushima, R., Oikawa, H., Nagai, S., Kamiyama, T., Baba, K., Miyazono, A., Kosada, Y., Kaga, S., Matsuyama, Y., Suzuki, T. (2018). Toxin profiles of okadaic acid analogues and other lipophilic toxins in Dinophysis from Japanese Coastal Waters. Toxins (Basel). 10(11), 457. Retrieved from https://doi.org/10.3390/toxins10110457 .

US Center for Disease Control. (1973). Shellfish poisoning - Florida. *Morbidity Mortality Weekly Report*, 22(48), 397-398. Retrieved from https://stacks.cdc.gov/view/cdc/1843

US Food and Drug Administration. (1997). Poisonous or Deleterious Substances Food. *Federal Register*, 42(190), 52814-52819.

US Food and Drug Administration. (2000). Guidance for Industry: Action Levels for Poisonous or Deleterious Substances in Human Food and Animal Feed. Retrieved from https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-action-levels-poisonous-or-deleterio substances-human-food-and-animal-feed.

US Food and Drug Administration. (2011). Fish and Fishery Products Hazards and Controls Guidance 4th Edition. Retrieved from https://www.fda.gov/food/seafood-guidance-documents-regulatory-information/fish-and-fishery-products-hazards-and-controls-guidance-4th-edition

US Public Health Service (PHS). (1958). Proceedings: 1957 Conference on Shellfish Poison. U.S. PHS, Washington, D.C. 125 pages. Retrieved from https://babel.hathitrust.org/cgi/pt?id=uc1.31822005678131&view=1up&seq

Watkins, S.M., & Reich, A., Fleming, L.E., Hammond, R. (2008). Neurotoxic shellfish poisoning. *Marine Drugs*, *6(3)*, 431-455. Retrieved from: https://doi.org/10.3390/md6030431.

Wekell, J.C., & Hurst, J., Lefebvre, K.A. (2004). The origin of the regulatory limits f PSP and ASP toxins in shellfish. *Journal of Shellfish Research*, 23(3), 927-9 Retrieved from: https://www.researchgate.net/publication/285809374_The_origin_of_the_re atory_limits_for_PSP_and_ASP_toxins_in_shellfish

Wiese, M., & D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A. (2010). Neurotoxic alkaloids: saxitoxin and its analogs. *Marine Drugs*, *8(7)*, 2185-2211. Retrieved from https://doi.org/10.3390/md8072185.

~~Marine biotoxins may be ingested by molluscan shellfish feeding on toxic dinoflagellates. Dinoflagellates in their vegetative stage flourish seasonally when water conditions are favorable. Toxic blooms of dinoflagellates or diatoms can occur unexpectedly or may follow predictable patterns. PSP, NSP and Domoic Acid poisoning, also known as ASP are the three (3) types of poisonings most commonly associated with oysters, clams, mussels and scallops in the United States.~~

~~Cases of paralytic shellfish poisoning, including several fatalities resulting from poisonous shellfish, have been reported from both the Atlantic and Pacific coasts. The minimum quantity of poison, which will cause intoxication in the susceptible person, is not known. Epidemiological investigations of paralytic shellfish poisoning in Canada have indicated 200 to 600 micrograms of poison will produce symptoms in susceptible persons. A death has been attributed to the ingestion of a probable 480 micrograms of poison. Investigations indicate that lesser amounts of the poison have no deleterious effects on humans. Growing areas should be closed at a level to provide an adequate margin of safety, since in many instances, toxicity levels will change rapidly.~~

~~A review of the literature and research dealing with the source of the poison, the occurrences, and distribution of poisonous shellfish physiology and toxicology, characteristics of the poison, and prevention and control of poisoning has been prepared.~~

~~In Gulf coast areas, toxicity in shellfish has been associated with red tide outbreaks caused by massive blooms of the toxic dinoflagellate, *Karenia brevis* (formerly *Ptychodiscus brevis*). Toxic symptoms in mice suggest a type of NSP rather than symptoms of PSP. The most common public health problem associated with *Karenia brevis* blooms is respiratory irritation; however, NSP associated with *Karenia brevis* blooms have been reported in Florida. Uncooked clams from a batch eaten by a patient with neurotoxic symptoms were found to contain 118 mouse units per 100 grams of shellfish meat.~~

~~Toxic dinoflagellates or diatoms are indigenous to most coastal and estuarine waters on the Atlantic, Gulf, and Pacific coasts of America, as well as in many other parts of the world. Blooms of these organisms can occur unexpectedly and rapidly. This phenomenon occurred in New England in 1972 when shellfish suddenly became toxic in a previously unaffected portion of the coastline and resulted in many illnesses. During 1991 and 1992, there was a spread of domoic acid producing organisms throughout the world including the detection of high~~

	~~numbers of the diatom *Pseudo-nitzschia pseudo-delcatissima* in Australia and *Pseudo-nitzschia pseudo-seratia* in California. Domoic acid was also recovered from shellfish in Washington and Oregon. All shellfish producing States or MOU countries must have a contingency plan that defines administrative procedures, laboratory support, sample collection procedures, and patrol procedures to be implemented on an emergency basis in the event of the occurrence of shellfish toxins. A model State contingency plan for control of marine biotoxins is provided in the NSSP Model Ordinance Guidance Documents, *Guidance for Developing Marine Biotoxin Contingency Plans* (ISSC/FDA, 2017).~~ ~~All States or MOU countries must monitor toxin levels to establish a baseline historical reference. Thereafter, States or MOU countries where shellfish toxins are likely to occur must monitor toxin levels on a routine basis to meet the approved area requirements for direct market harvesting. Experience with monitoring for shellfish toxins suggests that an effective program should include the following:~~ ~~Sampling stations should be located at sites where past experience has shown toxin is most likely to appear first.~~ ~~Samples should be collected of shellfish 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, mussels have been found to be useful for early PSP detection.~~ ~~The frequency and period for collection of samples should be based upon historical patterns. This assumes several years of baseline data in order to establish stations and sampling plans.~~ ~~An information network should be established between the health and marine resource communities and the Authority. Any toxin-like illnesses related to shellfish and environmental phenomena such as algal blooms, fish kills, or bird kills, which might indicate the early stages of an increase in toxin levels, should be rapidly communicated over the network.~~ ~~Sampling stations and frequency of sampling should be increased when monitoring data or other information suggests that toxin levels are increasing.~~ ~~Sample collection, sample transportation, and sample analysis procedures should be developed so that in an emergency sample results will be known within twelve (12) hours.~~ ~~When monitoring data or other information indicates that toxin levels have increased to the quarantine levels, growing area closures must be immediately implemented. The determination of which growing areas should be closed should include consideration of the rapidity with which toxin levels can increase to excessive levels and the inherent delays in the State sample collection procedures. It may be appropriate to close growing areas adjacent to known toxic areas until increased sampling can establish which areas are toxin-free and that toxin levels have stabilized.~~

~~Shellfish growing areas closed because marine biotoxins have exceeded quarantine levels may be reopened for growing after a sufficient number of samples and other environmental indices, if used, have established that the level of toxin will remain below quarantine levels for an extended period. For example, experience has shown that appropriate reopening criteria include a minimum of three (3) samples collected over a period of at least fourteen (14) days. These samples should show the absence of PSP or levels below 80 micrograms per 100 grams.~~

~~**A.** Contingency Plan.~~

~~The suitability of some areas for harvesting shellstock is periodically influenced by the presence of toxigenic micro-algae. Recent increases in toxigenic micro-algae distribution dictate that a more comprehensive series of public health controls be adopted. The need exists to make contingency plans to address the contamination of a growing area by toxigenic micro-algae or a disease outbreak caused by marine biotoxin. This contingency plan must describe administrative procedures, laboratory support, sample collection procedures, and patrol procedures to be implemented on an emergency basis in the event of the occurrence of marine biotoxin in shellstock. The primary goal of this planning should be to ensure that maximum public health protection is provided in growing areas subject to marine biotoxin contamination. For a discussion of marine biotoxin disease and its management in shellfish growing areas, see the NSSP Model Ordinance Guidance Documents: *Guidance for Developing Marine Biotoxin Contingency Plan* (ISSC/FDA, 2017).~~

~~**B.** Marine Biotoxin Monitoring.~~

~~The primary purpose of a marine biotoxin monitoring program is to prevent illness or death among the shellfish consuming public. The monitoring program should use the "indicator station" and "critical species" concepts to develop an early warning system to prevent harvest of biotoxin contaminated shellstock. For a full discussion, see the NSSP Model Ordinance Guidance Documents: *Guidance for Developing Marine Biotoxin Contingency Plan* (ISSC/FDA, 2017).~~

~~**C.** Closed Status of Growing Areas.~~

~~In the event of a toxigenic micro-algae bloom, shellstock growing areas shall be placed in the closed status for harvesting to prevent human consumption of biotoxin contaminated shellfish. The biotoxin level governing the need to place the growing area in the closed status will vary depending on the species of toxigenic micro-algae and the species of bivalve shellfish. Since the ability to concentrate biotoxins varies among species, it is possible for one (1) species in a growing area to have safe levels of biotoxin while another species in the same growing area will have dangerous biotoxin concentrations. In this situation, the Authority may permit the harvesting of one (1) species with no adverse public health consequences while prohibiting the harvest of another species. In these situations, the Authority must closely monitor the growing area and develop a sufficient database for use in making this determination.~~

	~~The Authority must develop criteria, which must be met before a growing area can be returned to the open status for harvesting. These criteria should integrate public health, conservation, and economic considerations. The criteria should also employ a sufficient number of samples and other environmental indices, if used, to establish that the level of toxin will remain, for an extended period of time, at levels safe for human consumption. For additional discussion concerning biotoxin contamination of shellstock, see the NSSP Model Ordinance Guidance Documents: *Guidance for Developing Marine Biotoxin Contingency Plan* (ISSC/FDA, 2017).~~ ~~**D.** Heat Processing.~~ ~~Heat treatment can reduce the toxicity of some biotoxins. When heat treatment is used, the Authority must require that the processor provide adequate demonstration of the destruction of the biotoxin and adequate controls to assure that the end product is safe for human consumption.~~ ~~**E.** Records.~~ ~~Good record keeping is essential to the successful management of a Marine Biotoxin Contingency Plan. Appropriate records of monitoring data, evaluation reports, and closure and reopening notices should be compiled and maintained by the Authority. This information is important in defining the severity of the problem, as well as for a retrospective evaluation of the adequacy of the entire control program.~~
13. Public Health Significance	Marine biotoxins can cause injury, illness, or death. More clearly presented information will assist NSSP participants in understanding the public health reasons for marine biotoxin contingency and management plans.
14. Cost Information	None

Proposal No. 19-124

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Kimberly Stryker
3. Affiliation	State of Alaska Department of Environmental Conservation
4. Address Line 1	555 Cordova Street
5. Address Line 2	
6. City, State, Zip	Anchorage, AK 99501
7. Phone	907-269-7583
8. Fax	907-269-7510
9. Email	Kimberly.stryker@alaska.gov
10. Proposal Subject	Marine Biotoxin Control – Guidance Document
11. Specific NSSP Guide Reference	Section IV Guidance Documents Chapter II. Growing Areas Chapter IV. Shellstock Growing Areas .02
12. Text of Proposal/ Requested Action	**.02 Guidance for Developing Marine Biotoxin Contingency and Management Plans.** Regardless of whether a growing area has a history of toxin-producing phytoplankto being able to detect occurrences and take appropriate action to prevent contaminated product from entering commerce is an important part of marine biotoxin control. There are two types of plans defined in the NSSP MO for the control of marine biotoxins: a *contingency plan* and a *management plan*. The *contingency plan* is primarily for reactive management to an illness outbreak or emergence of a toxin-producing phytoplankton in a growing area that has not historically occurred before. The contingency plan is only appropriate for a shellfish Authority that has no history or reason to expect toxin-producing phytoplankton in th growing areas. The primary goal of the contingency plan is to detect emerging toxins and to outline response activities necessary to prevent additional illnesses (if illness h already occurred) and protect the public's health. The *management plan* is primarily for proactive management of marine biotoxins in growing areas with a history of toxin-producing phytoplankton and toxicity in shellfi and/or a previous illness event or outbreak. A management plan is required for a shellfish authority that has a history of toxin-producing phytoplankton, toxicity in shellfish and/or an illness event or outbreak attributed to their growing areas. A shellfish authority might have a management plan for certain marine biotoxins, lik PSP toxins, but a contingency plan for toxins like AZP toxins. **General Plan Elements** Whether the authority is developing a plan to manage biotoxins, or a contingency pla for the unexpected, the plan should address the following elements: • Statutory and/or Regulatory Authorities • Resource/Growing Areas and Species

- Communication
- Control & Response
- Growing Area Reopening Criteria
- Recordkeeping
- Post Event Actions
- Plan Testing, Post Event Activities

Recommended General Plan Guidelines

**Statutory and/or Regulatory Authorities*

The authority should prepare a summary of the laws and regulations in the state (or MOU country) that allow the authority to promptly and effectively take actions to prevent or remove potentially toxic shellfish from commerce in the event of a marine biotoxin event, including:

1. close a growing area to harvest;
2. embargo shellfish that has not entered commerce;
3. prevent harvesting of contaminated species;
4. provide for embargo and/or recall of any potentially toxic shellfish already o the market; and
5. withdraw interstate shipping permits.

**Resource/Growing Areas and Species*

As is the case in several aspects of the NSSP MO, the plan should include a list or reference to a list of locations of classified shellfish growing areas and the species present in the area. This is especially important if the authority intends to implement species-specific biotoxin closures as part of the plan.

**Communication*

Information-sharing among government and non-government agencies is critical as p of an effective biotoxin plan, whether contingency or management. As such, the authority should establish and formalize channels of communication with appropriat partner agencies (e.g., wildlife, epidemiology, local health, public safety, public heal and environmental), research or academic organizations (e.g., marine biologists), adjacent shellfish control authorities, industry, and other similar partners in advance any serious biotoxin event.

Information to be communicated includes that which is relevant to early warning as v as control and response, including:

1. abnormal environmental phenomenon that may be associated with a shellfish growing area (e.g., bird, fish, or marine mammal die-offs or abnormal behavior, or water discoloration);
2. occurrences of toxic phytoplankton blooms;
3. toxin-like illness reports in humans;
4. growing area closures (specifically, disseminating information on occurrences and/or toxicity in shellfish meats to adjacent states, industry and local health agencies);

5. coordination of control activities taken by state and federal agencies or departments and district, regional, or local health authorities (e.g., patrol legal actions); and
6. consumer educational outreach during growing area closure periods.

This aspect of the plan may include references to Memoranda of Understanding and tables that outline each partner's roles and responsibilities, and procedures that defin how agencies will maintain contact lists. Model press releases, email notifications, a similar templates may also be useful.

**Control and Response Activities*

An authority's plan should include the following elements to address control and response activities:

1. Growing Area Closure Criteria

   An authority's plan (either contingency or management) should define the circumstances under which the authority will place a growing area in the clo status due to marine biotoxin contamination. The criteria should integrate pu health and economic considerations. Principle considerations include

   * The rapidity with which toxin levels can increase to excessive levels
   * 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 (it may be appropriate to close harvesting areas adjacent to known toxic areas u increased sampling can establish which areas are toxin free and that toxin levels have stabilized); and
   * The type of harvesting restrictions to be invoked (all species or spec species).

   The biotoxin level governing the need to place the growing area in the close status may vary depending on the species of phytoplankton and the species o bivalve shellfish. Since the ability to concentrate biotoxins varies among species, it is possible for one species in a growing area to have safe levels of biotoxin while another species in the same growing area will have dangerous biotoxin concentrations. In this situation, the authority may allow the harves of one species with no adverse public health consequences while prohibiting harvest of another species. In these situations, the authority must closely monitor the growing area and develop a sufficient database for use in makin this determination.

2. Administrative Actions

   The authority should specify the administrative procedures, including timeframes, necessary to place growing areas in the closed status, identify potentially contaminated shellfish products, determine the distribution of the products, and initiate embargo and/or recall activities.

3. Other Control Activities.

   If the authority's statutes or regulation do not allow for a certain administrati action and/or the authority must seek a court order or other legal action, the authority should define the procedures and timeframes, where applicable.

The authority should also refer to, or describe patrol activities relative to growing area closures due to marine toxins.

**Growing Area Reopening Criteria*

The authority's plan should describe how the authority determines that shellfish for commercial harvest in a growing area are safe for harvest and distribution into commerce for human consumption following an event. The protocol should reflect th authority's consideration of the public's health, and economic consequences.

A system of representative samples and other environmental indices are typically use to establish detoxification curves indicating that the level of toxin or cell counts have decreased to acceptable levels. Several authorities require that three (3) samples collected over a period of fourteen (14) days show results below the quarantine limit before reopening the affected area.

**Routine Monitoring Program*

A routine surveillance monitoring program (also referred to as an early warning phytoplankton and/or shellfish-monitoring program) is recommended as part of a marine biotoxin control plan to detect the presence of a "bloom." In describing this program, the authority should include:

1. Geographic Distribution of Primary Sampling Stations
   For both phytoplankton and shellfish monitoring plans, primary sampling stations (also referred to as indicator or sentinel stations) should be located a sites where toxin is most likely to first appear, based either on past experienc or knowledge of site conditions. The geographic distribution for collection o samples should take into consideration the randomness of toxic algal blooms For these reasons, several years of baseline data are often necessary in order establish stations. To facilitate knowledge transfer, it is advisable that the authority describe its rationale in selecting sampling sites.
2. Determination of Species to be Sampled
   For a monitoring plan, sampling design should always take into account wha commercially-harvested species are present in the growing area and samples should be collected of species which are most likely to reveal the early prese of toxin and are most likely to show the highest toxin levels. For example, mussels have been found to be useful for early detection of an event.
3. Frequency and Timing of Sample Collection
4. Just as location of sampling sites should be carefully considered, the authorit should establish the frequency and period for collection of samples in order t identify an event as early as possible. Historical occurrences and fluctuations coastal phytoplankton populations due to the influence of meteorological and hydrographic events are important considerations. For example, a large rain storm may cause nutrient loading in coastal waters and trigger a toxic phytoplankton bloom or a hurricane may drive offshore phytoplankton bloom onshore. As well, uptake rates for various species of shellfish being tested is critical in terms of timing.
5. Sample Collection Procedures
6. 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.

7. Identification of Laboratories/Analysts;
   Biotoxin sample results must be provided by an NSSP conforming lab that is utilizing an approved or limited use method. For checklist requirements and additional guidance regarding laboratory evaluation for conformance, see Chapter II Growing Areas. For NSSP requirements, see Section II MO, Chap I Shellfish Sanitation Program, @.03(B).

   The Authority should consider where they can access sample processing for biotoxins that occur or may occur within their jurisdiction, and identify alternative laboratory support, should that support become necessary.

8. Description of Testing Methods, Which May Include Approved Limited Use and Approved Methods
   To control marine biotoxins, the authority must evaluate the concentration of toxin present in the shellfish. In the case of NSP, phytoplankton must be monitored as well as shellfish. Approved and limited use methods are listed the NSSP Guidance Documents.

9. Establishment of Appropriate Screening Levels
   Though the NSSP establishes the toxin levels in shellfish at which a growing area must be closed, many programs implementing early warning systems include phytoplankton cell counts. Additionally, shellfish toxin levels that a below the regulatory levels may trigger emergency or expanded testing, or precautionary closures. Growing areas should be closed at a level that provid an adequate margin of safety, since in many instances, toxicity levels will change rapidly and the time between sampling and results should be conside Precautionary closures can be made in order to prevent the harvest of potentially toxic shellfish while sample results are being collected and processed.

10. Procedures to Expand Sampling if Toxin Levels or Cell Counts Indicate a Harmful Algal Bloom.
    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 station and/or increase the frequency of sampling for marine biotoxins. The procedu 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 an emergency shellfish sampling program

**Recordkeeping*

Records generated as part of a marine biotoxin program may be important in definin the severity of an event, as well as for retrospectively evaluating the adequacy of the

entire control program.

The NSSP requires certain biotoxin-related records be maintained. As such, authority plan should define records to be generated, reviewed, and maintained. Required reco include:

* Monitoring data, including shellfish and phytoplankton and water sample analyses results, relating to levels of marine biotoxins in each growing area;
* Closure and reopening notices;
* Investigation-related documents, including sample results;
* Recall-related records, including public warnings, notification to other states involved in the recall, FDA, and ISSC, recall status reports in accordance with Section II, Chapter II Risk Assessment and Risk Management, @.01(I); and
* Evaluation reports, which may include analyses of trends and detoxification curves.

An authority may also consider maintaining

- Records of reported illnesses that include data on the incidence of illness and appropriate case history data; and
- Pertinent environmental observations.

Whenever possible, the authority's servicing laboratory should archive shellfish homogenates for additional analysis.

**Plan Testing, Post Event Activities*

The authority should test the plan periodically to ensure prompt implementation in th event it is needed. As well, the authority should routinely review data post-event to improve aspects of the authority's plan. Because historical information plays such a critical role in the authority's plan, authorities are highly encouraged to document rationale for significant changes.

Heat Processing.

In shellfish growing areas where low levels of PSP routinely occur, harvesting for thermal processing purposes may be an alternative to consider. Thermal processing, as defined by applicable FDA regulations (21 CFR 113), will reduce the toxin concentration of certain toxins in the shellfish via dilution, not destruction.

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; and must require that the processor provide adequate demonstration of the destruction of the biotoxin and adequate controls to assure that the end product is safe for human consumption.

~~NSSP guidance documents provide the public health principles supporting major components of the NSSP and its Model Ordinance, which includes the requirement~~

	the program . NSSP *Model Ordinance* requirements apply only to interstate commerce although most states apply the requirements intrastate. For the most up-date and detailed listing of requirements, the reader should consult the most recent edition of the Model Ordinance. **Introductin** Shellfish are filter feeders and, therefore, they have the ability to concentrate toxic phytoplankton from the water column when present in shellfish growing waters. T toxins produced by certain species of phytoplankton can cause illness and death in humans. Toxins are accumulated in the viscera and/or other tissues of shellfish and are transferred to humans when the shellfish are eaten (Gordan *et al*., 1973). These toxins are not normally destroyed by cooking or processing and cannot be detected taste. The presence of toxic phytoplankton in the water column or traces of their to in shellfish meat does not necessarily constitute a health risk, as toxicity is depende on concentration (dose) in the shellfish. To protect the consumer, the Authority m evaluate the concentration of toxin present in the shellfish or the toxic phytoplankte concentration in the water column against the levels established in the NSSP Model Ordinance to determine what action, if any, should be taken. While there is a wide range of methodologies developed for screening and confirmat of toxic phytoplankton and their toxins, methods must be adopted into the NSSP if th are to be implemented for the confirmation of toxins for making decisions to reopen growing areas. Additionally, there are screening methods that have been evaluated b the ISSC and found fit for purpose for the NSSP, thereby providing confidence in the methods for specific screening purposes. Toxin methods fall into two categories in t NSSP: Approved Methods for Marine Biotoxin Testing (Section IV. Guidance Documents Chapter II Growing Areas .14 Table 2.) and Approved Limited Use Methods for Marine Biotoxin Testing (Section IV. Guidance Documents Chapter II Growing Areas .14 Table 4.). These methods range from mouse bioassays to immunochromatography and other antibody based platforms to chemical analytical methods such as high performance liquid chromatography (HPLC). Information available in the referenced Tables above provides references for the methods and, as applicable, and limitations placed on the use of the method within the NSSP. For tox that have no method adopted into the NSSP, best available science is employed. There are five (5) types of shellfish poisonings which are specifically addressed in th NSSP Model Ordinance: Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP), Amnesic Shellfish Poisoning (ASP), also known as Domoic Acid poisoning, Diarrhetic Shellfish Poisoning (DSP) and Azaspiracid Shellfish Poisoning (AZP). Of these five (5) types of shellfish poisoning, PSP, NSP and ASP are the mo dangerous PSP and ASP can cause death at sufficiently high concentrations. In addition, ASP can cause lasting neurological damage. PSP is caused by saxitoxins produced by the dinoflagellates of the genus *Alexandrium* (formerly *Gonyaulax*). Th dinoflagellate *Pyrodinium bahamense* is also a producer of saxitoxins. NSP is caus by brevetoxins produced by the dinoflagellates of the genus *Karenia* (formerly *Gymnodinium*). ASP is caused by domoic acid and is produced by diatoms of the genus Pseudonitzchia. Certain *Dinophysis* spp. and *Prorocentrum* spp. produce okadaic acid and dinophysis toxins that cause DSP. *Azadinium* spp. is the producer o azaspiracids, which cause AZP.Both *Alexandrium* and *Karenia* can produce "red tide i.e. discolorations of seawater caused by blooms of the algae; however, they may als

~~reach concentrations that may result in toxic shellfish without imparting any water discoloration. Toxic blooms of these dinoflagellates can occur unexpectedly or follo predictable patterns. The unpredictability in occurrence of toxic blooms was demonstrated in New England in 1972 when shellfish suddenly became toxic in a previously unaffected portion of the coastline and resulted in many illnesses (Schwal 1973). Historically, *Alexandrium* blooms have occurred between April and October along the Pacific coasts from Alaska to California and in the Northeast from the Canadian Provinces to Long Island Sound (U.S. Public Health Service, 1958); but th patterns may be changing. The blooms generally last only a few weeks and most shellfish (with the exception of some species of clams and scallops, which retain the toxin for longer periods) clear themselves rapidly of the toxin once the bloom dissipates. NSP has occurred from the Carolinas and extends throughout the Gulf Coast states. It shows no indication of regular recurrence and shellfish generally tak longer to eliminate the toxin (Liston, 1994). DSP and AZP cause similar symptoms mostly related to diarrhea and abdominal pain. DSP toxin-producing phytoplankton have been documented to occur off the coasts of Washington (Trainer et al. 2013) an Texas (Deeds et al. 2010) as well as off the coast in the northeast (e.g., Massachuset [Tong et al. 2015]).While AZP has occurred in the U.S., the contaminated shellfish v imported (Klontz et al. 2009). Harvesting closures in the U.S. have not been documented due to AZP toxins.~~

~~The minimum concentration of PSP toxin that will cause intoxication in susceptible persons is not known. Epidemiological investigations of PSP in Canada, however, ha 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 PSP toxin. Investigations indicate that lesser amounts of the toxin have no deleterio effects on humans. Shellfish growing areas should be closed at a PSP toxin level, wh provides an adequate margin of safety, since in many instances PSP toxicity levels c change rapidly.~~

~~The NSSP Model Ordinance requires that growing areas be placed in the closed statu when the PSP toxin concentration is equal to or exceeds the action level of 80 micrograms per 100 grams of edible portion of raw shellfish (FDA, 1977; FDA, 198~~

~~In shellfish growing areas where low levels of PSP routinely occur, harvesting for thermal processing purposes may be an alternative to consider. Thermal processing as defined by applicable FDA regulations (21 CFR 113) will reduce PSP toxin concentration of the shellfish via dilution, not destruction. 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.~~

~~In Gulf coast areas, toxicity in shellfish has been associated with red tide outbreaks caused by massive blooms of the toxic dinoflagellate, *Karenia brevis*. The most common public health problem associated with *Karenia* blooms is respiratory irritation; however, neurotoxic shellfish poisonings associated with *Karenia brevis* blooms have been reported in Florida (Center for Disease Control, 1973 [a] and [b] Uncooked clams from a batch eaten by a patient with neurotoxic symptoms were found to contain 118 mouse units per 100 grams of shellfish meat. The NSSP Mod Ordinance mandates that growing areas be placed in the closed status when any NS toxin is found in shellfish meat at or above 20 MU per 100 grams of shellfish, or w~~

~~the cell counts for members of the genus *Karenia* in the water column equal or exce~~
~~5,000 cells per liter of water.~~

~~ASP is caused by domoic acid, which is produced by diatoms of the genus *Pseudonitzachia*. Blooms of *Pseudonitzachia* are of varying intensity, duration and extent.. During the 1991-1992 incident in Washington and the 2015 event on the w coast from Washington to California, high toxin levels persisted for several months (Liston, 1994; McCabe et al. 2016). There was also an extensive event in the Northeast from Maine to Rhode Island in 2016, with different regions showing vary toxicity and species dominance within the bloom. The event started in late Septem in eastern Maine and ended in October; however, Rhode Island experienced anothe bloom in February of 2017.The NSSP Model Ordinance requires that growing area placed in the closed status when the domoic acid concentration is equal to or exceed 20 parts per million raw shellfish.~~

~~The suitability of some growing areas for shellfish harvesting is periodically influenced by the presence of marine biotoxins such as those responsible for PSP, NSP, ASP, DSP and AZP. The occurrence of these toxins is often unpredictable, a the potential for them to occur exists along most coastlines of the United States and other countries having shellfish sanitation Memoranda of Understanding (MOU) agreements with the United States. As a result, states or countries with MOUs with the U.S. need to have management plans and/or contingency plans to address shellf borne intoxications.~~

~~Controlling Marine Biotoxins in Shellfish~~

~~There are two types of plans defined in the NSSP MO for the control of marine biotoxins~~

~~The contingency plan must describe administrative procedures, laboratory support, sample collection procedures, and patrol procedures to be implemented on an emergency basis in the event of the occurrence of shellfish toxicity (Wilt, 1974) The primary goal of this planning should be to ensure that maximum public health protection is provided. To achieve this goal the following objectives should be met~~

- ~~*An early warning system should be developed and implemented.~~
- ~~*Procedures should be established to define the severity of occurrences.~~
- ~~*The state or MOU country should be able to respond effectively to minimize illness.~~
- ~~*Adequate intelligence and surveillance information should be gathered a evaluated by the Authority.~~
- ~~*Procedures should be instituted to return the Biotoxin contaminated areas to th open status of their growing area classification.~~

~~Under the certification provisions of the NSSP, FDA and receiver states should hav the assurance that shellfish producing states or MOU countries are taking and can t adequate measures to prevent harvesting, shipping, and consumption of toxic shellf To provide this assurance, the NSSP requires the Authority to develop and adopt a marine Biotoxin contingency plan for all marine and estuarine shellfish growing are The Authority's plan should specify how each of the objectives listed above will be accomplished. This document provides recommended guidelines to be used in~~

preparing a plan to meet these objectives.

Recommended Contingency Plan Guidelines

- The process for precautionary closures:
- A sampling plan that considers water samples to evaluate t extent and intensity of the bloom
- A sampling plan that considers species specific shellfish sampling
- Access to screening tests; both rapid and approved method
- Trained staff to carry out sample collection and testing if necessary
- A reopening criteria

The Marine Biotoxin Management Plan

The marine biotoxin management plan is primarily for proactive management of marine biotoxins based on a history of toxin-producing phytoplankton and toxicity shellfish and/or a previous illness event or outbreak. The management plan must describe an early warning system, administrative procedures, laboratory support, sample collection procedures, patrol procedures to be implemented and reopening criteria (Wilt, 1974). A management plan is required for a shellfish Authority that a history of toxin-producing phytoplankton, toxicity in shellfish and/or an illness ev or outbreak attributed to their growing areas. A shellfish Authority might have a management plan for certain marine biotoxins like PSP toxins but a contingency pl for toxins like AZP toxins. The primary goal of the management plan should be to prevent illnesses from toxic shellfish and ensure that maximum public health protection is provided. To achieve this goal the following objectives should be met

- An early warning system should be developed and implemented.
- Procedures should be established to define the severity of occurrences.
- The Authority should be able to respond effectively to minimize illness.
  - Adequate intelligence and surveillance information should be gathe and evaluated by the
  - Authority.
  - Procedures should be instituted to return the biotoxin contaminated area the open status of their
  - growing area classification.

** Provide an early warning system:*

1. Communication procedures should be established with other appropriate agencies to rapidly report to the Authority any abnormal environmental phenomenon that might be associated with shellfish growing areas such as bird or fish kills, water discoloration or abnormal behavior of shellfish or marine scavengers.
2. The Authorities should establish procedures for health agencies to report an toxin-like illnesses.
3. An early warning phytoplankton and/or shellfish monitoring program shoul be implemented.

   These monitoring programs should use the "key station" (for both

phytoplankton and shellfish monitoring) and "critical species" concepts (fo shellfish monitoring).

* Sampling stations should be located at sites where past experience ha shown toxin is most likely to appear first.
* When 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 th highest toxin levels. For example, mussels have been found to be usefu for early PSP detection.
* The frequencies and periods for collection of samples should be established recognizing the randomness of PSP blooms. This assumes several years of baseline data in order to establish stations and samplin plans.
* Frequency of sampling should be adequate to monitor for fluctuation coastal phytoplankton populations.

4. Channels of communication concerning shellfish toxicity should be establis with other states, countries (in the case of MOU countries), FDA, and other responsible officials. A marine Biotoxin control official should be designa by the Authority to receive and distribute all marine Biotoxin related information. Consultation with adjacent jurisdictions, marine biologists and other environmental officials might also be useful (Felsing, 1966; Quayle, 1969; Prakash *et al*., 1971).

** Define the severity of the problem:*

1. A procedure should be established to promptly expand the sampling program for marine Biotoxins in the event of increased toxicity/cell count any indicator monitoring stations identified within the plan. Sampling stations and frequencies of sampling should be increased when monitoring data or other information suggests that toxin levels are increasing. T procedure should include plans for obtaining the additional resources necessary to implement the expanded sampling and laboratory analysis program.
2. Information should be available concerning the location of commercial shellfish resource areas and species present in the state.
3. Criteria should be developed to define the circumstances under which grow areas will be placed in the closed status because of marine Biotoxin contamination. The criteria should integrate public health, conservation, a economic considerations. Principal items of concern include consideration the rapidity with which toxin levels can increase to excessive levels, the 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 safe zone), and the type of harvesting restrictions to be invoked (all species or specific species). It may be appropriate to close harvesting areas adjacent t known toxic areas until increased sampling can establish which areas are to free and that toxin levels have stabilized.
4. Procedures should be established to promptly identify which shellfish produ or lots might be potentially contaminated, and to determine the distribution of these products or

lots.

** Respond effectively to minimize illness:*

1. A summary should be provided citing the laws and regulations in the state (MOU country) that promptly and effectively allow the Authority to restrict harvesting, withdraw interstate shipping permits, and to embargo/recall any potentially toxic shellfish already on the market in the event of a marine Biotoxin event. The plan should clearly define the timeframe involved in taking appropriate legal action.
2. The administrative procedures necessary to place growing areas in the close status, to withdraw interstate certification of dealers, and to embargo and recall shellfish should be delineated. The timeframe necessary to accompli these actions should also be specified.
3. A plan should be developed which will define what type of patrol program necessary to properly control harvesting in toxin contaminated growing are The program should be tested to ensure prompt implementation in the even is needed.
4. Procedures should be developed to promptly disseminate information on th occurrences of toxic phytoplankton blooms to the industry and local health agencies. It is helpful to establish relationships and procedures with other agencies such as the state CDC and Poison Control and authorities in advar of any serious biotoxin event.
5. Procedures should be established to coordinate control activities taken by st and federal
   agencies or departments and district, regional, or local health authorities.

** Return growing areas to the open status of their NSSP classification:*

1. Once a growing area is placed in the closed status because of marine Biotox contamination, a procedure should be instituted to gather data necessary to decide when the area can be returned to the open status of its classification. system of representative samples to establish detoxification curves should b part of this procedure.
2. The Authority should develop a set of criteria that must be met before a growing area can be returned to the open status. These criteria should integrate public health, conservation, and economic considerations, and employ a sufficient number of samples and other environmental indices, if used, to establish that the level of toxin or cell counts are below the closure level. For example, experience has shown that appropriate reopening criter for PSP include a minimum of three (3) samples collected over a period of least fourteen (14) days. These samples should show the absence of PSP o levels below 80 micrograms per 100 grams of shellfish tissue.
3. A program of consumer education should be continued as long as any area remains in the closed status because of marine Biotoxin contamination.

References
Title 21 CFR Part 7
References

	1. Center for Disease Control (a). 1973. Shellfish Poisoning - Florida. *Morbid. Mortal. Weekly Rep*.22(48):397-398. 2. Center For Disease Control (b). 1973. Neurotoxic Shellfish Poisoning - Florida. *Morbid. Mortal.Weekly Rep*. 22(48):397-398. 3. Felsing, W.A., Jr. 1966. Proceedings of Joint Seminar on North Pacific Cla September 24-25,1965. U.S. Public Health Service, Washington, D.C. 4. Food and Drug Administration. 1977. Poisonous or Deleterious Substances Food. *FederalRegister* 42(190):52814-52819. 5. Food and Drug Administration. 1985. Action Levels For Poisonous or Deleterious Substances in Human Food and Animal Feed. U.S. Department of Health and Human Services, Public Health Service, Washington, D.C. 20204. 1 pages. 6. Gordon, K., M.D., *et al*. 1973. Shellfish Poisoning. *Morbid. Mortal. Weekly Rep*. 22, (48):397-398. 7. Liston, J. 1994. Association of *Vibrionaceae*, natural toxins, and parasites v fecal indicators, p.215-216. In Hackney, C.R. and M.D. Pierson (eds.), *Environmental Indicators and Shellfish Safety*. Chapman and Hall, New York, 8. Prakash, A., J.C. Medcof, and A. D. Tennant. 1971. Paralytic shellfish poisoning in easternCanada. Bulletin 177, Fisheries Research Board of Canada. Ottawa, Canada. 9. Quayle, D.B. 1969. Paralytic shellfish poisoning in British Columbia. Bulle 168, FisheriesResearch Board of Canada. Ottawa, Canada. 10. Schwalm, D.J. 1973. The 1972 PSP outbreak in New England. FDA Report Boston, MA. U.S.Food and Drug Administration, Washington, D.C. 11. U.S. Public Health Service (PHS). 1958. Proceedings: 1957 Conference on Shellfish Poison. U.S.PHS, Washington, D.C. 125 pages. 12. Wilt, D.S. (ed). 1974. Proceedings of Eighth National Shellfish Sanitation Workshop. January 16-18. New Orleans, LA. National Technical Information Services (PB8-6 236916/AS), U.S. Dept. of Commerce, Springfield, VA. 158 p
13. Public Health Significance	Marine biotoxins can cause injury, illness, or death. More clearly presented guidance will assist control authorities in developing marine biotoxin contingency and management plans.
14. Cost Information	None

Proposal No. 19-125

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☐ Growing Area b. ☒ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Executive Office
3. Affiliation	Interstate Shellfish Sanitation Conference
4. Address Line 1	209 Dawson Road
5. Address Line 2	Suite 1
6. City, State, Zip	Columbia, SC 29223
7. Phone	(803) 788-7559
8. Fax	(803) 788-7576
9. Email	issc@issc.org
10. Proposal Subject	*Karenia brevis* Guidance
11. Specific NSSP Guide Reference	Section IV Guidance Documents – Chapter II. Growing Areas
12. Text of Proposal/ Requested Action	**.02 Guidance for Developing Marine Biotoxin Plans** Introduction Shellfish are filter… There are a… There are five… Both *Alexandrium* and… The minimum concentration… The NSSP Model… In shellfish growing… In Gulf coast… areas, toxicity in shellfish has been associated with red tide outbreaks caused by massive blooms of the toxic dinoflagellate, *Karenia brevis*. The most common public health problem associated with *Karenia* blooms is respiratory irritation; however, neurotoxic shellfish poisonings associated with *Karenia brevis* blooms have been reported in Florida (Center for Disease Control, 1973 [a] and [b]). Uncooked clams from a batch eaten by a patient with neurotoxic symptoms were found to contain 118 mouse units per 100 grams of shellfish meat. The NSSP Model Ordinance mandates that growing areas be placed in the closed status when any NSP toxin is found in shellfish meat at or above 20 MU per 100 grams of shellfish, or when the cell counts for ~~members of the genus~~ *Karenia brevis* in the water column equal or exceed 5,000 cells per liter of water.
13. Public Health Significance	The 5,000 cell count standard applies to *Karenia brevis* only
14. Cost Information	

Proposal No. 19-126

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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	240-402-24001
8. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	MPN-Real-Time PCR for Enumeration of Vibrio vulnificus in Oysters
11. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter II. Growing Areas .14 Approved NSSP Laboratory Tests.

12. Text of Proposal/ Requested Action

5. Approved Methods for Vibrio Enumeration

	Vibrio Indicator Type:	**Application: PHP Sample Type: Shucked**	**Application: Reopening**
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**
MPN-Real Time PCR[7]	***Vibrio parahaemolyticus (V.p.)***	**X**	**X**
Direct Plating Method[8]	***Vibrio parahaemolyticus (V.p.)***		**X**
MPN-Real Time PCR[9]	***Vibrio vulnificus (V.v.)***	**X**	

Footnotes:

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

[2] 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.

[3] 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.

[4] 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 the vvh gene for total *V. vulnificus* as described in Kinsey et al., 2015.
13. Public Health Significance	This MPN-real-time PCR method provides results in as little as 24 h from receipt of sample. The current NSSP methods for enumeration of *Vv* have limitations: the traditional MPN requires a minimum of 3 days and the SYBR Green PCR is only validated on an instrument platform which is no longer supported by the manufacturer. This method provides an additional option for laboratories to maintain the same level of testing as has been maintained in the program.
14. Cost Information	This method costs ~$100 per sample for laboratory consumables, supplies, and reagents. Most equipment needed for testing is standard microbiology equipment, but purchase of a heat block (~$400) and/or centrifuge (~$2,500) may be necessary. Purchase of a real-time PCR instrument will be required ($30,000-$45,000). Additional costs for a laboratory would vary based on their operational overhead and labor.

Name of the New Method	MPN-Real-Time PCR Method for the Detection of Vibrio Vulnificus from Oysters
Name of the Method Developer	USFDA Gulf Coast Seafood Laboratory
Developer Contact Information	USFDA Gulf Coast Seafood Laboratory, 1 Iberville Drive, Dauphin Island, AL 36528 Jessica.jones@fda.hhs.gov

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
Clearly define the need for which the method has been developed.	Y	Currently, the most common NSSP method used to detect *Vibrio vulnificus* (Vv) in oysters is MPN-culture. The method is time consuming and laborious taking a minimum of four full days to produce a result. A quicker method uses Real-time PCR for detection, currently the only NSSP approved Real-time PCR utilizes Sybr green: a non-specific DNA binding molecule, which negates the ability to multiplex thus is not permissive of the use of an internal control to assure the reaction integrity. The ability to use an internal control adds a level of reliability the use of a non-specific binder like Sybr Green cannot. Additionally, the Sybr Green method is validated for use with the Smart Cycler by Cepheid which, as of December 2018, will no longer be supported by the manufacturer. The MPN Real-time PCR method for Vv detection in oysters will utilize the AB7500 Fast, the same instrument which the NSSP-approved MPN Real-time PCR methods for Vp utilizes. Further, this method uses a specific probe targeting the vvh gene of Vv and includes an internal control in a single assay. This assay is rapid and robust producing highly reliable results in 24-36 hours.
What is the intended purpose of the method?	Y	Approved NSSP method for enumeration of Vv from oysters.

Is there an acknowledged need for this method in the NSSP?	Y	There is current methodology. This assay is quicker than the aproved culture methods and more robust than the existing real-time PCR method.
What type of method? i.e. chemical, molecular, culture, etc.	Y	MPN enrichment with molecular confirmation.

B. Method Documentation		
1. Method documentation includes the following information:		
Method Title	Y	MPN-Real-Time PCR Method for the Detection of Vibrio vulnificus from Oysters
Method Scope	Y	This method is for the detection of *Vibrio vulnificus* from oysters using the AB7500 Fast real-time PCR platform.
References	Y	Campbell, M.S., Wright, A.C., 2003. Real-time PCR analysis of *Vibrio vulnificus* from oysters. Appl Environ Microbiol 69, 7137-7144 Jones, J.L., Kinsey, T.P., Johnson, L.W., Porso, R., Friedman, B., Curtis, M., Wesighan, P., Schuster, R., Bowers, J.C., 2016. Effects of Intertidal Harvest Practices on Levels of *Vibrio parahaemolyticu*s and *Vibrio vulnificus* Bacteria in Oysters. Appl Environ Microbiol 82, 4517-4522. Kaysner, C., DePaola, A., 2004. *Vibrio*, Bacteriological Analytical Manual, 8th ed. Nordstrom, J.L., Vickery, M.C., Blackstone, G.M., Murray, S.L., DePaola, A., 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. Appl Environ Microbiol 73, 5840-5847.
Principle	Y	This method is uses an MPN format for enumeration based on molecular (PCR) detection of the vvh gene specific to Vv.

Any proprietary aspects	Y	The AB7500 Fast is a proprietary real-time PCR platform developed by Applied Biosystems and sold through ThermoFisher Scientific. The optical plates and caps or film used are proprietary to the instrument.
Equipment required	Y	Equipment is listed in Appendix A.
Reagents required	Y	Media and reagents are listed in Appendix B.
Sample collection, preservation and storage requirements	Y	Shellstock samples are bagged immediatley upon collection and labeled with collector's name, the source of harvest, sampling stations, time, and date. Samples are placed in dry storage (ice chest or equivalent) maintained between 2°C and 10°C with ice or cold packs for transport. A layer of towels, bubblewrap, or another appropriate substance will separate shellfish from contact with ice or cold packs. If collected samples are frozen (such as IQF), direct contact with ice or cold packs is not permitted. Immediately upon arrival of sample(s) to the laboratory, date, time, and initials of receiver are documented. The temperature of three shellfish, each from a separate location within each shipping container, is measured by opening the sell enough to insert a temperature probe into the meat of the shellfish. If IQF samples are received, assure samples are frozen. Store at less than -15°C until ready to process. Temperatures are taken immediately after defrosting as described above. The shellfish is discarded after temperature is measured. Once temperature of the samples upon intake is established, the samples are placed under refrigeration for not longer than 36h after collection, unless processed immeditely. Storage is documented. If processing IQF samples, samples are defrosted under refrigeration for no longer than 36h.
Safety requirements	Y	Basic Personal Protection Equipment (PPE) is needed. A chain mail glove may

		be worn during shucking. Blending is done in a biosafety hood or the blender is placed in a splash shielded containter or blender box. All biological waste is autoclaved and disposed of according to state regulations.
Clear and easy to follow step-by-step procedure	Y	Detailed procedure including sample preparation, MPN, PCR, and data analysis is included in Appendix C.
Quality control steps specific for this method	Y	Appropriately diluted process controls are used (Vv ATCC 33816 and Vp F11-3A). Appropriately diluted Internal Amplification Control (IAC) DNA is included in all PCRs. Manual review of amplification curves is conducted.

C. Validation Criteria		
1. Accuracy / Trueness	Y	Result: 110% Data: Table 1 Spike Range: -0.35 to 6.54 Log CFU/g
2. Measurement uncertainty	Y	Result: -0.57 to 0.044 log MPN/g Data: Table 1 Spike Range: -0.35 to 6.54 Log CFU/g
3. Precision characteristics (repeatability)	Y	Results: Variance ratio is *not significant*, based on least square regression. Calculated variability of the MPN method is 0.39, with a lower 95% CI of 0.32. The theoretical variability is 0.32. Data: Table 2, Figure 1 Spike Range: 0.38 to 5.54 Log CFU/g
4. Recovery	Y	Result: 110% Is the one way ANOVA to determine the consistency of recovery significant? No. Data: Table 2 Spike Range: 0.38 to 5.54 Log CFU/g
5. Specificity	Y	*V. alginolyticus*: SI_{avg} = -1.28, p=0.42 *V. cholerae*: SI_{avg} = 1.26, p=0.09 *V. fluvialis*: SI_{avg} = -2.41, p=0.79 *V. parahaemolyticus*: SI_{avg} = 7.49, p=0.07

		Data: Table 3 Range: 0.52 to 1.53 Log CFU/g
6. Working and Linear ranges	Y	Pearson's r: 0.97 Line equation: log(MPN) = 0.44 + 0.93 x log(Plate Count) Is Pearson's r significant?:Yes Data: Table 4 and Figure 2 Range: -0.62 to 6.54 Log CFU/g
7. Limit of detection	Y	Result: 2.75 95% CI: 1.95, 3.88 Data: Table 4 and Figure 1 Range: -0.62 to 6.54 Log CFU/g
8. Limit of quantitation / Sensitivity	Y	Result: 0.3 MPN/g Data: Table 4 and Figure 1 Range: -0.62 to 6.54 Log CFU/g
9. Ruggedness	Y	Is there a significant difference between samples? Not under conditions tested. Data: Table 5 Range: 0.52 to 4.88 Log CFU/g
10. Matrix effects	Y	Effects of oyster matrix on the performance of the method was taken into consideration by using various sources of oysters for this study. Appendix D.
11. Comparability (if intended as a substitute for an established method accepted by the NSSP)	Y	No statistically significant difference between test and accepted methods. ($p<0.05$) Data: Table 6

D. Other Information		
1. Cost of the method	Y	Cost per sample for MPN: $1.05 Cost per sample for PCR: $20.55 Cost only includes reagents and consumables, infrastructure and personnel were not taken into account.
2. Special technical skills required to perform the method	Y	It is recommended that analysts have some formal training in molecular techniques or PCR, specifically.
3. Special equipment required and associated cost	Y	AB7500 FAST: $34,060.00 AB7500 FAST annual maintenance contract: $5,777.00
4. Abbreviations and acronyms defined	Y	Abbreviations and Acronyms are listed in appendix E.
5. Details of turn around times	Y	Results can be reported within 28h of

(time involved to complete the method)		sample receipt.
6. Provide brief overview of the quality systems used in the lab	Y	The laboratory adheres to the quality system standards of FDA/CFSAN, as well as those of the NSSP.

Submitters Signature	Date:
Submission of validation data and draft method to committee	Date:
Reviewing members:	
Accepted	Date:
Recommendations for further work	Date:

A. Validation Criteria

Data were generated using 20 separate lots of PHP oysters spiked with appropriate dilution(s) of a log phase culture of *Vibrio vulnificus*. Spike levels were determined by plate counts on TSA. Unless otherwise stated data was handled and analyzed as recommended in the SLV Documents for MPN Based Microbiological Methods on the ISSC website, with the exception of correcting for background using the blank sample data. The correction was not made because the levels in the blank samples were extremely low (near the LOD) and the it was more appropriate, from a statistical perspective, to not make the adjustment. For samples not detected, ½ the theoretical LOD was substituted for those values. For samples greater than the upper limit of the test, the values for the upper limit was used.

Table 1. Data used for determination of Accuracy/Trueness and Measurement Uncertainty.

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spiked Sample (Log MPN/g)
1	-0.35	0.31	-0.25
2	4	ND*	3.33
3	1.19	ND	1.4
4	2.92	-0.45	3.17
5	1.38	ND	1.8
6	1.06	-0.52	2.36
7	2.74	-0.52	2.64
8	4.78	ND	4.96
9	4.84	ND	5.75
10	3	ND	3.38
11	6.54	ND	6.16
12	1.11	ND	1.63
13	6.08	0.36	5.36
14	4.88	ND	5.62
15	-0.19	ND	-0.15
16	2.57	ND	2.36
17	0.97	ND	1.92
18	1.53	ND	1.17
19	1.88	-0.45	2.16
20	0.52	-0.13	0.50

*ND=Not Detected

Table 2. Data used for determination of Precision and Recovery. Samples A and B are replicate analyses of the spiked homogenate.

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spiked Sample A (Log MPN/g)	Spiked Sample B (Log MPN/g)
1	0.66	0.31	0.87	1.36
1	2.66	0.31	2.96	2.63
1	4.66	0.31	4.63	5.31
3	1.19	ND	1.63	1.17
3	3.19	ND	1.72	2.96
3	5.19	ND	3.96	5.17
5	0.38	ND	0.63	0.96
5	2.38	ND	2.63	2.17
5	4.38	ND	3.96	4.36
7	0.74	-0.52	1.32	1.31
7	2.74	-0.52	2.96	2.32
7	4.74	-0.52	4.97	5.35
9	0.84	ND	2.04	1.33
9	2.84	ND	3.66	3.38
9	4.84	ND	5.87	5.62
11	1.54	ND	1.63	1.63
11	3.54	ND	3.63	3.36
11	5.54	ND	5.34	5.62
13	1.08	0.36	1.36	1.96
13	3.08	0.36	2.96	3.16
13	5.08	0.36	5.18	4.97
15	0.81	ND	0.63	0.87
15	2.81	ND	2.63	3.96
15	4.81	ND	4.97	4.63
17	0.97	ND	1.97	1.87
17	2.97	ND	4.38	3.97
17	4.97	ND	5.87	5.887
19	0.88	-0.45	1.63	0.87
19	2.88	-0.45	3.36	3.36
19	4.88	-0.45	5.62	5.62

*ND=Not Detected

Figure 1. Plot of data from Table 2 by different concentrations (Low, Medium, and High). An alternative approach from ISSC recommendations to evaluating precision was used as a generalized least square regression with heterogenous variance structure was deemed a more appropriate test to estimate variance components for method error at different concentrations and then test whether or not method error varies significantly by concentration level. The output estimates of the variance components of the fit of two different models and then a comparison of those fits. One model has different parameters for method variation for each level (L, M, H) and the other constrains that variation to be the same across levels. The 1st model (null) estimates a common method error SD as 0.387 (same as the nested ANOVA). The 2nd model (full) estimates different method error SDs as 0.3217, 0.4688 and 0.3558 at levels L, M, and H respectively. Both models fit the same main effects (Levels nested within Samples) to remove that variation from what remains to determine method error estimates. A likelihood ratio test is used to compare the difference in the fit between the two models. The test statistic is the likelihood ratio between the two models and this is distributed as a Chi-square with 2 degrees of freedom (the difference in the number of parameters between the two models, 3 vs 1 variance parameters). The test statistic has a value of 1.58 and the p-value is 0.54 indicating no significant difference between the fits and hence no strong statistical evidence that method error varies across levels (L, M, H). The MSE for the residuals is 0.15. This corresponds to a SD of 0.39, which is only slightly higher than the theoretical method error SD (0.32), with a lower 95% confidence limit of 0.32.

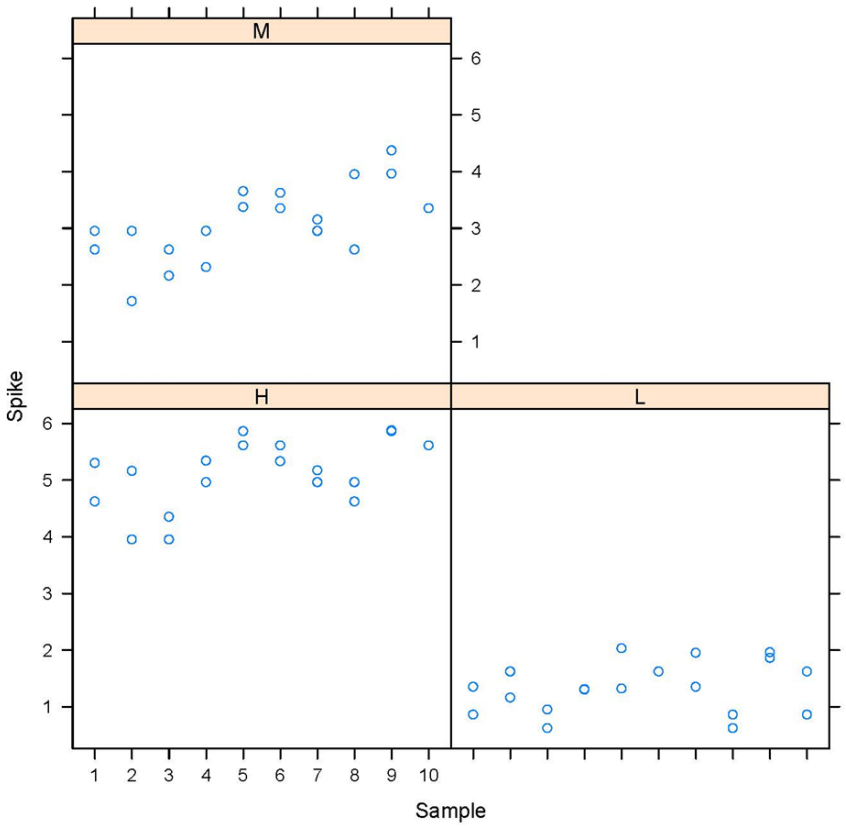


Table 3. Data used for determination of Specificity. Spike samples A-E are replicate analyses of the homogenate spiked only with Vv. Dual spike samples A-E are replicate analyses of the same homogenate spiked with Vv and the interfering organism.

Sample	Interfering Organism	Interfering Organism Plate Count (Log CFU/g)	*Vibrio vulnificus* Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spike Sample A (Log MPN/g)	Spike Sample B (Log MPN/g)	Spike Sample C (Log MPN/g)	Spike Sample D (Log MPN/g)	Spike Sample E (Log MPN/g)	Dual Spike Sample A (Log MPN/g)	Dual Spike Sample B (Log MPN/g)	Dual Spike Sample C (Log MPN/g)	Dual Spike Sample D (Log MPN/g)	Dual Spike Sample E (Log MPN/g)
6	*Vibrio parahaemolyticus*	5.49	1.06	-0.52	2.36	2.38	2.38	2.38	2.66	1.63	0.31	0.19	1.32	0.19
12	*Vibrio cholerae*	6.75	1.11	ND	1.63	1.63	1.63	1.63	1.96	1.36	0.96	1.36	1.63	1.63
18	*Vibrio fluvialis*	6.83	1.53	ND	1.17	1.36	1.63	1.36	1.86	1.96	1.96	1.63	-0.03	0.06
20	*Vibrio alginolyticus*	6.17	0.52	-0.13	0.45	0.96	0.31	0.45	0.45	0.96	0.44	0.43	-0.04	0.3

Table 4. Data used for determination of Working and Linear Ranges, Limit of Detection, and Limit of Quantitation/Sensitivity. Samples A and B are replicate analyses of the spiked homogenate. The LOQ is determined by the amount of inoculum used in the lowest dilution of the MPN, so long as the LOD is not statistically different than 1. As tested with a starting inoculum of 1g, the LOD of this method is 0.3 MPN/g

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Spike Sample A (Log MPN/g)	Spike Sample B (Log MPN/g)
1	-0.35	0.31	-0.45	-0.04
1	0.66	0.31	0.87	1.36
1	1.66	0.31	1.63	3.06
1	2.66	0.31	2.96	2.63
1	4.66	0.31	4.63	5.31
1	5.66	0.31	5.92	6.16
3	0.19	ND	0.86	0.63
3	1.19	ND	1.63	1.17
3	2.19	ND	2.36	2.87
3	3.19	ND	1.72	2.96
3	5.19	ND	3.96	5.17
3	6.19	ND	6.16	6.16
5	-0.62	ND	0	-0.45
5	0.38	ND	0.63	0.96
5	1.38	ND	1.63	1.96
5	2.38	ND	2.63	2.17
5	4.38	ND	3.96	4.36
5	5.38	ND	4.97	4.63
7	-0.25	-0.52	0.87	0.19
7	0.74	-0.52	1.32	1.31
7	1.74	-0.52	1.96	2.96
7	2.74	-0.52	2.96	2.32
7	4.74	-0.52	4.97	5.35
7	5.74	-0.52	5.92	6.16
9	-0.15	ND	0.31	0.17
9	0.84	ND	2.04	1.33
9	1.84	ND	1.87	2.04
9	2.84	ND	3.66	3.38
9	4.84	ND	5.87	5.62
9	5.84	ND	5.87	6.16
11	0.54	ND	0.36	0.63
11	1.54	ND	1.63	1.63
11	2.54	ND	2.36	2.87
11	3.54	ND	3.63	3.36

11	5.54	ND	5.34	5.62
11	6.54	ND	6.16	6.16
13	0.08	0.36	0.36	0.63
13	1.08	0.36	1.36	1.96
13	2.08	0.36	2.36	2.17
13	3.08	0.36	2.96	3.16
13	5.08	0.36	5.16	4.97
13	6.08	0.36	6.16	4.56
15	-0.19	ND	-0.45	0.16
15	0.81	ND	0.63	0.87
15	1.81	ND	1.45	0.54
15	2.81	ND	2.63	3.96
15	4.81	ND	4.97	4.63
15	5.81	ND	5.92	6.16
17	-0.03	ND	0.96	1.17
17	0.97	ND	1.96	1.87
17	1.97	ND	2.97	2.97
17	2.97	ND	4.38	3.97
17	4.97	ND	5.87	5.87
17	5.97	ND	6.16	6.16
19	-0.12	-0.45	-0.04	0.17
19	0.88	-0.45	1.63	0.87
19	1.88	-0.45	1.96	2.36
19	2.88	-0.45	3.36	3.36
19	4.88	-0.45	5.62	5.62
19	5.88	-0.45	6.16	6.16

Figure 2. Plot of data from Table 4 for determination of LOD/LOQ.

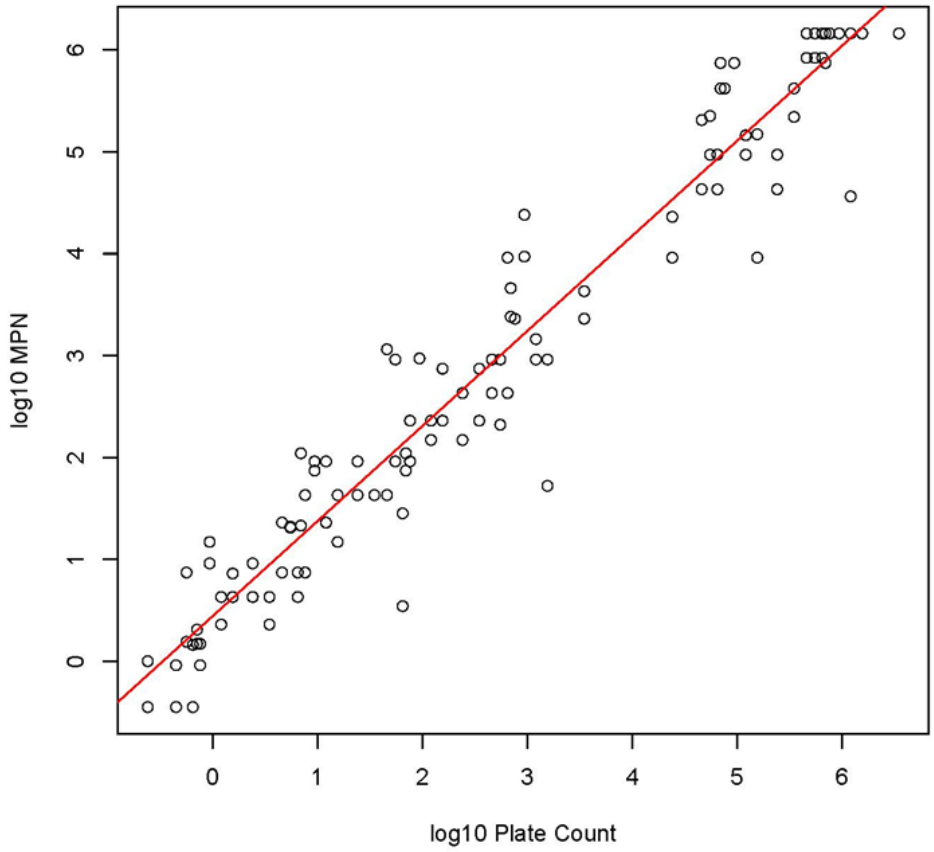


Table 5. Data used for determination of Ruggedness. Results reported as log MPN/g of *V. vulnificus* for each variation of the method SOP.

Sample	Plate Count (Log CFU/g)	Sample Blank (Log MPN/g)	Media/ Reagents		Oysters shucked and held prior to blending				RT 30 m Post-blending	MPN incubation			Boil prep time		Master Mix stored frozen				Master Mix thawed and re-frozen			Master Mix at RT 0.5-1.5h
			Lot 1	Lot 2	4C 1h	4C 3h	RT 30m	RT 1h		35C >24h	RT 18-24h	39C 18-24h	5m	30m	5d	3d	2d	1d	4X	3X	2X	
2	4.00	0.52	3.33	5.04	3.38	4.04	3.66	4.04	4.04	5.04	4.04	3.66	4.04	4.04	3.33	3.06	3.33	3.33	3.33	3.33	3.33	2.54
4	2.92	-0.45	3.17	3.17	2.66	3.04	3.38	2.36	3.17	2.97	3.38	3.38	3.17	3.38	3.17	3.17	3.17	3.17	3.17	3.17	3.17	3.17
6	1.06	-0.52	2.36	2.36	0.06	-0.45	0.31	1.87	0.36	0.54	1.53	0.63	2.66	2.38	2.66	2.38	2.38	2.38	2.66	2.66	2.38	2.38
8	4.78	ND	4.96	4.63	4.38	417	4.38	4.63	4.63	4.59	4.38	4.63	4.63	4.96	4.63	4.63	4.63	4.63	4.63	4.63	4.63	4.63
10	3.00	ND	3.38	3.38	2.97	2.38	3.66	2.63	3.16	3.17	2.96	2.63	3.36	3.36	3.36	3.36	3.36	3.36	2.96	3.36	3.36	3.36
12	1.11	ND	1.63	1.63	1.63	0.87	1.86	1.36	1.63	1.3	1.17	1.16	1.63	1.63	1.96	1.63	1.63	1.63	1.63	1.63	1.63	1.63
14	4.88	ND	5.62	5.34	4.97	4.97	4.97	4.97	4.97	5.34	4.63	4.63	5.62	5.62	5.62	5.62	5.62	5.62	5.62	5.62	-0.52	5.62
16	2.57	ND	2.36	2.36	1.36	1.96	2.17	2.36	2.63	2.63	2.63	3.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36	2.36
18	1.53	ND	1.17	1.36	1.63	1.36	1.86	1.36	1.63	1.63	1.63	2.17	1.32	1.63	1.96	1.63	1.63	1.96	1.96	1.96	1.96	1.96
20	0.52	-0.13	0.45	0.96	0.31	-0.04	0.96	0.19	-0.04	1.17	0.58	0.36	0.32	0.17	0.45	0.45	1.63	0.45	0.45	1.63	0.45	0.45

Table 6. Data used for determination of Comparability. Samples of with naturally incurred *V. vulnificus* were analyzed by both the test (MPN-real-time PCR) and NSSP (MPN-culture, with DNA probe confirmation) methods.

Sample	Real-Time PCR (Log MPN/g)	DNA Probe (Log MPN/g)
AL18-121	3.87	4.17
AL18-122	4.17	3.31
AL18-123	4.17	3.31
AL18-130	3.96	3.17
AL18-131	3.96	2.96
AL18-132	4.06	**>4.06**
AL18-139	3.96	3.96
AL18-148	1.98	3.96
AL-1	3.63	3.96
AL-2	2.63	3.32
SC-1	1.63	2.36
SC-2	2.36	2.36
SC-3	0.58	3.16
NC-1	2.45	3.36
NC-2	3.63	3.63
NC-3	3.17	3.17
NC-4	2.96	3.63
NC-5	2.63	3.63
NC-6	2.63	3.63
NC-7	2.36	4.31
VA-1	3.63	3.63
VA-2	-0.45	2.63
VA-3	2.44	2.17
WA-1	ND	1.36
WA-2	ND	-0.03
WA-3	ND	1.36
WA-4	ND	-0.52
WA-5	ND	1.32
CA-1	ND	-0.04
CA-2	ND	ND
OR-1	ND	ND
OR-2	ND	ND

Appendix A. Equipment Required.

Blender
Oyster knife
Shucking knife
Nitrile or Latex gloves
Soap
Stiff bristled brush
Chain mail glove (optional)
Bone cutting forceps (optional)
Test tubes (FisherScientific, 14-961-32, or equivalent)
Tube closures (FisherScientific, 14-957-92K, or equivalent)
Test tube racks (FisherScientific, 14-809-64, or equivalent)
Sterile stripettes (FisherScientific, 07-200-574, or equivalent) or pipet tips
Pipette-Aid or micropipettor (capable of 1000 µl)
Balance with a sensitivity of at least 0.01g
Incubator capable of maintaining 35±2°C
Heat block (95-100°C) or boiling water bath
Eppendorf 5415D centrifuge or equivalent (capable of >10,000x*g*)
Microcentrifuge tubes (USA Scientific, 1620-2799, or equivalent)
Mini-centrifuge (USA Scientific, 2631-0006, or equivalent)
AB 7500 Fast System (Life Technologies, Foster City, CA)
MicroAmp Fast Optical 96 Well Reaction Plate 0.1mL (Cat# 4346907) or MicroAmp Fast 8-tube strips (0.1 mL) (Cat# 4358293)
MicroAmp Optical 8-Cap Strip (Cat# 4323032) or Optical Adhesive Film (Cat# 4311971)
Micropipettors (volume ranges from 0.1 – 1000 µl)
Filtered, DNase/RNase-free pipette tips
Refrigerator capable of maintaining 2-8°C
Freezer capable of maintaining <-15°C
Stripfuge or 96 well plate centrifuge
Ice bucket (optional)
Tube and plate racks
PCR hoods with UV light

Appendix B. Media and Reagents Required.

APW, prepared according to BAM manual, Chapter 9, Vibrio (M10).
PBS, prepared according to BAM manual, Chapter 9, Vibrio (R59).
Platinum *Taq* DNA polymerase kit (ThermoFisher,10966026) Invitrogen, Carlsbad, CA):
Includes Taq, PCR Buffer, 50mM $MgCl_2$
PCR Nucleotide Mix (DNTP's) (Sigma Aldrich, 11814362001)
ROX reference dye (ThermoFisher,12223012)
Internal Amplification Control (IAC) DNA (BioGX, Birmingham, AL)
PCR-grade water (Ambion AM9937, or equivalent)
Crushed ice (optional)
Tris pH 8.0 (ThermoFisher, AM9855G, or equivalent)
Oligonucleotide primers (desalted) – see Table
Nuclease-style probes (HPLC purified) – see Table

	Sequence (5' to 3')	Modifications
*vvh*F	TGTTTATGGTGAGAACGGTGACA	--
*vvh*R	TTCTTTATCTAGGCCCCAAACTTG	--
vvh Probe	CCGTTAACCGAACCACCCGCAA	5Cy5-3IAbRQSp[a]
IAC 46G	GACATCGATATGGGTGCCG	--
IAC 186R	CGAGACGATGCAGCCATTC	--
IAC Probe	TCTCATGCGTCTCCCTGGTGAATGTG	56-JOEN-3IABkFQ[b]

[a] Iowa Black RQ-Sp
[b] Iowa Black FQ

Appendix C. Detailed Procedure.

1. **Shellfish Preparation:**
    a. Scrape off growth and loose material from shell and scrub shell stock with sterile stiff brush under running water.
    b. Place clean shellstock on clean towels or absorbent paper.
    c. Change gloves and brushes between samples.
    d. Protective chain mail glove can be used under a latex or nitrile glove; outer gloves should be changed between samples and disinfected with alcohol immediately prior to analysis.
    e. Tare a sterile blender.
    f. Using a sterile oyster knife, insert the point between the shells on the ventral side, about ¼ the distance from the hinge to the bill; alternately, knife can be inserted after making small opening with sterile bone cutting forceps.
    g. Cut adductor muscle from upper flat shell and pry the shell wide enough to drain shell liquor into the blender.
    h. The upper shell can then be pried loose at hinge and discarded.
    i. The whole animal (including adductor muscle) should be transferred to the sterile blender after severing the adductor muscle connection to the lower shell.
    j. A minimum of 12 animals is used.
    k. blend for 60-120 sec. If sample requires dilution, an equal weight of sterile PBS is used. After blending, homogenized sample is further processed within 20 minutes.
2. **MPN for Vibrio Analysis**
    a. Prepare a 1:10 dilution of the homogenate by transferring 1g (weighing is required for accurate transfer) of the homogenate to 9 mL of PBS.
        i. If diluent was used, transfer 2 g of 1:1 homogenate to 8 mL of PBS. Additional 10-fold dilutions can be prepared volumetrically (i.e., 1 mL of 1:10 to 9mL of PBS for a 1:100 dilution).
        ii. Volume of PBS is critical, so tubes must be aseptically filled after sterilization of diluent.
    b. Transfer 1g of homogenate to APW, in triplicate (this should be done by weight to ensure accurate transfer).
    c. Inoculate 1 ml portions of the 1:10, 1:100, 1:1000, 1:10,000, 1:100,000, and 1:1,000,000 dilutions (from step 2.a.) into APW, in triplicate, for as many dilutions as deemed necessary for the sample.
    d. Inoculate appropriate process controls into properly labeled tubes of APW.
    e. Leave one APW tube un-inoculated as a blank.
    f. Incubate APW overnight (18-24h) at 35 ±2°C.
    g. Confirm presence of Vv in each turbid tube by Real-Time PCR as described below.
    h. Determine MPN estimate for each sample using the draft "Dilution Selection Tool" to select appropriate dilutions. Use the standard table or calculator tool available in the BAM, Appendix 2 and report as MPN/g of shellfish.

3. **Preparation of DNA Extracts**
    a. Transfer 1mL from each MPN tube with visible growth (turbidity) to a microcentrifuge tube.
    b. Boil (heat to 95-100°C) the sample aliquots from APW tubes, including the process controls and blank, for 10 min. Ensure that one set of process controls is included with each set of samples in a heat block.
    c. Immediately plunge into ice until cold, or freeze at <-15°C.
    d. If extracts were previously frozen, ensure they are completely thawed (not exceeding room temperature) before proceeding.
    e. Centrifuge samples for 2 min at >10,000 x *g*. Use 2µL of supernatant as template in the real-time PCR reaction as detailed below.
    f. DNA extracts can be stored at 4°C for up to 3d or at <-15°C for up to 6 months.
4. **Preparation of PCR**
    a. Prepare mastermix in the clean hood and using aerosol resistant pipette tips. Use DNAse and RNAse free consumables.
        i. Refer to the Table below for component concentrations.
        ii. Briefly mix tubes of individual components.
        iii. Briefly centrifuge the tubes (2-3 sec) in a mini centrifuge.
        iv. Combine components (except for IAC DNA) into an appropriately sized tube.

PCR Mastermix

Component	Units	Final Concentration	Vol/Rxn (µL)
PCR H2O	--	--	12.22
PCR Buffer	X	1.000	2.500
$MgCl_2$	mM	5.000	2.500
dNTPs (mixed equal conc of each)	mM	0.300	0.750
Forward Primer *vvh*F	µM	0.300	0.750
Reverse Primer *vvh*R	µM	0.300	0.750
Forward Primer IAC 46F	µM	0.075	0.188
Reverse Primer IAC 186R	µM	0.075	0.188
Probe *vvh* Cy5	µM	0.200	0.500
Probe IAC JOE	µM	0.150	0.375
Platinum Taq	Units/µL	1.120	0.220
ROX (passive reference dye) 1:1 dilution	--	0.03	0.060

    b. After the mastermix is compiled, move to a template hood and add the appropriate amount of IAC DNA to the mastermix. Use an IAC concentration that will amplify between 24-29 cycles.
    c. The completed mastermix should be used the day of preparation or frozen until use. Mastermix can be frozen at this point or after it has been aliquoted into the reaction tubes or wells.

d. Flick mastermix tube to mix. If previously frozen as a single tube, ensure the mastermix is completely thawed (not exceeding room temperature).
e. Briefly centrifuge mastermix (2-3 sec) in a mini centrifuge.
f. Add 23µL of mastermix to each reaction tube or well. If previously frozen after aliquoting to individual tubes or wells, ensure the mastermix is completely thawed (not exceeding room temperature) and then briefly centrifuge (2-3 sec) before proceeding.
g. Add 2µL of supernatant from each boiled DNA extract sample (including process controls and APW blank) to a reaction tube or well.
h. Add 2µL of positive control template (boiled cells of strain VV ATCC 33816) to a reaction tube or well as a PCR positive control.
i. Add 2µL of PCR-grade water to a tube or well as a PCR negative control.
j. Centrifuge sample tubes or 96-well plate briefly (2-3 sec) to ensure reagents and sample are settled to the bottom.
k. Load sample tubes or 96-well plate to instrument and start cycling with the cycling parameters listed in Table below.
l. The read stage for the instrument should be programmed to the extension phase.

Cycling Parameters

	Temp (°C)	Time (s)	
Initial Denature	95	60	--
Denature	95	15	
Anneal	57	15	x45
Extend	72	25	

5. **Data Analysis:**
   a. For results analysis, default instrument settings will be used, except the threshold is set at 0.02 and background end cycle set from 3 to 10 on the AB7500.
   b. Positive/negative results will be recorded based on the instrument determinations. Analyst will review amplification data for all samples and can record a positive/negative determination discrepant with the instrument output if supported by the raw fluorescence data.
   c. If both the IAC and target are negative, the reaction should be considered invalid, and the sample re-tested.
   d. If the negative PCR control reaction is positive, all positive samples in the same run must be considered invalid, and can be re-tested.
   e. If the positive PCR control reaction is negative, all negative samples in the same run must be considered invalid, and can be re-tested.

Appendix D. Source of matrix for spike samples.

Sample	PHP Type	Date of Harvest	Location of Harvest	Process Date	Vv Strain
1	HPP	2017-10-15	Area 3. LA	2017-10-18	07-2405
2	HPP	2017-10-21	Area 5. LA	2017-10-25	K4776
3	IQF	2017-05-17	Area 8. LA	2017-05-28	R844-G9
4	Irradiated	2017-11-02	Area 3. LA	2017-11-07	R19-C1
5	HPP	2017-11-13	Area 19. LA	2017-11-15	K4633
6	IQF	2017-06-12	Area 9. LA	2017-06-15	R84-F1
7	HPP	2017-12-03	Area 3. LA	2017-12-06	07-2405
8	Irradiated	2018-01-03	Area 3. LA	2018-01-07	K4776
9	HPP	2018-04-15	Area 19. LA	2018-04-18	R844-G9
10	IQF	2018-01-14	Area 3. LA	2018-01-18	R19-C1
11	HPP	2018-05-19	Area 19. LA	2018-05-23	K4633
12	IQF	2018-03-07	Area 9. LA	2018-03-08	R84-F1
13	HPP	2018-06-17	Area 12. LA	2018-06-20	07-2405
14	IQF	2017-12-01	Area 3. LA	2017-12-04	K4776
15	HPP	2018-07-01	Area 3. LA	2018-07-05	R844-G9
16	IQF	2017-12-16	Area 3. LA	2017-12-18	R19-C1
17	HPP	2018-07-29	Area 3. LA	2018-08-01	K4633
18	IQF	2017-12-16	Area 3. LA	2017-12-18	R84-F1
19	HPP	2018-08-12	Area 3. LA	2018-08-16	07-2405
20	IQF	2017-12-20	Area 9. LA	2017-12-21	K4776

Appendix D. Abbreviations and Acronyms.

APW – Alkaline Pepton Water
ATCC – American Tissue Culture Collection
BAM – Bacteriological Analytical Manual
CFU – Colony Forming Unit
DNA – Deoxyribonucleic Acid
EDTA- Ethylene diamine tetraacetic acid
IAC – Internal Amplification Control
MPN- Most Probable Number
NPC – Negative Process Control
NSSP – National Shellfish Sanitation Program
PBS- Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PPC – Positive Process Control
RNA- Ribonucleic Acid
Tris – tris(hydroxymethyl)aminomethane
Vv- Vibrio vulnificus

Proposal No. 19-127

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Leanne J. 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	St. Petersburg, FL 33701
7. Phone	727-502-4891
8. Fax	
9. Email	leanne.flewelling@myfwc.com
10. Proposal Subject	Modification of the MARBIONC Brevetoxin ELISA Standard Operating Procedures
11. Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas. 14 Approved NSSP Laboratory Tests 4. Approved Limited Use Methods for Marine Biotoxin Testing
12. Text of Proposal/ Requested Action	In 2017, the ISSC approved the MARBIONC Brevetoxin ELISA as a Limited Use Method under the NSSP (Proposal 17-107). The Standard Operating Procedure (SOP) for the MARBIONC Brevetoxin ELISA submitted as a part of the supporting documents for Proposal 17-107 specifies that quantification of sample dilutions is restricted to those dilutions falling within the linear portion of the standard curve, which is specified as the range of concentrations that yield 20-70% inhibition in the assay. One of the QA/QC criterion in the SOP requires that the variation (%CV) of concentrations calculated from sample dilutions falling within this range must be <20%. This proposal is to modify the MARBIONC ELISA SOP to: a) narrow the range for quantifying sample dilutions to 30%-70%, b) update the QA/QC criteria to reflect this change, and c) make minor additions and corrections to the text of the SOP. The modified SOP with proposed changes is provided in Appendix A. Data and justification for the proposed changes are provided in Appendix B.
13. Public Health Significance	The approval of this ELISA as a Limited Use Method for testing to support the NSSP has enabled rapid testing for NSP, which has enhanced the protection of public health by enabling more frequent NSP testing. Revising the SOP and QA/QC criteria will help to minimize avoidable QA/QC failures while still controlling for errors and protecting public health.
14. Cost Information	N/A

MARBIONC Enzyme-linked Immunosorbent Assay (ELISA) for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish

Principle of Analysis

In this indirect competitive ELISA based on Naar et al. (2002), a 96-well ELISA plate is coated with protein-linked brevetoxin, and any remaining binding sites in the wells are blocked. Polyclonal goat anti-brevetoxin antibodies are then incubated with samples or standards in the plate wells. The antibodies will react with the brevetoxins in the samples or standards or will be immobilized on the plate. Antibodies that are not attached to the plate after incubation are washed out during subsequent rinses. Antibodies immobilized on the plate are detected through steps linking the antibodies to horseradish peroxidase (HRP)-linked secondary antibodies and addition of an HRP substrate (3,3'5,5'-Tetramethylbenzidine [TMB]), which yields a blue color (Amax = 370 nm and 652 nm) that changes to yellow (Amax = 450 nm) upon addition of a sulfuric acid stop solution. The intensity of this color is inversely proportional to the amount of brevetoxin that was present in the well during incubation. Using this method, one ELISA plate can be used to quantitatively assay five shellfish samples. For qualitative (+/-) screening, more samples can be run on one plate (up to 40).

Included in MARBIONC ELISA Kit (store in freezer):

- Reagent A BSA-linked PbTx-3
- Reagent C Goat anti-brevetoxin Ab
- Reagent D HRP-linked anti-goat secondary Ab
- Brevetoxin standard (PbTx-3, 10 μg)

Reagents required but not included (Brands and product numbers are for convenience. Unless otherwise noted, equivalents are acceptable)**:**

- Methanol (ACS grade or better)
- Reagent B: Superblock Blocking Buffer (Thermo Scientific 37545)
- Phosphate Buffered Saline, pH 7.4 (PBS, Sigma P-3813)
- Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 (PBS-Tween, Sigma P-3563)
- Gelatin (Sigma G-6144)
- 3,3'5,5'-Tetramethylbenzidine (TMB, Sigma T0440)
- Sulfuric acid stop solution (H_2SO_4, 0.5M)
- Nanopure water (or equivalent quality water)

Consumables needed:

- Disposable glass test tubes
- Disposable plastic dilution tubes (96-well cluster format)
- 15-ml and 50-ml graduated polypropylene centrifuge tubes
- Nunc flat-bottom polystyrene 96-well Maxisorp Immunoplates (**substitution NOT recommended**)
- Microplate sealing film
- Assorted pipet tips
- Solution basins
- Aluminum foil

Equipment needed:

Basic laboratory glassware (beakers, 1-L graduated cylinders, bottles, 10-ml volumetric flask)
Balance capable of measuring to 0.1g
Number 10 sieve
Laboratory blender
Vortex mixer
Centrifuge capable of 3,000*xg*, with rotor for 15 ~~ml~~ml or 50 ml centrifuge tubes
Microplate reader with filter for measurement at 450 nm
Multichannel pipettor (100-300 µl), individual pipettors (10-1000 µl)
Orbital microplate shaker
Refrigerator (4°C)/freezer (-20°C)

Pre-Assay Preparation

In advance:

PbTx-3 for positive control. Each set of kit reagents (15-plate supply) comes with 10 µg of PbTx-3 for use as a positive control.
Stock solution (1 µg/ml): Dissolve in 10 ml of 100% methanol. Store at -20°C. (May be stored for up to 1 year.)
Working solution (100 ng/ml): From this stock, dilute 1 ml to 10 ml with 100% methanol. Store at -20°C. (May be used for several months.)

80% aqueous methanol. Add 800 ml of methanol to a 1L graduated cylinder and bring to 1L with Nanopure water (or equivalent quality water). Good for up to 1 year.

5% gelatin stock solution. Dissolve 5 g gelatin in 100 ml Nanopure water - stir on heated stir plate until clear. Portion into 15-ml centrifuge tubes and refrigerate. Good for several weeks at 4°C.

SuperBlock - Dissolve 1 pouch in 200 ml Nanopure water. Portion 50-ml aliquots into 50-ml centrifuge tubes and refrigerate. Good for several weeks at 4°C.

PBS, pH 7.4 1 L - Dissolve 1 pouch of PBS powder in 1 L of Nanopure water. (Unused buffer may be stored for no more than one week at 4°C.)

PBS-Tween (0.05% Tween), pH 7.4 1L - Dissolve 1 pouch of PBS-Tween powder in 1 L of Nanopure water. (Unused buffer may be stored for no more than one week at 4°C.)

Make fresh daily:
PGT (PBS, 0.05% Tween, 0.5% gelatin) - Immerse a tube of stock gelatin in warm water for a few minutes to liquefy. Pour 5 ml gelatin into a 50-ml centrifuge tube and fill to 50 ml with PBS-Tween. Make one tube per plate.

Shellfish Sample Preparation (follows requirements for the NSP mouse bioassay)

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°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 shell 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 brevetoxins. Samples must be processed within 24 hours of shucking.

Rapid Extraction of Shellfish for Brevetoxins

1. Weigh 1.0 g of homogenized shellfish into a 15-~~mL~~ ml or 50-ml polypropylene centrifuge tube.
2. Add 9 ~~mL~~ ml of 80% aqueous methanol, and cap tightly.
3. Vortex for 2 minutes at highest speed.
4. Centrifuge at a minimum of 3000xg for 10 minutes.
5. Pour off supernatant into clean, labeled graduated 15-~~mL~~ ml centrifuge tube.
6. Bring the volume of the supernatant to 10mL with 80% methanol.
7. Vortex for 15 seconds to mix.
8. Transfer to a clean labeled glass vial and store at -20°C until assayed.

ELISA Protocol

****IMPORTANT NOTE**** Kit Reagents A, C, and D are diluted in a glycerol solution to prevent freezing. To avoid pipetting error due to viscosity, only place the very tip of the pipet into the vial to withdraw the desired amount. DO NOT PRE-RINSE THE TIP. Submerge the tip into the buffer when dispensing, and rinse the tip several times with buffer to ensure complete transfer.

Step 1 - Reagent A
Shake vial of Reagent A gently by hand. Dilute Reagent A. 1:300 (or as specified in kit instructions) in **PBS**. (For 1 plate, add 40 µl of A to 12 ml **PBS**; for 2 plates, add 80 µl A to 24 ml **PBS**).

Fill each well of a 96-well Maxisorp Immunoplates with 100 µl of diluted Reagent A. Cover with microplate sealing film, and incubate on a plate shaker for 1 hour at room temperature. After 1 hour, pour liquid from plate and rinse each well 3 times with 300 µl **PBS**. (**No Tween for this step**.)

Step 2 - Reagent B
Fill each well with 250 µl of Reagent B-Blocking Buffer. Cover with microplate sealing film, and incubate on plate shaker for 30 minutes at room temperature. Pour the liquid from the plate and rinse each well 3 times with 300 µl PBS-Tween.

Step 3 - Sample and positive control dilutions *(This step can be done while Step 1 and 2 are incubating.)*

Note: Sample extracts and PbTx-3 working solution should be brought to room temperature before diluting.

Arrange dilution tubes in a rack according to plate layout - see below. Eight (8) tubes are needed for each sample or positive control.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Pos Ctrl (PbTx-3)
A	tube A	tube A	tube A	tube A	tube A	tube A
B	tube B	tube B	tube B	tube B	tube B	tube B
C	tube C	tube C	tube C	tube C	tube C	tube C
D	tube D	tube D	tube D	tube D	tube D	tube D
E	tube E	tube E	tube E	tube E	tube E	tube E
F	tube F	tube F	tube F	tube F	tube F	tube F
G	tube G	tube G	tube G	tube G	tube G	tube G
H	tube H	tube H	tube H	tube H	tube H	tube H

Leave dilution tubes in row **A** empty. To all other tubes in rows **B-H** (for both samples and Pos Ctrl) add 250 µl of PGT. For each sample, add 975µl of PGT to a small glass test tube. Add 25 µl of sample extract to the tube, and vortex briefly to mix. Transfer 250 µl of this diluted extract into dilution tube **A**. Withdraw another 250 µl from the glass tube, place into tube **B**, and vortex to mix. Then withdraw 250 µl from tube **B**, place into tube **C**, and vortex to mix. Continue this **serial dilution** for tubes **D** through **G**. **DO NOT DILUTE INTO TUBE H.** Do this for each sample.

Positive Control (PbTx-3)
To make the positive control, add 950µl of PGT to a small glass test tube. Add 50 µl of brevetoxin working solution (at 100 ng PbTx-3/ml) to the tube (50 µl PbTx-3 + 950 ul PGT= 5 ng PbTx-3/ml). (This is sufficient for up to two plates.) For each plate, transfer 250 µl of diluted PbTx-3 into dilution tube **A**. Withdraw another 250 µl from the glass tube and place into tube **B**, and vortex to mix. Then withdraw 250 µl from tube **B**, place into tube **C**, and vortex to mix. Continue this **serial dilution** for tubes **D** through **G**. **DO NOT DILUTE INTO TUBE H.**
(Tube H are PGT only and will serve as Reference Wells for maximum absorbance in the absence of brevetoxin.)

Step 4 - Transfer Samples On to Plate

After the plate has been blocked and washed (after Step 2 is complete), use a multichannel pipette to transfer the diluted samples and standards to the plate.

Fill wells of the microplate with 100 µl of each tube **in duplicate** (side by side wells), according to the figure below.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Pos. Ctrl.	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	1:400	PbTx-3	5 ng/ml
B	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	1:800	PbTx-3	2.5 ng/nl
C	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	1:1600	PbTx-3	1.25 ng/ml
D	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	1:3200	PbTx-3	0.625 ng/ml
E	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	1:6400	PbTx-3	0.31 mg/ml
F	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	1:12800	PbTx-3	0.156 ng/ml
G	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	1:25600	PbTx-3	0.078 ng/ml
H	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT	PGT

Step 5 - Reagent C
Dilute Reagent **C** 1:300 (or as specified in kit instructions)
(For 1 plate, add 40 µl of ~~A~~ C to 12 ml PGT; for 2 plates, add 80 µl ~~A~~ C to 24 ml PGT)
To each well add 100 µl of diluted Reagent C. Cover with microplate sealing film, and shake the plate on the plate shaker for 90 minutes at room temperature. Pour the liquid from the plate and rinse each well 3 times with 300 µl PBS-Tween.

Step 6 - Reagent D
Dilute Reagent **D** 1:800 (or as specified in kit instructions)
(For 1 plate, add 15 µl of D to 12 ml PGT; for 2 plates, add 30 µl D to 24 ml PGT.)
Fill each well with 100 µl of diluted Reagent D. Cover with microplate sealing film, and incubate on a plate shaker for 1 hour at room temperature.
(When you get to this step – aliquot 12 ml of TMB per plate into a 15 or 50-ml centrifuge tube and warm to room temperature. Keep the tube in the dark (do not expose to light).
After 1 hour, pour liquid from plate and rinse each well 3 times with 300 µl PBS-Tween. **Then rinse each well one time with 300 µl PBS to ensure no Tween remains on the plate.**

Step 7 - TMB
Fill each well with 100 µl of TMB. Cover the plate with a piece of aluminum foil and incubate for 5-7 minutes (or until a blue color develops in the reference wells). Stop the reaction by adding 100 µl of 0.5M H_2SO_4 to each well. The blue color in the wells should turn yellow. Read the plate at 450 nm.

Note: The stop time may vary with kit reagent lots and bottles of TMB. The timing of the final step should be standardized with each new lot of kit reagents and each new lot of TMB to achieve maximum optical densities (at 450 nm) of 1.0 ± 30%.

Calculations

Presence of brevetoxin in the sample will prevent color development in the well. Toxin can be quantified by converting absorbance values to % color inhibition and comparing to the positive control.

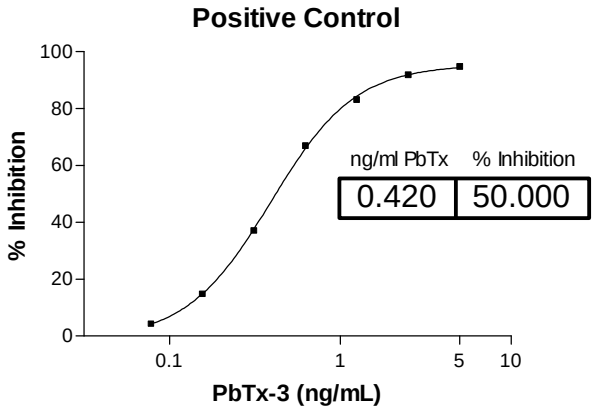
1. Average the values of the duplicate wells for each dilution, and determine the % color inhibition using the following equation:

   % inhibition = [1 - (Avg of dups/Amax)] x 100%

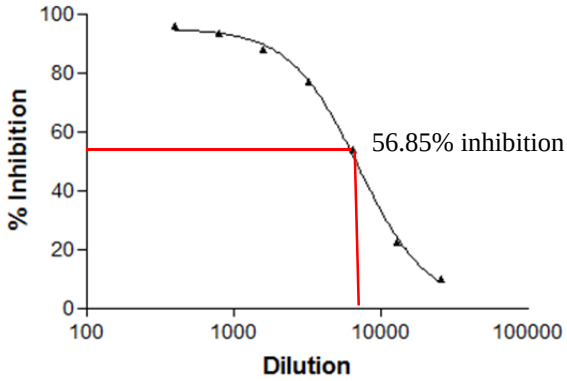
   where Amax is the average absorbance of the reference wells (PGT only) oriented below the sample or standard dilutions.

2. Using the 4-parameter logistic (4PL) curve in a curve-fitting program like Prism or SigmaPlot, fit a curve to the positive control with ng toxin/ml on the x-axis (log scale), and % inhibition on the y-axis (linear scale).
3. Determine the concentration for sample dilutions falling within the linear portion of the standard curve.
4. Multiply the concentration by the sample dilution and divide by 1000 to obtain PbTx-3 eq. results in ppm.

Example Standard Curve (50% inhibition = 0.42 ng PbTx-3/ml)
The control curve should be steep. On the linear part of the curve, the space between the dilutions (on the y-axis) is large. There should be clear plateaus at the top and bottom of the curve.



Example Sample Serial Dilution
Sample curves plotted with dilution on the x-axis (log scale), and % inhibition on the y-axis (linear scale) should have the same features. There should be a clear plateau either at the top or the bottom (or both). Shallow curves with no plateaus or linear curves with little space between points indicate interference in the assay, and results should be discarded.



For a sample with % inhibition of 56.85% at dilution of 1:6,400, the interpolated concentration = 0.495 ng/mL

[PbTx-3 eq] = 0.495 ng/ml x 6400 = 3168 ng/ml or 3.17 ppm

Quality Control Criteria

Acceptance of **assay results** is dependent on meeting the following criteria:

- Absorbance of reference wells must be (Amax) ≥ 0.6. (Optimal absorbance is 1.0 ± 30%.)
- %CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (~~20~~30-70% inhibition) must be < 20%.

If either ~~criteria~~ criterion is~~are~~ not met, re-run the ELISA plate.

Acceptance of **sample results** is dependent on meeting the following criteria:

- %CV of raw absorbance of duplicate wells for sample dilutions used for quantitation (within the linear range of the assay; ~~20~~30-70% inhibition) must be < 20%.
- %CV of calculated concentrations of different sample dilutions within the linear range of the assay must be < 20%. (A 20% or greater disparity between the calculated concentrations of two different dilutions of the same sample indicates assay interference or dilution error.)

If either criterion~~a~~ is~~are~~ not met, re-run the sample.

In 2017, the ISSC approved the MARBIONC Brevetoxin ELISA as a Limited Use Method under the NSSP (Proposal 17-107). The Standard Operating Procedure (SOP) for the MARBIONC Brevetoxin ELISA submitted as a part of the supporting documents for Proposal 17-107 specifies that quantification of sample dilutions is restricted to those dilutions falling within the linear portion of the standard curve (defined as 20%-70% inhibition). Sample dilutions with signals falling within this portion of the standard curve are used to quantify the brevetoxin concentration in the sample. One of the specified QA/QC criterion requires that the %CV of sample dilutions within this range must be <20%. Since its acceptance as a Limited Use Method, we have conducted numerous assays. Based on our results, we are proposing to narrow the specified range for quantifying sample dilutions to 30%-70% and to modify the QA/QC criteria to reflect this change. Additionally, we have made some minor corrections and additions to the SOP.

Basis for Proposed Modifications

We propose to narrow the specified range for quantifying sample dilutions to 30%-70% and to modify the QA/QC criteria to reflect this change.

Competitive ELISAs yield sigmoidally-shaped standard curves (Figure 1), and the rate of change of signal vs. concentration varies across the range of standard concentrations. The steep vertical portion of the curve exhibits large signal changes with small concentration changes, and the shallow horizontal portions of the curve exhibit small signal changes with large concentration changes. Because of this concentration dependence, accurate quantification in a competitive ELISA requires that sample dilutions fall within the relatively narrow, steep portion of the curve, which has the most reliable concentration dependence. Quantification is most accurate closer to the center of the curve.

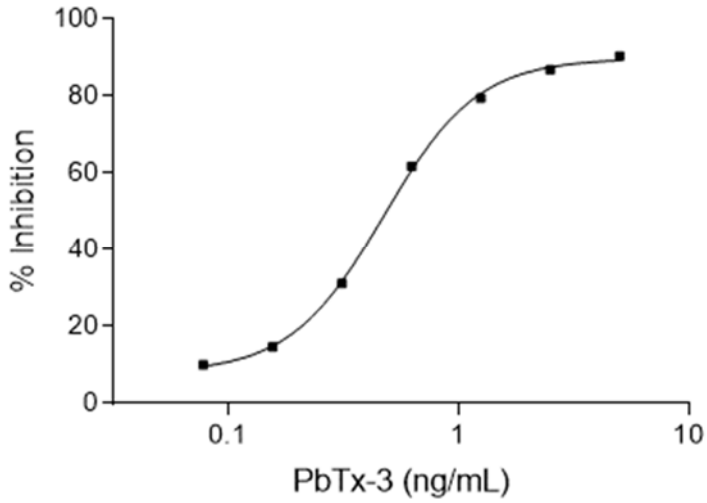


Figure 1. Example of a typical standard curve using the MARBIONC Brevetoxin ELISA

Signals (well absorbance measurements) are first normalized to zero controls, which provide maximum absorbance values, and are expressed as percent of the maximum (A/Amax) or, inversely, as percent inhibition (1-A/Amax). In general, concentration estimates can be obtained from signals that fall within 20% to 80%; however, to achieve variability between dilutions (calculated as %CV) of less than 20%, it is often necessary to use values obtained for a narrower portion of the curve (Sasaki and Mitchell 2002).

As a part of the SLV for the MARBIONC Brevetoxin ELISA, bend points from 60 different standard curves were calculated according to Sebaugh and McCray (2003), and the average of these bend points were used to help define the linear portion of the assay standard curve (20%-70% inhibition). In practice, the linear portion of the standard curve can vary slightly from assay to assay (Figure 2).

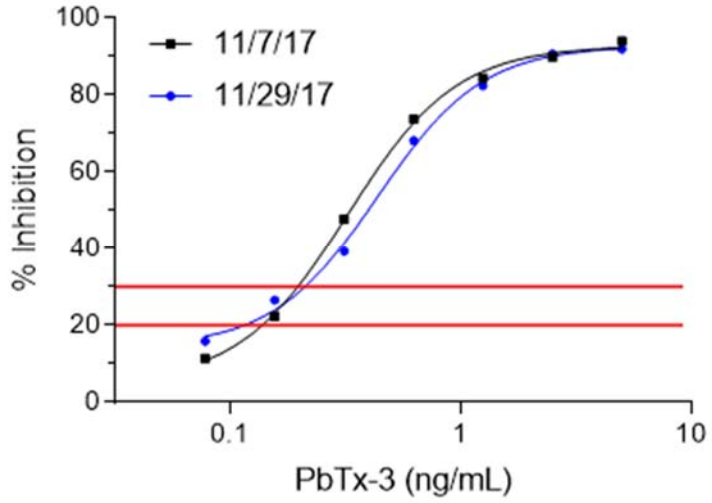


Figure 2. Calibration curves from 2 ELISA assays on different days. Red lines at 20% and 30% illustrate loss of linearity below 30% inhibition on the 11/29/17 standard curve.

Data from 326 shellfish samples tested since the method was approved in October 2017 were examined to determine the effect on variation and outcome of sample results (pass or fail sample QA/QC requirements) when the range for acceptable sample dilution signal was modified from 20%-70% to 30%-70% (Table 1). In most cases (n=281), assay results yielded two dilutions per sample that fell between 20% and 70%. Separating samples with at least one dilution between 20% and 30% from samples where all dilutions were ≥ 30% created two separate sample sets with no overlap. For 45 samples, assay results yielded three dilutions between 20% and 80% (with one dilution between 20% and 30% in 44 of the 45 samples). For these samples, %CV of dilutions was calculated for each pair of adjacent dilutions, and if either comparison yielded %CV ≥ 20% , the sampled failed QC. (For some samples, only a single sample dilution fell within the 20%-70% range. Those samples are not included the data presented.) Full data on sample dilutions included here are provided in Table 2 at the end of Appendix B.

While the range specified in currently accepted QA/QC criteria (20%-70%) yield acceptable variation between sample dilutions most of the time, narrowing this range does make a difference. Unacceptably high %CVs occurred in 17% of samples for which one of two dilutions fell between 20%-30% compared to only 8% unacceptably high %CVs in samples with dilutions between 30%-70%. Samples that had three dilutions within the 20%-70% range were rarer, and 27% of these samples had unacceptably high %CVs.

Table 1. Summary of shellfish samples (hard clams and oysters) included in this proposal.

ELISA dilution results	Number of samples	%CV of dilutions <20% pass QC	%CV of dilutions ≥ 20% fail QC
One of two dilutions between 20%-30%	179	148 (83%)	31 (17%)
Two dilutions between 30%-70%	193	177 (92%)	16 (8%)
Three dilutions between 20%-70%	45	33 (73%)	12 (27%)

An unpaired t-test also demonstrates a clear statistical difference ($p = 0.0024$) between the %CVs calculated for each of the two data sets for samples with two dilutions in the linear range.

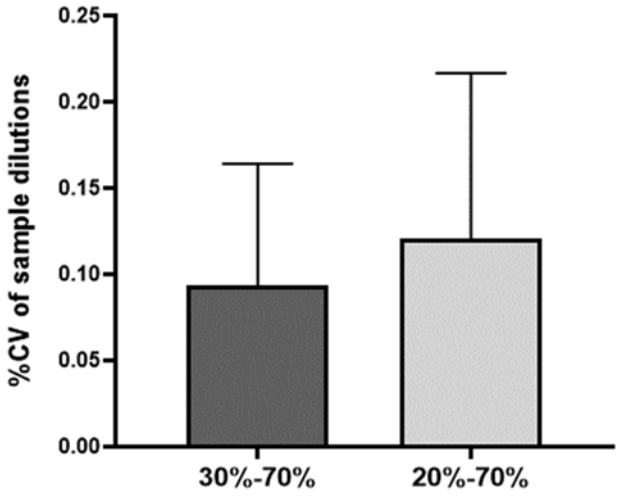


Figure 3. Mean %CV calculated for samples that had two dilutions between 30%-70% (left bar, n=193) and those with one of two dilutions between 20%-30% (right bar, n=179). Means are statistically different (unpaired t-test, $p = 0.0024$). Error bars indicate standard deviation (in the positive direction only for clarity).

The sigmoidal curves generated in a competitive ELISA are similar to those generated in receptor binding assays. In the Approved PSP RBA, the quantitative range specified is 0.2-0.7 B/B_0 (percent of maximum binding). If expressed in terms of binding inhibition ($1-B/B_0$), this

would be 0.3-0.8. VanDolah stated in that submission (Proposal 13-114) that they selected a more conservative cutoff (at the lower end of the curve) than the 0.8 B/B_0 (or 0.2 binding inhibition) frequently used in receptor assays because quantification was unacceptably variable at that cutoff. Our reasoning in this proposal is similar.

High sample variation between dilutions are most often due to analyst error, matrix effects generated by the sample, or poor quantification at the extreme ends of the linear portion of the standards curve. These are the reasons for imposing QA/QC criteria that specify the acceptable range for quantification and the acceptable variation (%CV) between dilutions that fall within that range. Matrix effects were not seen in any of the species studied in the SLV at the starting sample dilutions of 400, which is the minimum dilution specified in the protocol. Therefore, we feel that any unacceptable variation between dilutions is more likely due to interpolation error or analyst error. Revising the acceptable range of quantification to a more conservative one as proposed will minimize interpolation error and avoidable QC failures while still controlling for assay quality.

Minor corrections and additions to the SOP

- Corrections to the protocol are indicated in Appendix A and include grammatical corrections, minor changes for consistency in how units are expressed (e.g. ml vs mL), and correction of typos.
- Glassware was added to "Equipment needed" on Page 2.
- For the first step of the extraction on Page 3, the option of using 50 ml centrifuge tubes was added. The size of the centrifuge tubes used for this step of the extraction is not critical. This change adds flexibility. (In "Equipment needed" on Page 2 the rotor required was therefore also made optional.)
- At "Step 7" on Page 5, additional guidance was provided on when to stop the color development. This reinforces the note at the bottom of the page that the timing can change with reagents.
- Quality control criteria were modified as described above.

References cited:

Sasaki D. and R.A. Mitchell (2002). How to obtain reproducible quantitative ELISA results. Oxford Biomedical Research, Inc. https://www.oxfordbiomed.com/sites/default/files/2017-02/How%20to%20Obtain%20Reproducible%20Quantitative%20ELISA%20results.pdf

Sebaugh, J. L. and P. D. McCray (2003). Defining the linear portion of a sigmoid-shaped curve: bend points. Pharmaceutical Statistics 2: 167-174.

Table 2. Samples included in the analyses for this proposal with the dilutions used, calculated sample concentrations, and %CVs of paired dilutions. Dilutions <30% inhibition are highlighted in blue, and unacceptably high %CVs are highlighted in red.

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T17-1239	1600	64.3%	1.33	2.13		
T17-1239	3200	35.4%	0.54	1.73	1.93	14.5%
T17-1240	800	53.6%	0.97	0.78		
T17-1240	1600	29.8%	0.45	0.73	0.75	4.9%
T17-1241	1600	64.7%	1.37	2.20		
T17-1241	3200	27.7%	0.42	1.34	1.77	34.1%
T17-1333	800	64.1%	1.51	1.21		
T17-1333	1600	36.5%	0.55	0.87	1.04	22.9%
T17-1383	800	65.6%	1.63	1.30		
T17-1383	1600	45.3%	0.75	1.19	1.25	6.1%
T17-1383	3200	22.4%	0.34	1.08	1.14	7.3%
T17-1384	800	69.8%	2.02	1.61		
T17-1384	1600	52.9%	0.97	1.55	1.58	2.6%
T17-1384	3200	21.5%	0.32	1.04	1.30	28.2%
T17-1389	3200	48.3%	0.32	1.02		
T17-1389	6400	29.5%	0.19	1.24	1.13	13.7%
T17-1390	3200	51.1%	0.34	1.09		
T17-1390	6400	28.8%	0.19	1.21	1.15	7.4%
T17-1391	6400	46.2%	0.30	1.93		
T17-1391	12800	21.6%	0.15	1.89	1.91	1.8%
T17-1526a	1600	60.5%	0.86	1.38		
T17-1526a	3200	24.5%	0.35	1.13	1.26	14.2%
T17-1526b	1600	61.7%	0.99	1.58		
T17-1526b	3200	30.0%	0.46	1.47	1.52	5.2%
T17-1527a	1600	59.0%	0.83	1.33		
T17-1527a	3200	27.6%	0.39	1.24	1.29	4.9%
T17-1527b	1600	61.1%	0.97	1.56		
T17-1527b	3200	31.6%	0.48	1.53	1.54	1.0%
T17-1528a	1600	63.7%	0.94	1.50		
T17-1528a	3200	31.1%	0.43	1.38	1.44	5.9%
T17-1528b	1600	56.6%	0.87	1.39		
T17-1528b	3200	26.8%	0.42	1.34	1.36	2.8%
T17-1529a	1600	56.8%	0.79	1.26		
T17-1529a	3200	25.3%	0.36	1.16	1.21	6.0%
T17-1529b	1600	54.4%	0.82	1.32		
T17-1529b	3200	25.8%	0.41	1.30	1.31	1.2%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T17-1530	3200	67.3%	0.64	2.05		
T17-1530	6400	47.0%	0.36	2.33	2.19	10.4%
T17-1530	12800	23.2%	0.15	1.91	2.12	14.2%
T17-1531	12800	63.4%	0.57	7.29		
T17-1531	25600	32.1%	0.23	5.88	6.58	15.2%
T17-1532	6400	63.5%	0.57	3.65		
T17-1532	12800	36.4%	0.27	3.41	3.53	4.8%
T17-1535	12467.2	49.8%	0.39	4.91		
T17-1535	24934.4	25.5%	0.17	4.26	4.59	10.0%
T17-1535b	12467.2	59.0%	0.51	6.42		
T17-1535b	24934.4	32.0%	0.22	5.49	5.96	11.0%
T17-1536	6233.6	52.4%	0.42	2.63		
T17-1536	12467.2	30.9%	0.22	2.73	2.85	10.2%
T17-1536	24934.4	21.1%	0.13	3.18	2.95	10.7%
T17-1536b	6233.6	51.0%	0.40	2.52		
T17-1536b	12467.2	33.5%	0.24	2.95	2.92	13.0%
T17-1536b	24934.4	20.4%	0.13	3.28	3.11	7.6%
T17-1538	97.4	69.3%	0.61	0.06		
T17-1538	194.8	40.8%	0.27	0.05	0.06	7.8%
T17-1538	389.6	24.2%	0.16	0.06	0.06	10.8%
T17-1549	800	64.1%	0.51	0.41		
T17-1549	1600	41.1%	0.27	0.44	0.42	4.7%
T17-1549	3200	21.2%	0.15	0.47	0.45	5.1%
T17-1555	12800	58.5%	0.44	5.69		
T17-1555	25600	28.6%	0.21	5.28	5.48	5.3%
T17-1558	40000	68.8%	0.59	23.70		
T17-1558	80000	43.5%	0.28	22.36	25.14	4.1%
T17-1558	160000	28.7%	0.18	29.36		19.2%
T17-1559	80000	65.3%	0.52	41.94		
T17-1559	160000	37.2%	0.23	37.59	39.76	7.7%
T17-1566	6400	64.9%	0.77	4.90		
T17-1566	12800	37.8%	0.31	4.01	4.45	14.2%
T17-1570	3200	63.4%	0.72	2.31		
T17-1570	6400	37.3%	0.31	1.97	2.14	11.3%
T17-1574	12800	59.5%	0.63	8.04		
T17-1574	25600	33.7%	0.28	7.06	7.55	9.2%
T17-1576	3200	57.2%	0.41	1.31		
T17-1576	6400	28.7%	0.18	1.17	1.24	7.8%
T17-1578	6400	51.8%	0.35	2.24		

T17-1578	12800	23.6%	0.16	1.99	2.12	8.5%
Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T17-1583	6400	66.1%	0.66	4.20		
T17-1583	12800	41.2%	0.32	4.12	4.16	1.3%
T17-1585	12800	62.2%	0.58	7.40		
T17-1585	25600	30.2%	0.23	6.00	6.70	14.7%
T17-1586	80000	40.8%	0.36	28.41		
T17-1586	160000	22.2%	0.22	34.45	31.43	13.6%
T17-1587	6400	68.2%	0.75	4.81		
T17-1587	12800	39.9%	0.35	4.45	4.63	5.3%
T17-1587	25600	22.4%	0.20	5.22	4.84	11.1%
T17-1588	1600	61.8%	0.62	0.98		
T17-1588	3200	41.0%	0.36	1.14	1.06	10.6%
T17-1591	80000	38.6%	0.34	26.93		
T17-1591	160000	20.3%	0.20	32.39	29.66	13.0%
T17-1592	80000	49.6%	0.44	35.00		
T17-1592	160000	25.6%	0.24	38.28	36.64	6.3%
T18-0001	12800	64.3%	1.52	19.51		
T18-0001	25600	36.4%	0.62	15.87	17.69	14.6%
T18-0002	12800	63.0%	1.26	16.07		
T18-0002	25600	30.1%	0.51	12.99	14.53	15.0%
T18-0006	12800	66.3%	1.40	17.91		
T18-0006	25600	34.0%	0.57	14.64	16.27	14.2%
T18-0007	12800	63.3%	1.47	18.87		
T18-0007	25600	30.3%	0.50	12.83	15.85	27.0%
T18-0008	12800	65.7%	1.30	16.64		
T18-0008	25600	44.0%	0.74	18.90	17.77	9.0%
T18-0014	80000	54.5%	0.44	34.99		
T18-0014	160000	24.7%	0.17	26.62	30.80	19.2%
T18-0058	12800	49.7%	0.65	8.33		
T18-0058	25600	21.1%	0.28	7.26	7.79	9.6%
T18-0059	12800	52.4%	0.68	8.73		
T18-0059	25600	23.7%	0.24	6.14	7.44	24.6%
T18-0060	12800	63.1%	0.98	12.56		
T18-0060	25600	33.5%	0.42	10.63	11.59	11.8%
T18-0061	12800	64.0%	1.02	13.00		
T18-0061	25600	27.2%	0.28	7.24	10.12	40.2%
T18-0070a	6400	59.3%	0.86	5.48		
T18-0070a	12800	25.7%	0.26	3.38	4.43	33.5%
T18-0070b	8000	51.2%	0.83	6.66		
T18-0070b	16000	21.6%	0.49	7.88	7.27	11.9%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-0071	6400	64.3%	1.03	6.60		
T18-0071	12800	28.8%	0.36	4.59	5.60	25.5%
T18-0071b	4000	63.9%	0.90	3.61		
T18-0071b	8000	41.1%	0.41	3.27	3.44	6.9%
T18-0088	6400	61.0%	1.06	6.77		
T18-0088	12800	21.1%	0.36	4.63	5.70	26.7%
T18-0089	6400	62.2%	0.97	6.18		
T18-0089	12800	25.6%	0.44	5.68	5.93	6.0%
T18-0089b	6400	59.8%	0.77	4.95		
T18-0089b	12800	21.2%	0.17	2.19	3.57	54.7%
T18-0091	12800	56.3%	0.68	8.74		
T18-0091	25600	32.2%	0.30	7.56	8.15	10.3%
T18-0135	3200	68.1%	1.22	3.90		
T18-0135	6400	38.4%	0.56	3.61	3.76	5.4%
T18-0135	12800	23.0%	0.37	4.70	4.16	18.5%
T18-0136	3200	66.3%	1.05	3.35		
T18-0136	6400	34.7%	0.49	3.13	3.24	4.9%
T18-0139	12800	53.9%	0.82	10.50		
T18-0139	25600	24.8%	0.39	9.96	10.23	3.8%
T18-0143	3200	55.8%	0.86	2.76		
T18-0143	6400	31.7%	0.48	3.04	2.90	6.9%
T18-0145	3200	65.0%	1.10	3.52		
T18-0145	6400	35.9%	0.49	3.17	3.34	7.4%
T18-0146	3200	64.5%	1.05	3.35		
T18-0146	6400	30.7%	0.49	3.13	3.24	4.8%
T18-0147	6400	47.7%	0.67	4.30		
T18-0147	12800	22.7%	0.33	4.22	4.26	1.3%
T18-0148	6400	55.0%	0.83	5.29		
T18-0148	12800	22.1%	0.40	5.10	5.20	2.5%
T18-0149	12800	66.2%	1.15	14.68		
T18-0149	25600	29.7%	0.41	10.62	12.65	22.7%
T18-0150	12800	66.0%	1.09	13.99		
T18-0150	25600	35.9%	0.55	14.04	14.02	0.3%
T18-0151	400	52.8%	0.75	0.30		
T18-0151	800	25.2%	0.38	0.30	0.30	0.1%
T18-0151b	200	64.7%	1.20	0.24		
T18-0151b	400	47.5%	0.73	0.29	0.27	14.1%
T18-0151b	800	21.0%	0.35	0.28	0.29	2.5%
T18-0155	3200	64.5%	1.11	3.57		

T18-0155	6400	28.2%	0.47	2.99	3.28	12.4%
Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-0156	3200	67.8%	1.10	3.51		
T18-0156	6400	30.0%	0.43	2.72	3.12	17.9%
T18-0157	3200	54.7%	0.86	2.76		
T18-0157	6400	21.0%	0.39	2.48	2.62	7.6%
T18-0164	6400	43.6%	0.66	4.21		
T18-0164	12800	23.8%	0.38	4.83	4.52	9.8%
T18-0165	1600	66.0%	1.21	1.93		
T18-0165	3200	39.9%	0.62	1.98	1.96	2.0%
T18-0166	1600	65.6%	1.25	2.01		
T18-0166	3200	40.1%	0.60	1.92	1.96	3.2%
T18-0167	1600	67.5%	1.26	2.02		
T18-0167	3200	41.1%	0.64	2.04	2.03	0.5%
T18-0168	1600	64.0%	1.18	1.89		
T18-0168	3200	34.9%	0.52	1.67	1.78	8.9%
T18-0186	3200	60.4%	1.02	3.25		
T18-0186	6400	27.0%	0.45	2.88	3.07	8.6%
T18-0187	3200	50.4%	0.79	2.52		
T18-0187	6400	21.1%	0.34	2.21	2.36	9.5%
T18-0188	6400	57.4%	0.94	6.00		
T18-0188	12800	25.8%	0.44	5.58	5.79	5.1%
T18-0189	6400	56.8%	0.95	6.05		
T18-0189	12800	20.5%	0.34	4.32	5.19	23.7%
T18-0226	3200	49.4%	0.77	2.46		
T18-0226	6400	20.7%	0.35	2.24	2.35	6.5%
T18-0227	6400	47.4%	0.73	4.68		
T18-0227	12800	23.7%	0.39	4.98	4.83	4.5%
T18-0229	6400	49.8%	0.79	5.03		
T18-0229	12800	20.7%	0.42	5.37	5.20	4.7%
T18-0230	3200	62.4%	1.11	3.55		
T18-0230	6400	30.8%	0.48	3.08	3.31	10.1%
T18-0231	3200	63.3%	1.11	3.56		
T18-0231	6400	29.3%	0.51	3.27	3.41	6.2%
T18-0234	1600	61.6%	1.14	1.82		
T18-0234	3200	34.3%	0.49	1.57	1.70	10.3%
T18-0235	3200	45.3%	0.69	2.21		
T18-0235	6400	21.9%	0.39	2.52	2.36	9.3%
T18-0238	6400	71.5%	0.92	5.86		
T18-0238	12800	35.3%	0.42	5.38	5.62	6.1%
T18-0239	6400	71.3%	0.91	5.84		

T18-0239	12800	39.2%	0.45	5.82	5.83	0.3%
Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-0296	3200	69.0%	1.39	4.43		
T18-0296	6400	40.8%	0.56	3.58	4.01	15.0%
T18-0298	3200	62.5%	1.07	3.44		
T18-0298	6400	26.4%	0.37	2.37	2.90	25.9%
T18-0300	12800	66.7%	1.72	22.00		
T18-0300	25600	39.3%	0.70	17.98	19.99	14.2%
T18-0301	6400	69.0%	1.90	12.13		
T18-0301	12800	38.1%	0.68	8.68	10.41	23.4%
T18-0302	6400	54.3%	1.11	7.11		
T18-0302	12800	23.7%	0.42	5.40	6.25	19.3%
T18-0309	6400	68.9%	0.79	5.06		
T18-0309	12800	42.0%	0.36	4.64	4.85	6.1%
T18-0310	12800	53.8%	0.49	6.31		
T18-0310	25600	25.2%	0.23	5.85	6.08	5.4%
T18-0411	6400	65.3%	0.48	3.07		
T18-0411	12800	38.6%	0.24	3.08	3.08	0.2%
T18-0412	12800	48.2%	0.31	3.94		
T18-0412	25600	22.2%	0.14	3.64	3.79	5.5%
T18-0452	6400	61.5%	1.05	6.73		
T18-0452	12800	29.9%	0.45	5.76	6.24	10.9%
T18-0453	6400	62.1%	1.02	6.53		
T18-0453	12800	29.4%	0.40	5.06	5.79	18.0%
T18-0456	6400	53.4%	1.08	6.90		
T18-0456	12800	32.0%	0.57	7.31	7.10	4.1%
T18-0484	6400	63.8%	1.58	10.12		
T18-0484	12800	29.6%	0.56	7.22	8.67	23.7%
T18-0485	6400	59.8%	1.33	8.48		
T18-0485	12800	27.8%	0.60	7.67	8.08	7.2%
T18-0489	3200	69.5%	1.93	6.16		
T18-0489	6400	45.6%	0.91	5.85	6.00	3.7%
T18-0489	12800	20.6%	0.49	6.30	6.07	5.2%
T18-0491	3200	59.6%	1.32	4.21		
T18-0491	6400	33.9%	0.70	4.45	4.33	3.9%
T18-0553	6400	65.2%	1.45	9.26		
T18-0553	12800	38.8%	0.60	7.70	8.48	13.0%
T18-0554	1600	58.6%	1.14	1.82		
T18-0554	3200	27.3%	0.40	1.28	1.55	24.6%
T18-0555	1600	59.1%	1.10	1.75		
T18-0555	3200	28.6%	0.47	1.52	1.64	10.2%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-0557	1600	68.6%	1.50	2.40		
T18-0557	3200	45.4%	0.75	2.41	2.41	0.3%
T18-0562	1600	61.5%	1.26	2.02		
T18-0562	3200	35.6%	0.54	1.73	1.87	10.9%
T18-0563	1600	61.7%	1.18	1.90		
T18-0563	3200	33.6%	0.55	1.75	1.83	5.4%
T18-0564	3200	58.3%	1.34	4.28		
T18-0564	6400	33.1%	0.63	4.05	4.17	3.9%
T18-0565	3200	68.0%	1.64	5.25		
T18-0565	6400	41.4%	0.64	4.12	4.69	17.1%
T18-0601	3200	66.3%	1.41	4.50		
T18-0601	6400	36.0%	0.63	4.01	4.26	8.1%
T18-0602	3200	64.0%	1.18	3.76		
T18-0602	6400	29.0%	0.43	2.76	3.26	21.7%
T18-0724	1600	57.7%	1.16	1.85		
T18-0724	3200	29.9%	0.47	1.50	1.67	14.8%
T18-0725	1600	61.7%	1.33	2.13		
T18-0725	3200	38.0%	0.61	1.96	2.04	5.7%
T18-0726	1600	64.2%	1.86	2.98		
T18-0726	3200	48.2%	1.03	3.30	3.14	7.1%
T18-0727	3200	62.2%	1.35	4.32		
T18-0727	6400	29.2%	0.46	2.91	3.62	27.5%
T18-0729	1600	63.6%	1.82	2.91		
T18-0729	3200	49.8%	1.09	3.49	3.20	12.7%
T18-0729	6400	23.7%	0.39	2.50	2.99	23.5%
T18-0735	3200	63.8%	1.54	4.93		
T18-0735	6400	42.5%	0.70	4.46	4.69	7.1%
T18-0736	6400	48.8%	0.70	4.46		
T18-0736	12800	22.5%	0.29	3.71	4.09	13.0%
T18-0737	800	63.9%	1.55	1.24		
T18-0737	1600	48.0%	0.85	1.36	1.35	6.4%
T18-0737	3200	30.9%	0.46	1.46		5.4%
T18-0738	1600	63.3%	1.15	1.83		
T18-0738	3200	38.3%	0.51	1.62	1.72	8.9%
T18-0738	6400	22.4%	0.29	1.85	1.74	9.7%
T18-0739	1600	66.7%	1.74	2.79		
T18-0739	3200	44.5%	0.75	2.40	2.59	10.7%
T18-0739	6400	26.1%	0.38	2.43	2.41	1.1%
T18-0740	1600	65.4%	1.25	1.99		

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-0740	3200	39.9%	0.53	1.70	1.85	11.3%
T18-0741	3200	60.2%	1.34	4.28		
T18-0741	6400	34.8%	0.53	3.38	3.83	16.6%
T18-0742	3200	67.9%	1.39	4.45		
T18-0742	6400	40.7%	0.54	3.48	3.97	17.3%
T18-0743	3200	53.9%	0.74	2.38		
T18-0743	6400	26.5%	0.30	1.91	2.15	15.6%
T18-0744	3200	56.8%	0.65	2.07		
T18-0744	6400	27.2%	0.30	1.93	2.00	4.8%
T18-0745	3200	65.0%	1.09	3.49		
T18-0745	6400	36.5%	0.43	2.76	3.12	16.6%
T18-0746	3200	69.6%	0.93	2.96		
T18-0746	6400	32.7%	0.36	2.29	2.63	18.0%
T18-0747	3200	68.8%	1.91	6.11		
T18-0747	6400	44.3%	0.74	4.75	5.43	17.7%
T18-0748	3200	66.0%	1.28	4.10		
T18-0748	6400	36.0%	0.47	3.01	3.55	21.7%
T18-0749	1600	48.6%	0.63	1.01		
T18-0749	3200	23.5%	0.26	0.84	0.92	13.3%
T18-0750	1600	56.6%	0.64	1.03		
T18-0750	3200	25.8%	0.29	0.92	0.97	8.0%
T18-0751	3200	52.7%	0.81	2.60		
T18-0751	6400	23.4%	0.37	2.34	2.47	7.3%
T18-0752	3200	57.7%	1.13	3.63		
T18-0752	6400	28.1%	0.46	2.92	3.27	15.4%
T18-0754	3200	69.4%	1.37	4.38		
T18-0754	6400	43.7%	0.64	4.12	4.25	4.4%
T18-0755	3200	68.7%	1.34	4.27		
T18-0755	6400	46.7%	0.69	4.44	4.36	2.8%
T18-0756	1600	67.3%	1.27	2.03		
T18-0756	3200	32.6%	0.48	1.54	1.78	19.3%
T18-0757	1600	63.4%	1.38	2.22		
T18-0757	3200	33.1%	0.54	1.72	1.97	17.7%
T18-0758	1600	61.5%	1.13	1.81		
T18-0758	3200	33.4%	0.47	1.49	1.65	13.5%
T18-0759	1600	64.3%	1.40	2.23		
T18-0759	3200	33.2%	0.56	1.81	2.02	15.0%
T18-0760	1600	70.0%	0.99	1.59		
T18-0760	3200	36.0%	0.42	1.34	1.46	11.8%
T18-0761	1600	67.3%	0.90	1.44		

T18-0761	3200	33.7%	0.40	1.28	1.36	8.3%
Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-0762	1600	59.0%	0.72	1.15		
T18-0762	3200	26.3%	0.33	1.04	1.10	7.0%
T18-0763	1600	67.0%	0.90	1.44		
T18-0763	3200	33.3%	0.39	1.26	1.35	9.5%
T18-0765	1600	66.4%	1.27	2.03		
T18-0765	3200	42.8%	0.56	1.79	1.91	9.0%
T18-0766	1600	62.4%	0.81	1.30		
T18-0766	3200	40.0%	0.42	1.34	1.32	2.0%
T18-0767	800	66.8%	1.29	1.03		
T18-0767	1600	48.7%	0.67	1.07	1.05	2.4%
T18-0768	1600	56.9%	0.87	1.39		
T18-0768	3200	35.7%	0.45	1.44	1.41	2.2%
T18-0769	1600	59.8%	0.97	1.55		
T18-0769	3200	30.7%	0.38	1.23	1.39	16.3%
T18-0770	1600	63.1%	0.83	1.33		
T18-0770	3200	39.3%	0.41	1.31	1.32	1.4%
T18-0969	400	66.1%	1.12	0.45		
T18-0969	800	50.1%	0.63	0.50	0.48	8.4%
T18-0969	1600	22.7%	0.29	0.46	0.48	5.9%
T18-0978	3200	66.6%	1.10	3.51		
T18-0978	6400	29.6%	0.43	2.74	3.12	17.3%
T18-0979	3200	70.0%	0.99	3.18		
T18-0979	6400	36.0%	0.38	2.40	2.79	20.0%
T18-0980	800	65.4%	1.05	0.84		
T18-0980	1600	42.7%	0.58	0.93	0.89	7.4%
T18-0980	3200	20.4%	0.33	1.06	1.00	8.8%
T18-0982	800	68.7%	1.18	0.95		
T18-0982	1600	56.8%	0.82	1.31	1.13	22.9%
T18-0982	3200	26.9%	0.40	1.28	1.29	1.8%
T18-0983	1600	64.0%	0.82	1.30		
T18-0983	3200	33.0%	0.34	1.10	1.20	12.0%
T18-0984	800	47.8%	0.73	0.58		
T18-0984	1600	21.8%	0.32	0.52	0.55	8.6%
T18-0988	3200	59.0%	0.94	3.01		
T18-0988	6400	33.5%	0.45	2.86	2.94	3.6%
T18-0989	3200	66.8%	1.25	4.00		
T18-0989	6400	36.4%	0.41	2.61	3.30	29.8%
T18-1041	3200	58.0%	0.97	3.10		
T18-1041	6400	25.3%	0.42	2.69	2.90	9.9%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-1042	3200	69.5%	1.45	4.63		
T18-1042	6400	38.0%	0.53	3.36	4.00	22.4%
T18-1042	12800	26.0%	0.38	4.82	4.09	25.2%
T18-1043	800	55.5%	0.90	0.72		
T18-1043	1600	31.0%	0.48	0.77	0.75	5.3%
T18-1044	800	63.4%	1.11	0.89		
T18-1044	1600	35.1%	0.49	0.78	0.83	9.7%
T18-1045	1600	55.9%	0.91	1.45		
T18-1045	3200	27.5%	0.44	1.42	1.44	1.6%
T18-1046	1600	63.5%	1.12	1.79		
T18-1046	3200	36.4%	0.50	1.61	1.70	7.3%
T18-1438	1600	65.4%	1.64	2.62		
T18-1438	3200	45.9%	0.82	2.61	2.62	0.3%
T18-1439	1600	68.1%	1.55	2.49		
T18-1439	3200	48.2%	0.75	2.39	2.44	2.9%
T18-1439	6400	22.6%	0.33	2.11	2.25	8.6%
T18-1440	3200	29.3%	0.56	1.80	1.93	9.7%
T18-1440	3200	29.3%	0.56	1.80	1.93	9.7%
T18-1441	1600	60.4%	1.17	1.87		
T18-1441	3200	37.4%	0.51	1.62	1.75	9.9%
T18-1454	3200	68.0%	1.99	6.38		
T18-1454	6400	32.2%	0.55	3.52	4.95	40.9%
T18-1455	1600	66.0%	1.88	3.01		
T18-1455	3200	45.2%	0.82	2.62	2.82	9.7%
T18-1457	3200	61.2%	1.49	4.76		
T18-1457	6400	39.8%	0.68	4.37	4.56	6.0%
T18-1498	3200	69.1%	2.43	7.79		
T18-1498	6400	53.4%	1.11	7.13	7.46	6.3%
T18-1498	12800	20.5%	0.43	5.52	6.32	18.0%
T18-1499	6400	63.2%	1.65	10.56		
T18-1499	12800	32.5%	0.62	7.88	9.22	20.6%
T18-1500	1600	58.3%	1.32	2.12		
T18-1500	3200	33.6%	0.63	2.03	2.07	3.2%
T18-1518	400	59.0%	0.87	0.35		
T18-1518	800	31.6%	0.47	0.38	0.36	5.5%
T18-1639	800	61.0%	0.86	0.69		
T18-1639	1600	28.2%	0.41	0.65	0.67	4.3%
T18-1640	800	65.1%	0.96	0.77		
T18-1640	1600	34.3%	0.47	0.75	0.76	1.5%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-1945	1600	63.7%	0.77	1.24		
T18-1945	3200	38.3%	0.47	1.49	1.36	13.3%
T18-1946	1600	68.5%	1.01	1.62		
T18-1946	3200	39.9%	0.46	1.48	1.55	6.5%
T18-1950	6400	64.9%	0.90	5.74		
T18-1950	12800	35.3%	0.41	5.27	5.50	6.0%
T18-1953	3200	60.1%	0.71	2.28		
T18-1953	6400	23.3%	0.34	2.17	2.22	3.5%
T18-1954	3200	62.8%	0.84	2.69		
T18-1954	6400	21.9%	0.29	1.83	2.26	26.7%
T18-1990	1600	60.1%	0.82	1.31		
T18-1990	3200	36.9%	0.46	1.49	1.40	8.9%
T18-1991	1600	67.9%	0.99	1.59		
T18-1991	3200	38.3%	0.44	1.39	1.49	9.2%
T18-1992	3200	51.2%	0.65	2.08		
T18-1992	6400	27.4%	0.37	2.35	2.21	8.4%
T18-1993	3200	56.6%	0.70	2.24		
T18-1993	6400	26.2%	0.31	2.00	2.12	8.1%
T18-1994	3200	63.5%	0.90	2.89		
T18-1994	6400	39.9%	0.50	3.19	3.04	6.8%
T18-1995	1600	59.7%	0.81	1.30		
T18-1995	3200	42.1%	0.52	1.68	1.49	18.2%
T18-1996	1600	66.6%	1.00	1.60		
T18-1996	3200	46.5%	0.58	1.86	1.73	10.8%
T18-1998	800	60.1%	0.77	0.62		
T18-1998	1600	24.7%	0.30	0.48	0.55	18.3%
T18-2000	3200	69.3%	0.82	2.61		
T18-2000	6400	43.8%	0.36	2.32	2.46	8.3%
T18-2000	12800	23.2%	0.11	1.46	1.89	32.0%
T18-2012	1600	60.0%	0.87	1.40		
T18-2012	3200	34.9%	0.47	1.50	1.45	5.2%
T18-2013	1600	62.9%	0.65	1.04		
T18-2013	3200	38.9%	0.31	0.98	1.01	4.5%
T18-2013	6400	21.4%	0.08	0.51	0.74	44.6%
T18-2014	3200	68.2%	1.13	3.60		
T18-2014	6400	50.3%	0.68	4.34	3.97	13.1%
T18-2014	12800	22.9%	0.34	4.39	4.37	0.8%
T18-2015	6400	52.6%	0.47	3.04		
T18-2015	12800	24.0%	0.13	1.62	2.33	43.2%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-2016	1600	56.5%	0.79	1.27		
T18-2016	3200	33.0%	0.45	1.43	1.35	8.7%
T18-2017	1600	69.3%	0.82	1.31		
T18-2017	3200	46.1%	0.39	1.25	1.28	3.2%
T18-2017	6400	29.7%	0.20	1.29	1.27	2.3%
T18-2018	3200	62.0%	0.92	2.95		
T18-2018	6400	32.9%	0.45	2.86	2.90	2.3%
T18-2019	3200	65.6%	0.71	2.28		
T18-2019	6400	44.6%	0.37	2.37	2.33	2.8%
T18-2019	12800	22.3%	0.10	1.25	1.81	43.7%
T18-2031a	1600	53.8%	0.36	0.58		
T18-2031a	3200	23.2%	0.14	0.43	0.51	21.0%
T18-2031b	1600	58.5%	0.38	0.60		
T18-2031b	3200	30.3%	0.17	0.56	0.58	5.8%
T18-2031b	6400	20.1%	0.12	0.78	0.67	23.8%
T18-2032a	800	57.6%	0.41	0.33		
T18-2032a	1600	27.8%	0.16	0.26	0.29	16.5%
T18-2032b	800	59.7%	0.39	0.31		
T18-2032b	1600	35.7%	0.20	0.33	0.32	3.2%
T18-2032b	3200	21.2%	0.13	0.41	0.37	15.8%
T18-2272	3200	69.2%	1.92	6.16		
T18-2272	6400	37.0%	0.71	4.54	5.35	21.4%
T18-2273	3200	61.3%	1.36	4.37		
T18-2273	6400	35.2%	0.70	4.49	4.43	1.9%
T18-2351	3200	66.8%	1.09	3.48		
T18-2351	6400	39.9%	0.52	3.32	3.40	3.2%
T18-2352	3200	64.6%	1.20	3.85		
T18-2352	6400	38.7%	0.61	3.93	3.89	1.6%
T18-2353	3200	56.4%	0.80	2.57		
T18-2353	6400	32.9%	0.42	2.71	2.64	3.8%
T18-2354	3200	51.4%	0.83	2.66		
T18-2354	6400	31.3%	0.52	3.30	2.98	15.3%
T18-2355	800	65.3%	1.04	0.83		
T18-2355	1600	52.4%	0.72	1.16	0.99	23.2%
T18-2355	3200	25.9%	0.33	1.07	1.11	5.7%
T18-2356	800	67.6%	1.33	1.07		
T18-2356	1600	40.9%	0.65	1.03	1.05	2.2%
T18-2360	12800	69.3%	1.19	15.22		
T18-2360	25600	39.0%	0.54	13.71	14.46	7.4%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-2362	6400	66.0%	0.45	2.91		
T18-2362	12800	37.9%	0.20	2.61	2.76	7.6%
T18-2363	6400	69.3%	0.52	3.35		
T18-2363	12800	41.5%	0.26	3.29	3.32	1.3%
T18-2389	6400	61.3%	0.58	3.71		
T18-2389	12800	35.7%	0.26	3.37	3.54	6.8%
T18-2390	6400	65.1%	0.60	3.87		
T18-2390	12800	36.8%	0.26	3.34	3.60	10.5%
T18-2391	6400	54.6%	0.47	2.98		
T18-2391	12800	29.6%	0.22	2.76	2.87	5.5%
T18-2392	6400	62.5%	0.55	3.54		
T18-2392	12800	37.2%	0.26	3.37	3.46	3.5%
T18-2393	12800	50.1%	0.41	5.21		
T18-2393	25600	24.8%	0.18	4.66	4.93	7.8%
T18-2394	12800	55.2%	0.44	5.65		
T18-2394	25600	24.9%	0.18	4.57	5.11	14.9%
T18-2395	1600	67.5%	0.72	1.15		
T18-2395	3200	44.7%	0.35	1.11	1.13	3.0%
T18-2395	6400	21.8%	0.16	1.04	1.07	4.6%
T18-2396	1600	69.4%	0.71	1.13		
T18-2396	3200	56.2%	0.45	1.45	1.29	17.7%
T18-2396	6400	28.2%	0.20	1.28	1.37	9.2%
T18-2397	3200	53.7%	0.45	1.45		
T18-2397	6400	29.1%	0.21	1.36	1.41	4.5%
T18-2398	3200	64.5%	0.59	1.89		
T18-2398	6400	44.9%	0.33	2.10	2.00	7.3%
T18-2398	12800	21.5%	0.16	2.02	2.06	2.7%
T18-2406	400	54.0%	0.34	0.14		
T18-2406	800	29.6%	0.17	0.13	0.13	2.2%
T18-2407	400	55.8%	0.34	0.14		
T18-2407	800	29.3%	0.17	0.13	0.13	2.1%
T18-2412	6400	66.7%	0.61	3.91		
T18-2412	12800	43.2%	0.25	3.16	3.53	15.0%
T18-2413	6400	65.6%	0.50	3.19		
T18-2413	12800	32.9%	0.19	2.46	2.82	18.3%
T18-2414	3200	63.6%	0.54	1.72		
T18-2414	6400	39.1%	0.21	1.36	1.54	16.5%
T18-2415	3200	67.7%	0.54	1.72		
T18-2415	6400	43.7%	0.26	1.66	1.69	2.3%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T18-2416	12800	58.8%	0.44	5.65		
T18-2416	25600	27.7%	0.14	3.53	4.59	32.8%
T18-2485	6400	50.5%	0.37	2.37		
T18-2485	12800	22.5%	0.16	2.09	2.23	8.8%
T18-2486	6400	53.0%	0.53	3.39		
T18-2486	12800	28.0%	0.28	3.59	3.49	3.9%
T18-2566	1600	66.9%	1.35	2.15		
T18-2566	3200	55.9%	0.89	2.85	2.50	19.6%
T18-2566	6400	25.6%	0.36	2.28	2.56	15.7%
T18-2567	3200	62.5%	0.90	2.88		
T18-2567	6400	28.3%	0.40	2.56	2.72	8.4%
T18-2569	1600	68.4%	1.12	1.79		
T18-2569	3200	45.1%	0.58	1.85	1.82	2.2%
T18-2571	1600	62.6%	0.90	1.44		
T18-2571	3200	26.0%	0.38	1.21	1.33	12.2%
T18-2573	1600	66.5%	1.04	1.66		
T18-2573	3200	39.8%	0.52	1.65	1.65	0.4%
T19-0049	6400	62.9%	0.82	5.27		
T19-0049	12800	39.6%	0.40	5.13	5.20	2.0%
T19-0050	6400	66.9%	1.06	6.82		
T19-0050	12800	40.2%	0.44	5.65	6.23	13.2%
T19-0051	12800	52.6%	0.59	7.54		
T19-0051	25600	25.7%	0.25	6.48	7.01	10.6%
T19-0052	12800	50.7%	0.60	7.68		
T19-0052	25600	20.4%	0.23	6.01	6.85	17.3%
T19-0053	1600	67.8%	1.19	1.90		
T19-0053	3200	56.4%	0.75	2.41	2.15	16.8%
T19-0053	6400	25.7%	0.31	2.00	2.20	13.3%
T19-0054	3200	63.1%	1.03	3.29		
T19-0054	6400	38.3%	0.49	3.15	3.22	2.9%
T19-0055	3200	71.7%	1.49	4.78		
T19-0055	6400	45.7%	0.55	3.49	4.14	22.0%
T19-0055	12800	22.4%	0.28	3.59	3.54	1.8%
T19-0056	3200	66.3%	1.19	3.80		
T19-0056	6400	44.9%	0.58	3.72	3.76	1.5%
T19-0057	400	66.3%	0.93	0.37		
T19-0057	800	45.6%	0.49	0.39	0.38	2.9%
T19-0058	800	58.7%	0.68	0.54		
T19-0058	1600	29.9%	0.32	0.51	0.53	4.0%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T19-0059	1600	65.1%	0.90	1.43		
T19-0059	3200	47.6%	0.52	1.65	1.54	9.9%
T19-0059	6400	21.8%	0.22	1.43	1.54	9.9%
T19-0060	1600	67.6%	0.92	1.47		
T19-0060	3200	48.5%	0.51	1.64	1.56	8.1%
T19-0060	6400	23.5%	0.27	1.71	1.68	2.8%
T19-0061	6400	62.2%	0.90	5.77		
T19-0061	12800	42.8%	0.47	6.00	5.89	2.8%
T19-0062	6400	67.3%	0.89	5.69		
T19-0062	12800	43.5%	0.43	5.49	5.59	2.5%
T19-0063	12800	56.0%	0.71	9.11		
T19-0063	25600	23.6%	0.26	6.64	7.87	22.1%
T19-0064	12800	60.9%	0.70	8.92		
T19-0064	25600	29.3%	0.30	7.65	8.29	10.9%
T19-0065	12800	58.8%	0.79	10.08		
T19-0065	25600	25.1%	0.27	7.01	8.55	25.4%
T19-0066	12800	60.8%	0.70	8.90		
T19-0066	25600	26.7%	0.28	7.12	8.01	15.7%
T19-0067	3200	68.9%	1.27	4.07		
T19-0067	6400	43.6%	0.48	3.07	3.57	19.9%
T19-0067	12800	21.2%	0.24	3.04	3.06	0.6%
T19-0068	6400	52.2%	0.54	3.44		
T19-0068	12800	23.2%	0.25	3.22	3.33	4.6%
T19-0070	3200	58.4%	0.64	2.06		
T19-0070	6400	28.9%	0.30	1.89	1.97	6.0%
T19-0159	1600	54.7%	1.14	1.83		
T19-0159	3200	43.1%	0.79	2.53	2.18	22.9%
T19-0159	6400	21.9%	0.40	2.58	2.56	1.3%
T19-0160	1600	67.0%	1.43	2.29		
T19-0160	3200	48.0%	0.74	2.38	2.34	3.0%
T19-0185	400	63.3%	0.92	0.37		
T19-0185	800	40.1%	0.48	0.38	0.38	2.9%
T19-0187	1600	61.0%	0.86	1.37		
T19-0187	3200	32.6%	0.39	1.25	1.31	6.6%
T19-0191	3200	49.2%	0.89	2.86		
T19-0191	6400	28.6%	0.51	3.23	3.05	8.6%
T19-0192	3200	62.6%	0.94	3.01		
T19-0192	6400	36.6%	0.38	2.41	2.71	15.7%
T19-0192	12800	22.5%	0.21	2.68	2.55	7.6%

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T19-0193	1600	67.7%	1.81	2.90		
T19-0193	3200	43.3%	0.88	2.82	2.86	1.9%
T19-0193	6400	21.0%	0.44	2.80	2.81	0.5%
T19-0194	1600	62.4%	1.32	2.12		
T19-0194	3200	54.0%	1.04	3.31	2.72	31.1%
T19-0194	6400	22.7%	0.37	2.34	2.83	24.4%
T19-0194b	1600	66.2%	1.40	2.24		
T19-0194b	3200	46.1%	0.76	2.44	2.34	5.8%
T19-0196	3200	42.1%	0.59	1.89		
T19-0196	6400	24.0%	0.33	2.14	2.02	8.7%
T19-0197	6400	62.0%	1.05	6.75		
T19-0197	12800	52.0%	0.80	10.22	8.49	28.9%
T19-0198	12800	49.4%	0.74	9.44		
T19-0198	25600	21.2%	0.30	7.72	8.58	14.2%
T19-0199	12800	50.8%	0.77	9.92		
T19-0199	25600	21.5%	0.33	8.45	9.18	11.3%
T19-0201	6400	40.5%	0.60	3.82		
T19-0201	12800	22.3%	0.34	4.35	4.08	9.3%
T19-0202	3200	62.2%	1.12	3.58		
T19-0202	6400	48.2%	0.71	4.55	4.06	16.9%
T19-0202	12800	20.5%	0.29	3.77	4.16	13.3%
T19-0244	12800	52.0%	0.65	8.34		
T19-0244	25600	20.4%	0.25	6.32	7.33	19.5%
T19-0245	12800	60.4%	0.88	11.30		
T19-0245	25600	24.5%	0.30	7.57	9.43	27.9%
T19-0246	12800	62.3%	0.89	11.37		
T19-0246	25600	27.3%	0.32	8.13	9.75	23.5%
T19-0247	1600	66.7%	1.09	1.74		
T19-0247	3200	42.0%	0.51	1.63	1.69	4.5%
T19-0247	6400	20.6%	0.26	1.64	1.64	0.1%
T19-0267	1600	62.8%	1.11	1.78		
T19-0267	3200	33.7%	0.54	1.74	1.76	1.6%
T19-0268	1600	66.5%	0.97	1.56		
T19-0268	3200	39.5%	0.53	1.68	1.62	5.5%
T19-0316	12800	58.5%	0.43	5.45		
T19-0316	25600	28.8%	0.17	4.36	4.90	15.8%
T19-0317	12800	38.4%	0.43	5.56		
T19-0317	25600	23.1%	0.29	7.54	6.55	21.3%
T19-0318	12800	44.8%	0.47	6.07		

T19-0318	25600	22.1%	0.28	7.04	6.56	10.5%
Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T19-0319	12800	44.8%	0.45	5.73		
T19-0319	25600	20.2%	0.20	5.18	5.46	7.2%
T19-0320	12800	38.2%	0.43	5.55		
T19-0320	25600	22.0%	0.27	6.96	6.26	15.9%
T19-0321	12800	47.0%	0.55	7.10		
T19-0321	25600	23.2%	0.27	6.80	6.95	3.0%
T19-0323	1600	51.2%	0.62	0.98		
T19-0323	3200	28.7%	0.33	1.06	1.02	4.9%
T19-0324	1600	53.7%	0.69	1.11		
T19-0324	3200	27.1%	0.33	1.06	1.08	3.2%
T19-0450	6400	65.7%	0.70	4.49		
T19-0450	12800	32.4%	0.33	4.29	4.39	3.2%
T19-0451	6400	65.9%	0.70	4.51		
T19-0451	12800	35.5%	0.36	4.61	4.56	1.6%
T19-0452	1600	72.7%	0.85	1.37		
T19-0452	3200	36.7%	0.37	1.18	1.27	10.2%
T19-0459	12800	47.5%	0.49	6.21		
T19-0459	25600	22.8%	0.26	6.77	6.49	6.0%
T19-0460	6400	69.2%	0.77	4.92		
T19-0460	12800	33.1%	0.33	4.22	4.57	10.9%
T19-0461	6400	60.7%	0.65	4.17		
T19-0461	12800	24.4%	0.28	3.56	3.86	11.2%
T19-0462	6400	58.5%	0.59	3.76		
T19-0462	12800	22.3%	0.24	3.09	3.42	13.8%
T19-0463	1600	56.1%	0.59	0.94		
T19-0463	3200	22.2%	0.26	0.83	0.88	8.8%
T19-0464	1600	60.5%	0.61	0.98		
T19-0464	3200	28.3%	0.29	0.93	0.96	4.2%
T19-0465	6400	56.8%	0.56	3.55		
T19-0465	12800	25.0%	0.28	3.61	3.58	1.0%
T19-0469	6400	67.1%	1.14	7.27		
T19-0469	12800	43.0%	0.55	7.07	7.17	2.0%
T19-0578	6400	69.8%	1.02	6.55		
T19-0578	12800	41.9%	0.47	5.98	6.27	6.4%
T19-0603	12800	49.9%	0.60	7.68		
T19-0603	25600	20.3%	0.25	6.50	7.09	11.8%
T19-0605	3200	51.9%	0.63	2.02		
T19-0605	6400	21.5%	0.27	1.70	1.86	11.9%
T19-0645	3200	65.0%	1.01	3.24		

Sample ID	Assay Dilution	% inhibition [1-(A/Amax)]	Concentration (ng PbTx-3 eq/ml)	Sample ppm corrected for dilution	Mean ppm	%CV of adjacent dilutions
T19-0645	6400	33.0%	0.48	3.10	3.17	3.1%
T19-0646	3200	68.5%	1.07	3.42		
T19-0646	6400	41.7%	0.47	3.02	3.22	8.6%
T19-0647	6400	65.6%	1.03	6.59		
T19-0647	12800	27.9%	0.43	5.47	6.03	13.1%
T19-0648	6400	67.4%	1.02	6.55		
T19-0648	12800	40.4%	0.46	5.83	6.19	8.2%
T19-0691	1600	60.4%	0.66	1.05		
T19-0691	3200	28.6%	0.30	0.97	1.01	5.5%
T19-0693	12800	53.6%	0.56	7.11		
T19-0693	25600	22.8%	0.26	6.63	6.87	4.9%
T19-0694	12800	51.2%	0.50	6.43		
T19-0694	25600	22.5%	0.23	5.76	6.10	7.7%
T19-0695	6400	69.9%	0.87	5.55		
T19-0695	12800	36.5%	0.37	4.76	5.15	10.9%
T19-0814	12800	46.8%	0.52	6.71		
T19-0814	25600	24.7%	0.30	7.78	7.25	10.4%
T19-0816	12800	51.0%	0.51	6.53		
T19-0816	25600	24.9%	0.25	6.37	6.45	1.7%
T19-0817	12800	52.5%	0.52	6.62		
T19-0817	25600	20.0%	0.20	5.10	5.86	18.4%
T19-0818	3200	53.8%	0.64	2.05		
T19-0818	6400	27.2%	0.32	2.05	2.05	0.2%
T19-0819	3200	65.2%	0.86	2.75		
T19-0819	6400	30.7%	0.34	2.18	2.46	16.5%
T19-0821	6400	59.1%	0.68	4.37		
T19-0821	12800	26.3%	0.27	3.50	3.94	15.6%
T19-0822	12800	49.3%	0.52	6.69		
T19-0822	25600	20.6%	0.22	5.74	6.21	10.9%
T19-0823	12800	49.8%	0.53	6.78		
T19-0823	25600	23.7%	0.25	6.42	6.60	3.9%

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

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

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II
	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 III]

PART I – Quality Assurance			
ITEM			
CODE	**REF**		
		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).**
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 heterotropic 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~~21**		**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.** **An uninoculated APW blank will serve as the uninoculated 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~~21**		**3.2.3 The PCR forward and reverse primers and probes ~~are appropriate for the platform~~target.** **For Total and Pathogenic Vp Real-time PCR Method** Trh 627F: 5' ATA CCT TTT CCT TCT CCW GGT TC 3'~~tdh_269-20: 6FAM-5'-TGACATCCTACATGACTGTG-3'-MGBNFQ~~ Trh 731b R: 5' TTG TCC AGT AGT CAT CAA CGA TTG 3'~~trh_133-23: NED/TET-5'-AGAAATACAACAATCAAAACTGA-3'-MGBNFQ~~ Trh Glov R: 5' TTG TCC AAT AGT CCT CCA CAA TTG 3'~~tlh_1043: JOE/TEXAS-RED-5'-CGCTCGCGTTCACGAAACCGT-3'-BHQ2~~ WA IC F: 5' GGC GAA GCG AAT CTG GAA A 3'~~IAC_109: CY5-5'-TCTCATGCGTCTCCCTGGTGAATGTG-3'-BHQ2~~ WA IC R: 5' GGT GTA GTT GTG CGT GTA ATA TGA GA 3'~~trh_20F: 5'-TTGCTTTCAGTTTGCTATTGGCT-3'~~

			Orf8 F: 5' TCA CCT GAG GAC GCA GTT ACG 3'~~trh_292R: 5' TGTTTACCGTCATATAGGCGCTT 3'~~ Orf8 R: 5' TTC AAT TGT AGA ACC GCC AGC TA 3'~~tdh_89F:5' TCCCTTTTCCTGCCCCC 3'~~ Tlh-F: 5' CCG CTG ACA ATC GCT TCT C 3'~~tdh_321R: 5' CGCTGCCATTGTATAGTCTTTATC 3'~~ Tlh-R: 5' TTT GAT CTG GCT GCA TTG CT 3'~~tlh_884F: 5' ACTCAACACAAGAAGAGATCGACAA 3'~~ TDH-F: 5' TAT CCA TGT TGG CTG CAT TC 3'~~tlh_1091R: 5' GATGAGCGGTTGATGTCCAAA 3'~~ TDH-R: 5' CGA ACA ACA AAC AAT ATC TCA TCA GA 3'~~IAC_46F: 5' GACATCGATATGGGTGCCG 3'~~ Trh Probe: 6FAM 5' TAT TTG TYG TTA GAA ATA CAA CAA T 3' MGBNFQ~~IAC_186R: 5' CGAGACGATGCAGCCATTC 3'~~ 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 **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' MGB probe ~~vvhF 5' TGTTTATGGTGAGAACGGTGACA 3'~~ ~~vvhR 5' TTCTTTATCTAGGCCCCAAACTTG 3~~
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~~21		**3.2.5 Storage of thawed working stocks of primers and probes are stored between 2-8°C, not to exceed 2 weeks.~~Using molecular grade, nuclease free water, primer and probe stock solutions are diluted to produce a 0.01 mM working solution.~~**
C	~~14, 18~~21		**3.2.6 Storage of aliquoted working stocks of ~~Reconstituted~~ primers and probes ~~are~~ stored in a -20 °C manual defrost freezer does not exceed 1 year.~~for up to 5 freeze thaw cycles, not to exceed two years.~~**
C	21~~, 22~~		**3.2.7 Taqman Environmental Mastermix 2.0 is stored in -20°C manual defrost freezer until first use. ~~Platinum *Taq* DNA is stored in -20 °C manual defrost freezer until first use.~~ After first use, ~~can be~~it is stored between 2-8 °C.**
C	21~~, 22~~		**3.2.8 Internal control (IC) is stored in -20°C manual defrost freezer until first use.~~PCR reagents (dNTPs, buffer, MgCl2, fluorescent dyes) are stored in -20 °C manual defrost freezer until first use.~~ After first use, they ~~can be~~are 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, 21		**3.3.6 Two-hundred (200) µL~~One thousand (1000) µL~~ aliquots from each positive APW enrichment tube, including the process controls, are extracted.**
C	~~14, 18~~21		**3.3.7 For each run a specified amount of internal control (IC) is prepared such that each extracted well contains internal control DNA.~~Positive APW aliquots are placed in sterile microcentrifuge tubes and heated at 95-100 °C for 10 minutes.~~**
K	~~14, 18~~21		3.3.8 Extracts are refrigerated between 2-8°C and analyzed within 24 hrs. Frozen extracts are analyzed within 1 month of frozen storage.~~A set of positive and negative process controls are included with each batch of samples in a heating block/boiling bath.~~

C	**~~14, 18~~21**		**3.3.9 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.~~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~~21**		**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~~16, 18		3.4.3 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, 21**		**3.4.4 Eighteen (18) µL ~~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, 21**		**3.5.~~2~~1 Two (2) µL of DNA template is added to each reaction tube or plate well containing ~~23~~18 µL of Master Mix for a total PCR reaction volume of ~~25~~20 µL.**
C	**14, 19, 21**		**3.5.2 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, 21		3.5.3 Two (2) µL of molecular grade, nuclease free water is added to a reaction tube or plate well containing ~~23~~18 µL of Master Mix for each batch of Master Mix prepared as a no template control.
C	**14, 19, 21**		**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~~18 µL of Master Mix.**
C	**14, 19, 21**		**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~~18 µL of Master Mix.**
O	14, 19, 21		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~~18 µL of Master Mix as the positive PCR (amplification) control.
K	14, 19, 21		3.5.7 Immediately prior to loading the reaction tubes or plates into the instrument they are centrifuged for ~~3-5~~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		**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.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. U.S. Department of Commerce. 1976. *NBS Monograph 150*. U.S. Department of Commerce, Washington, D.C.
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.
8. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition. APHA, Washington, D.C.
9. Fisher, J. 1985. Measurement of pH. American Laboratory 16:54 – 60.
10. Association of Official Analytical Chemists (AOAC). 1999. AOAC *Methods Validation and Technical Programs – Criteria for Laboratories Performing Food Testing*. AOAC, Arlington, Va.
11. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA – 670/9-75-006. U.S. EPA, Cincinnati, Ohio.
12. Adams, W.N. 1974. NETSU. Personal Communication to Dr. Wallace Andrews, FDA.
13. U.S. Food and Drug Administration (FDA). 1995. *Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
14. Campbell, Mark, S. and Wright, Anita, C. Real-time PCR analysis of *Vibrio vulnificus* from oysters, Appl Environ Microbiol. 69, 12 (2003).
15. Wright, Anita, C., Garrido, V, Debuex, G, Farrell-Evans, M, Mudbidri, A, A. and Otwell, W, S. Appl Environ Microbiol. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. 73, 22 (2007).
16. Cepheid© product information. ~~Integrated DNA Technologies. Oligonucleotide Stability Study. 2014.~~
17. Section IV Guidance Documents, Naturally Occurring Pathogens, NSSP *Guide for the Control of Molluscan Shellfish*, 2009 Revision.
18. Nordstrom, J.L., M.C.L. Vickery, G.M. Blackstone, S.L. Murray, and A. DePaola. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic V. parahaemolyticus bacteria in oysters. Appl. Environ. Microbiol. 73(18):5840-5847.
19. Kinsey, T.P., K.A. Lydon, J.C. Bowers, J.L. Jones. 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): 1574-1580.
20. National Institute of Standards and Technology Special Publication 250-23, 128 pages (Sept. 1988) U.S. Government Printing office, Washington, D.C. Library of Congress Catalog Number: 88-6000580.
21. Washington State Public Health Laboratory. 2019. Vibrio parahaemolyticus and Vibrio vulnificus enumeration and detection through MPN and real-time PCR. WA PHL, Seattle, WA. ~~Integrated Solutions — Real-Time PCR Applications: Critical Factors for Successful Real-Time PCR. www.qiagen.com~~

~~22. FDA Reagent Stability Study, unpublished. 2013.~~

LABORATORY:	**DATE of EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY of NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	

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**

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:_____

LABORATORY:

Page	Item	Observation

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+). 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. Scope

Rapid and early detection of these pathogens will help the shellfish industry market oysters for consumption that are within regulatory limits for these pathogens, and ensure public health safety.

3. Reagents / Media

- Master Mix: TaqMan™ Environmental Master Mix 2.0; Thermo Fisher Cat. #4396838
- Molecular PCR grade water; Thermo Fisher Cat. #SH3053802 or equivalent
- TE buffer; Thermo Fisher Cat. #BP2473500 or equivalent
- Primers (See appendix A for sequences)
- Probes (See appendix A for sequences)
- Internal Control Plasmid
- MagNAPure 96 DNA and Viral NA Small Volume kit; Roche, Cat. # 06543588001
- Alkaline Peptone Water (APW); Prepared In-house
- Phosphate Buffer Saline (PBS); Prepared In-house

Record receipt of all PCR mastermix components in the Reagent Receipt Log (P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\Master Mix Documents\Updated Worksheets\Reagent Receipt Log). All reagents will be tracked by its lot number. The intent of the reagent receipt logbook is to allow for complete traceability. Additionally, lot numbers are recorded upon use on Master Mix Worksheet.

Prepare Primer and Probe mixes according to the Master Mix Worksheet (P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\Master Mix Documents\Mastermix Template). 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 5 years unless otherwise stated by manufacturers. Working concentrations of primers and probes 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 (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 B for Internal Control Plasmid info.

4. 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

4.1 Bacterial Cultures

- *Vibrio parahaemolyticus* (WA4647 and BAA-240, or equivalent)
- *Vibrio vulnificus* (ATCC 29307 or equivalent)

5. Equipment

- Non-mercury glass thermometer 0°C-10°C
- 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
- 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.

6. Safety Precautions

Vibrio species are pathogenic and should be handled following PHL safety guidelines and assay risk assessment.

UV light can seriously burn skin and eyes. Keep safety shield lowered when UV light is on. Always keep skin covered by lab coat and gloves

7. Specimen Information

Samples are to be shipped properly (adequate ice/cold packs) and temperature maintained between 0 and 10°C upon arrival. Once received and logged-in, the samples are to be placed under refrigeration unless processed immediately.

Samples received over 10°C are considered acceptable only under the following conditions:

The sample is shipped properly (adequate ice/cold packs) and was at an elevated temperature at collection and has had a short transit time (collected and received on the same day).

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.

8. Quality Control

- Instructions
    - Three process controls are included in every extraction run. To prepare the process control material, enrich *V. parahaemolyticus* WA4647 (*tlh*+, *tdh*+, *trh*+) and *V. vulnificus* ATCC 29307 (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, isolation, 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* (ATCC BAA-240, WA4647) and *Vibrio vulnificus* (ATCC 29307) 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.
- Certificates of analysis will be maintained in a binder within the Food lab for all pre-sterilized consumables.
- Disposable pipettes will be checked for accuracy and tested for sterility.
- Disposable pipettes (i.e. serological) used to inoculate samples and prepare reagents are checked for accuracy and 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.
  - Certificates of analysis (COA) for each new lot of pre-sterilized consumables will be maintained.
  - Each new lot of disposable pipettes (i.e. serological) will be tested for accuracy and checked for sterility.

- Acceptable Limits
  - See respective media, reagents, mastermix, or primer/probe QC guides for expected or acceptable results.
  - A successful PCR run should meet the following conditions:
    I. The positive controls should show clear amplification. If NO amplification is present in the positive controls for one or both multiplex's, determine the problem and re-run the sample.
    II. The negative process control should only show amplification for the exogenous internal control (IC). The no-template-control (NTC) should not show amplification for any of the targets.
    III. Creeping curves without a logarithmic increase are not considered true amplification. If amplification is present in a negative control, determine the source of contamination, thoroughly clean BSC and pipettes, and discard contaminated reagents and disposables.
  - Results will not be reported without acceptable QC results.
  - All certificates for pre-sterilized consumables will be checked for conformance and initialed by the laboratorian.

  - For all disposable pipette (i.e. serological) accuracy checks, a satisfactory accuracy is ±2% of volume tested.

- Corrective Action
  - PCR runs for which the NTC is positive or the positive control and/or internal control is negative should be repeated.
  - The Lead Microbiologist should be notified if any run fails QC.
  - If quality controls for mastermix, primers/probes, or disposable pipettes do not meet acceptable criteria, the QC fails and item shall not be used for testing.
  - Media/reagent quality controls not exhibiting the expected growth or reactions will be retested with fresh growth (18-24 hrs) organisms.
  - Samples tested with any failed media/reagent will be considered invalid and will be retested with new media/reagent which has passed quality control.

- Recording QC Data
  - Initial quality control data for media/reagents, mastermix, primers/probes, and disposable pipettes will be recorded on its respective QC worksheet. Records are maintained in a binder within the laboratory.
  - Record results for each PCR and Process controls by notating presence (+) or absence (–) on the sample worksheet.

9. Calibration

- **QuantStudio Dx PCR Workstation**

  Calibration kits are used to maintain the Real-Time PCR system with 384-Well Block. They include calibration plates to perform a spectral calibration with FAM™, VIC®, ROX™, SYBR® Green, TAMRA™, NED™ dyes, plates to perform region-of-interest (ROI) calibration, normalization calibration, and to run RNase P verification.

  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.

- **Micropipettor and Thermometers**

  Micropipettors are calibrated at appropriate volumes annually and checked for accuracy quarterly. At a minimum quarterly checks are performed at 100%, 50%, and 10% of nominal volume.

  Non-mercury glass thermometers will be sent out for annual calibration and checked for accuracy quarterly by a certifying vendor on-site. Long stem digital thermometers will be replaced yearly.

  "As Found" and/or "As left" calibration data for micropipettes or thermometers must indicate that the initial calibration or recalibration passed. Acceptable tolerance limits will be obtained as pre-determined by the manufacture or ISO 17025 accredited service by the certifying vendor.

If the "As found" calibration data for micropipettes or thermometers indicate that the calibration or recalibration failed, a PHL Quality Improvement (QI) Form must be filled out for all affected samples.

Calibration/re-calibration certificates for all micropipettes and thermometers will be checked for conformance and initialed by the Supervisor or Lead prior to use. Calibration certificates are maintained in a binder within the laboratory.

10. Procedure

10.1 Sample Accessioning

a. 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].
b. Oyster samples are removed from the shipping container and the sample submission form is located. At a minimum the sample submission forms must contain the following information: collector's name, harvest area, sampling station, time and date of collection.
c. A laboratory testing worksheet is generated for each sample.
d. Both the sample submission form and the testing worksheet are stamped with the appropriate laboratory number.
e. One oyster from each bag is opened to take tissue temperature. The temperature is recorded on the sample submission form.
f. The bag of oysters is labeled with its associated sample ID and placed into a 2-8°C refrigerator unless processed immediately.

10.2 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 is used for analysis.
f. After cleaning each oyster place the animal upside down on a clean paper towel lined tray. Ensure that you have labeled the tray with corresponding sample number.

Laying the oysters upside down will prevent the liquor (fluid inside a closed oyster) from draining out of the oysters while waiting to be shucked. Clean trays must be used for each sample.

g. Once cleaned, return the oysters to the refrigerator to dry or towel dry them for immediate shucking.

10.3 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.

10.4 Sample Processing- Setting up MPN

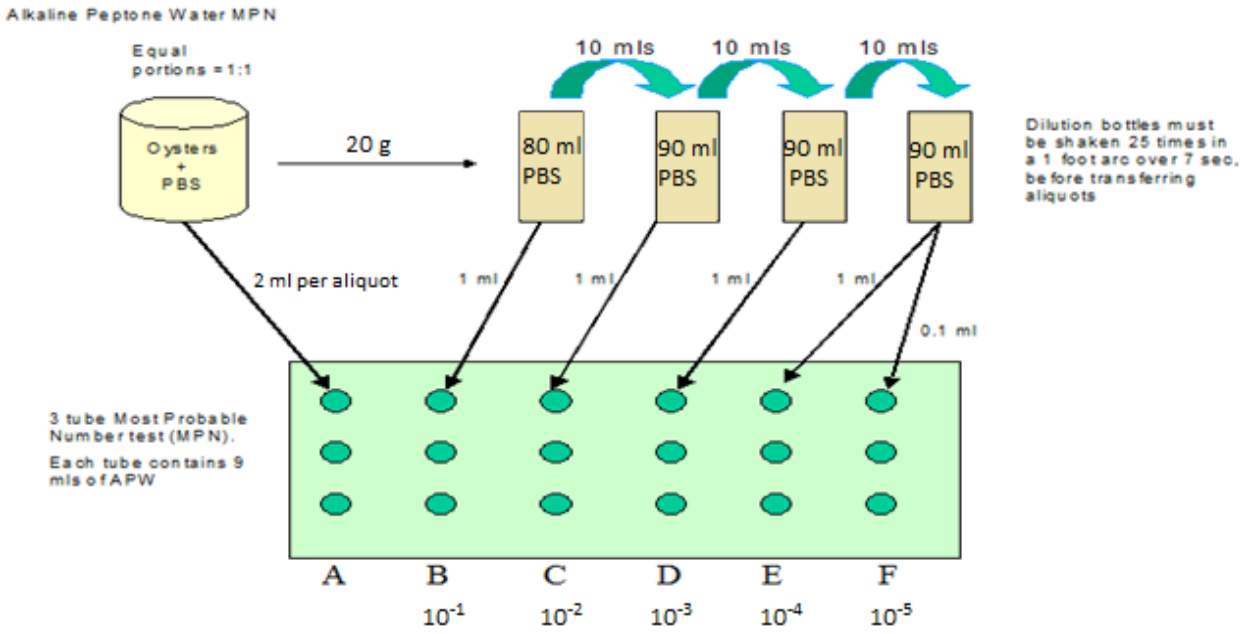
Enumeration in this assay is achieved by conducting an MPN (most probable number) analysis.

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).

The resulting homogenate should be relatively smooth. If the blender isn't generating a smooth homogenate, it is advisable to service the blender (replace blades).

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.

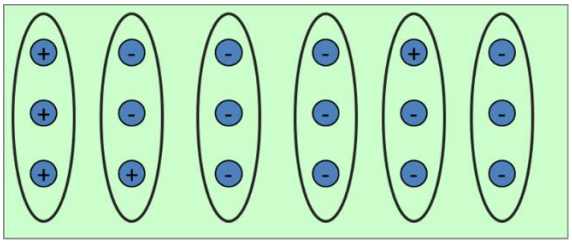
The initial 1:10 dilution is prepared gravimetrically with PBS (20 g of sample homogenate to 80 mL of PBS). Record the weight and volume used on the sample worksheet for the sample worksheet. All successive dilutions are prepared volumetrically.

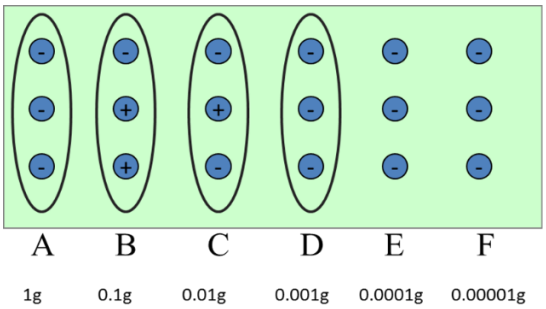


h. Incubate at 35°C± 1 for 18 to 24 hours. Write sample number, date, time, and analyst initial on the A1 tube, prior to placing in incubator.

10.5 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.

10.6 DNA Extraction

DNA extraction must be initiated within the 18-24 hour incubation window.

Reagent prep should be carried out in the Pre-PCR room in order to minimize the potential for contamination. 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. Due to the high number of sample tubes it is necessary to create a document to track the location of each one. This document is referred to as the "MagNA Pure Plate Map".
b. After the MagNA Pure Plate Map is created, 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.
c. Once loaded, seal the MagNA Pure cartridge with an adhesive cartridge seal.
d. Label the cartridge with the sample number, date, initials and label as "Pre-Extract". The specimen can now be loaded onto the MagNA Pure 96 instrument.
e. Confirm that the MagNA Pure 96 instrument and its linked computer are turned on.
f. Ensure that the correct MagNA Pure kit is selected "DNA/Viral SV 2.0".
g. Select the protocol "Pathogen Universal 200.3.1"
h. Sample volume should be entered as 200µL.
i. Elution volume should be entered as 100µL.

j. 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.
k. 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.
l. Click the "Stage Set-up" button.
m. Begin adding in the appropriate reagents/plastics in accordance with the outlined requirements on the MagNA Pure load screen (software).
n. Once the reagent trays are completely loaded and the tips are adequately filled, place the remaining trays back into the instrument.
o. Remove the cartridge seal from the processing cartridge and place into the instrument. Discard the seal into an autoclave waste container.

p. Ensure that all plastics, reagents and sample cartridges are in place and accounted for on the computer screen.
q. Close the door and press the "start extraction" button.
r. 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.
s. 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.

10.7 MagNAPure 96 Waste Removal and Decontamination.

a. Remove all soiled plastics, replace used tips, and wipe the trays with 10% bleach, isopropyl alcohol and RNAse Away using Manufacturer's suggested cleaning procedure. Run the UV decontamination protocol.
b. If waste bottle is full, follow MagNAPure 96 Waste Disposal Procedure listed below.
    I. Instrument will indicate waste container is full.
    II. Attach empty waste container to instrument and secure caps on full container.
    III. Carry full waste container with caps closed to the sink, set inside sink, and remove small cap.
    IV. Tip container onto side with small cap and allow to drain into sink. As it drains, you may need to tilt the container to ensure complete drainage of liquids.
    V. Spray sink and container with 70% Isopropanol.
    VI. Pour entire bottle of 70% Isopropanol (~ 500ml) into container, secure cap, and carefully invert to mix.
    VII. Allow Isopropanol to sit for 10 min.
    VIII. Spray sink and container again with 70% Isopropanol and wipe down container. Pour Isopropanol from inside container into the sink and secure caps.
    IX. Rinse sink and exterior of container with water.
    X. Spray exterior of container with 10% bleach, allow 3 minute contact time, and then rinse with water.
    XI. Container can be stored in lab with secured caps until next use.
c. For routine MagNA Pure 96 maintenance, follow the MagNA Pure 96 Daily Maintenance Log (for start-of-day and end-of-day instructions) and MagnNA Pure 96 Post Run Cleaning Log

instructions. Forms can be found in the Master Document Control or link to the following address:

P:\EHSPHL\PHL\MICRO\COMMON\ENTERICS - FOOD\QC\Media QC\501.4206.docx
P:\EHSPHL\PHL\MICRO\COMMON\ENTERICS - FOOD\QC\Media QC\501.4207.docx

10.8 PCR Mastermix Preparation

Mastermix preparation is performed in the Pre-PCR room, 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.
Note: A person who has previously in the same day worked with amplicon should not re-enter the Pre-PCR lab.

a. Prepare a PCR platemap using the MasterMix Prep worksheet. (P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\Master Mix Documents\Mastermix Template). Be sure to include positive and negative process controls, in addition to a positive and negative amplification control.
b. Using the worksheet 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.
f. Once the 384-well plate is loaded with mastermix, cover the plate with aluminum foil, place the plate in a biological transport container (sealed box), and transport to an available AirClean hood within the Food laboratory (Alternatively the Template Addition Room can be used).

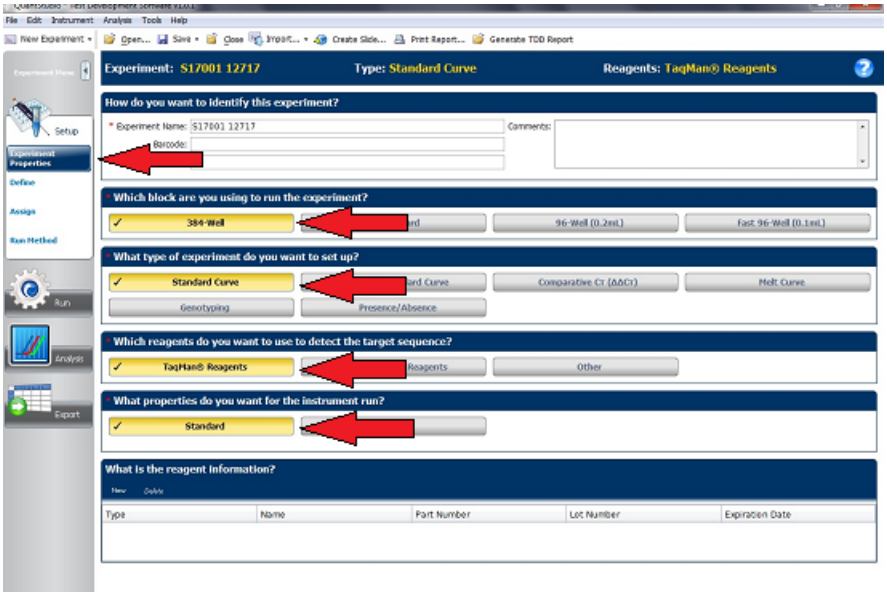
10.9 Template Addition

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.

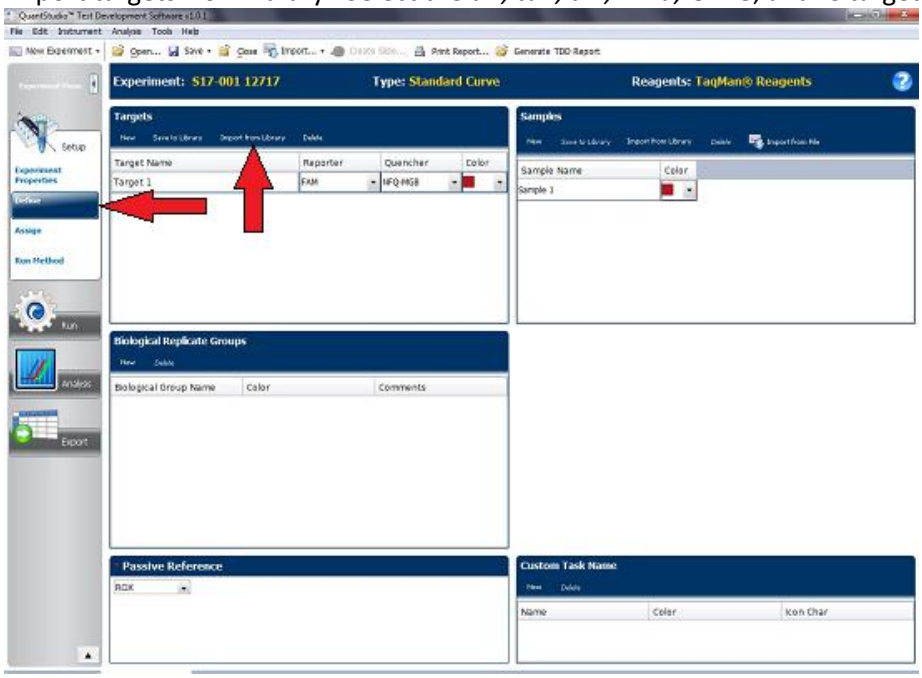
10.10 Setting up Real-Time PCR Station

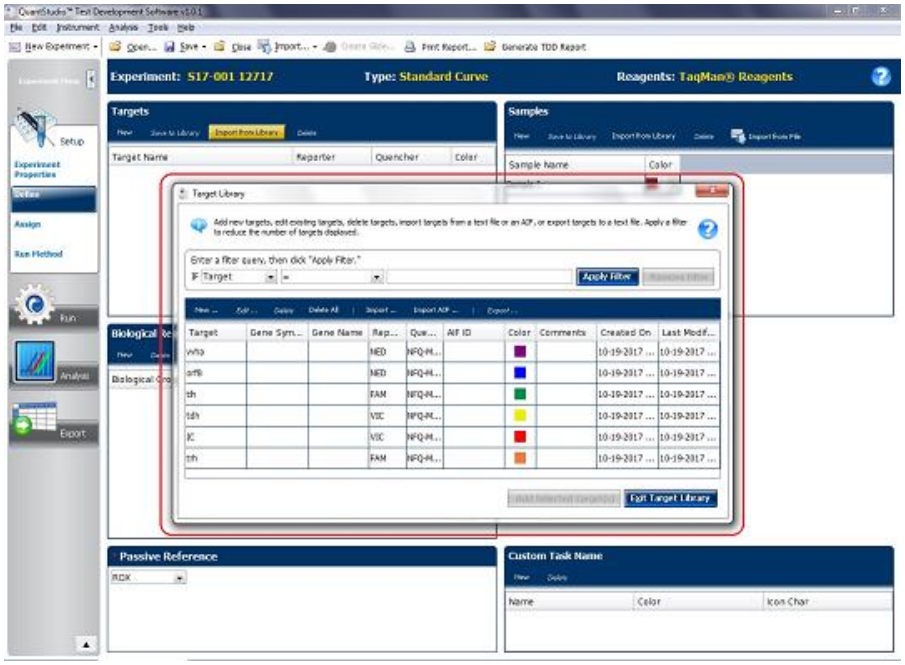
a. Turn on the Applied Biosystems® QuantStudio™ Dx™ instrument and the computer.
b. Open the Test Development software, under the File menu select “New Experiment”.

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.

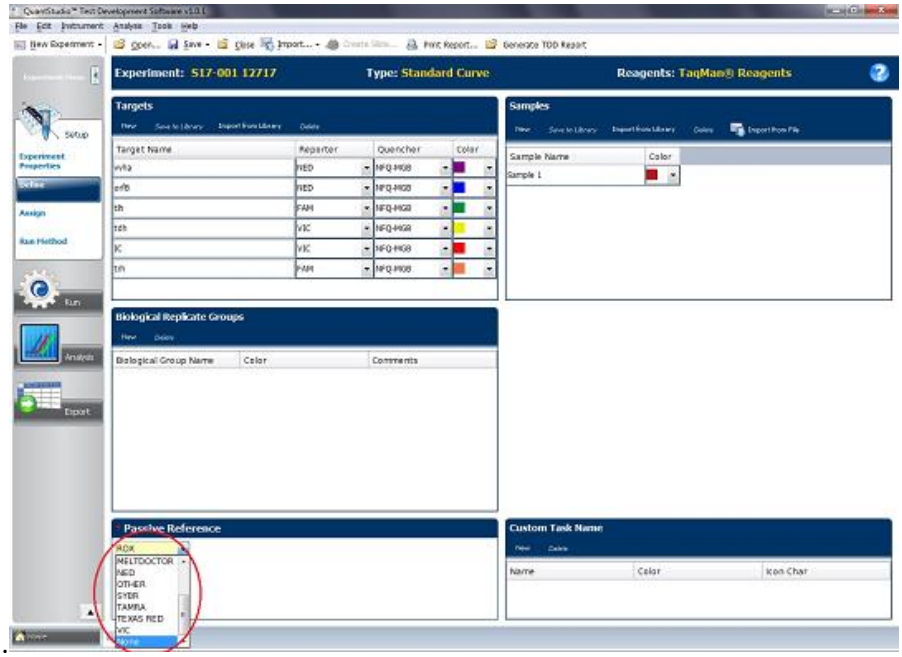


e. The "Define" tab is used to select the targets of detection. The targets are saved to the library. Import targets from library. Select the *tlh, tdh, trh, vvha,* ORF8, and IC targets.

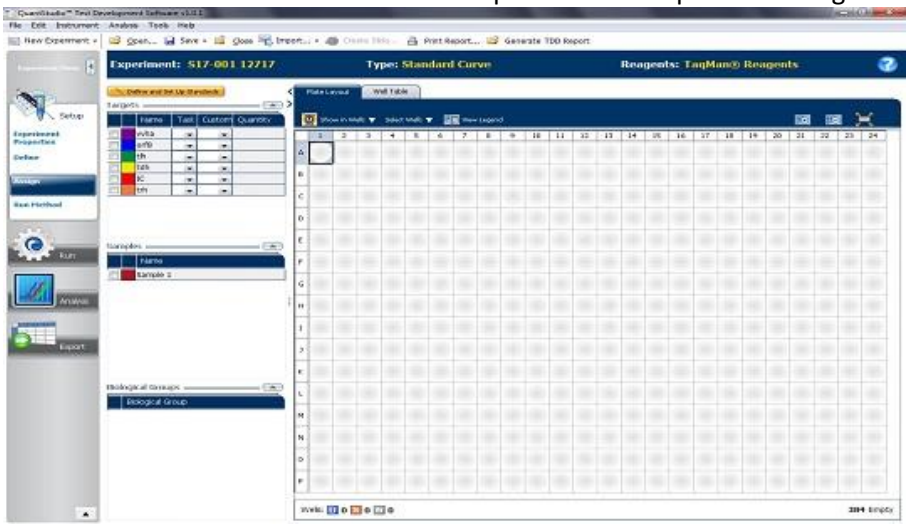




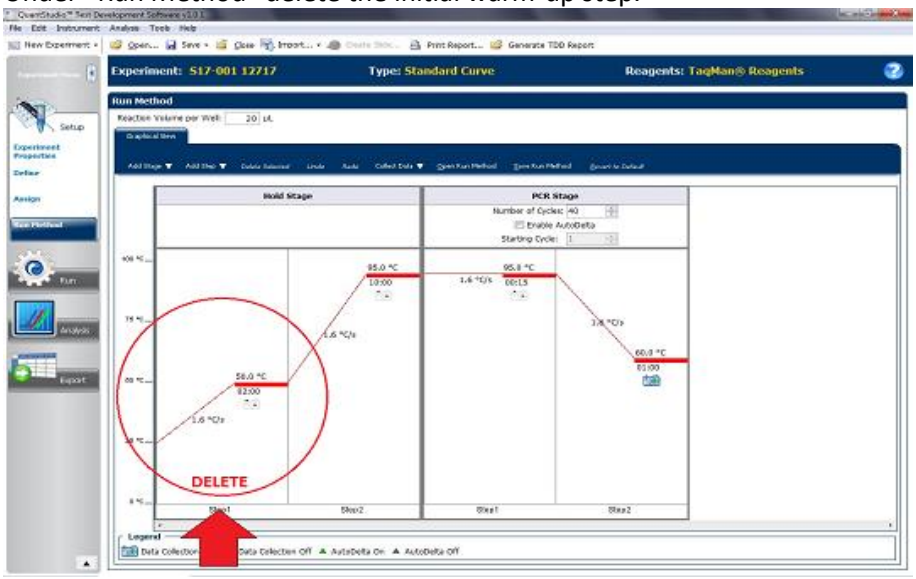
f. At the bottom of the screen, select "ROX" from the drop down menu regarding "Passive Reference"



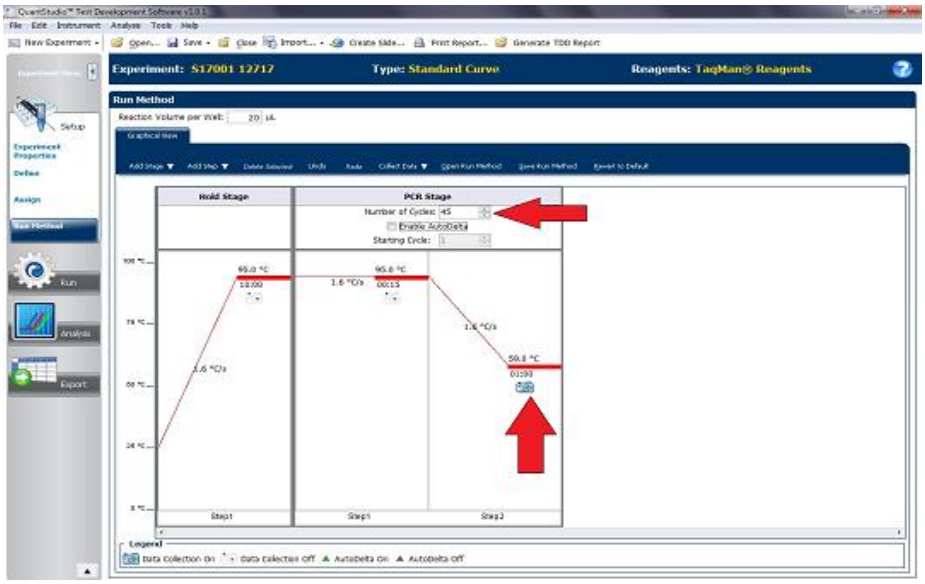
g. Select the "Assign" tab and assign the appropriate wells with the corresponding targets of interest. Be sure to double check the map and 384-well plate are in agreement.



h. Under "Run Method" delete the initial warm-up step.

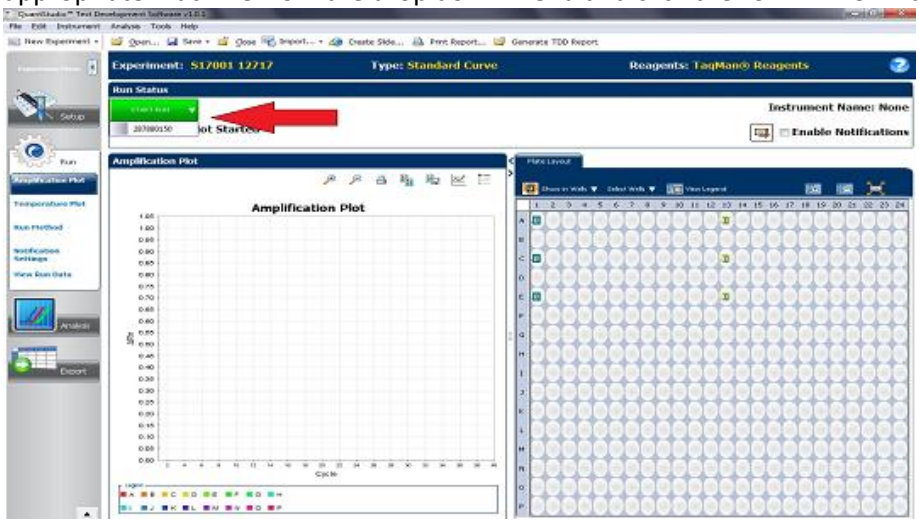


i. After doing so the parameters are the following:
   95° C for 10 minutes
   40 cycles
   95° C for 15 seconds
   59° C for 60 seconds



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.

11. Waste Management

The biological material is rendered non-hazardous through use of the MagNA Pure 96 System and associated reagents[ii]. The reference refers to the MagNA Pure Compact System, however; all of these instruments share the same buffer system, process, and concentrations. The remaining waste is considered flammable by Department of Ecology Standards and will be collected as hazardous waste for disposal.

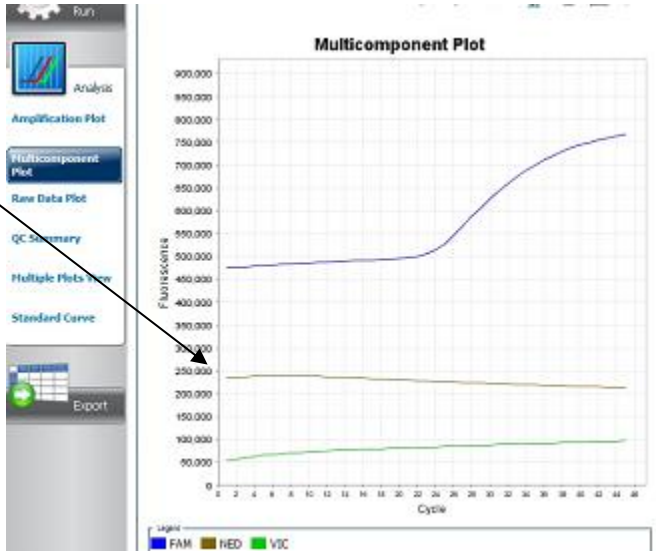
All other plastics and glassware containing the sample will be treated by autoclaving using appropriate conditions.

Date of disposal of each sample is recorded on the respective sample worksheet.

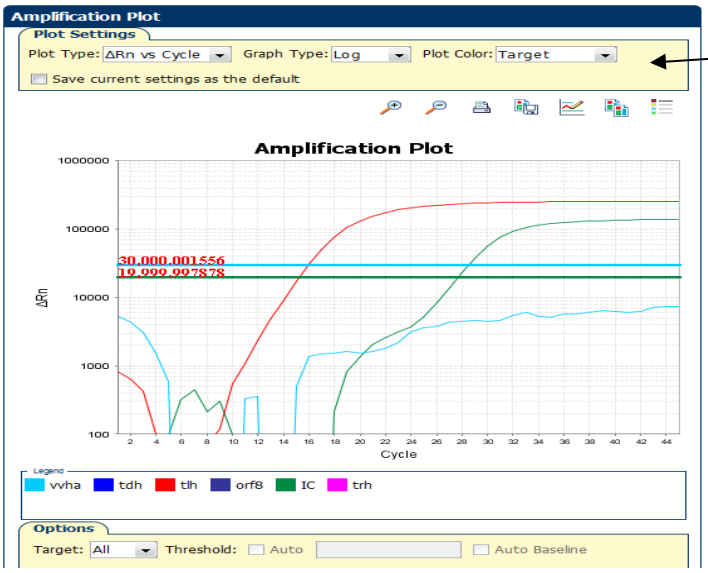
12. Interpretation

a. Once run is complete, remove plate from instrument and discard in appropriate waste receptacle.
b. Select "Analysis Settings" on the Amplification Plot screen.
c. 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.
d. Apply analysis settings and exit to Amplification Plot screen.
e. 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.
f. Select all wells in the plate by clicking in the upper left box of the plate layout.
g. 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.
h. 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



i. 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

12.1 Procedure for Abnormal Results

If abnormal results appear to be caused by cross contamination (i.e. late CT value) rerun real-time PCR in duplicate of suspected contaminated wells

If duplicate results are in agreement, report these results. If the duplicates differ, report the result that is in agreement with the original qPCR run.

Positive pathogenic markers (*tdh, trh,* ORF8) in absence of *Vibrio parahaemolyticus* marker (*tlh)*

I. Current findings do not support pathogenic markers being present without *Vp* being present. Real-time PCR reactions resulting in this situation should be re-run upon Lead Microbiologist discretion.
II. The presence of the *trh* gene in the absence of the *tlh* gene has been documented[i]. This is due to the *Vibrio parahaemolyticus trh* gene having 98% homology with the *trh* gene of *Vibrio alginolyticus*[i]. Any *trh* positive wells must be *tlh* positive as well.

12.2 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.

13. 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.

Unusual MPN indexes are typically due to contamination. It may be necessary to re-extract and/or re-run PCR. If this does not resolve the issue, further investigation is required to determine the source of contamination.

14. Reference Range

Reportable Range

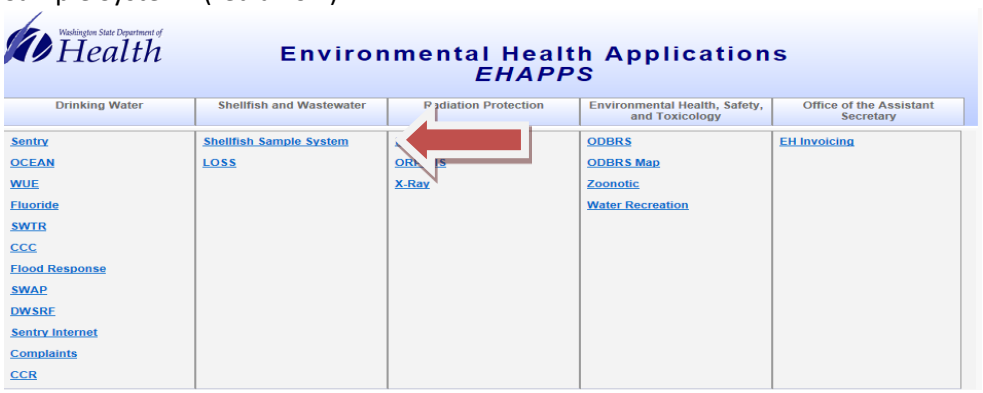
tlh: <0.36 MPN/g to >110,000 MPN/g
tdh: <0.36 MPN/g to >110,000 MPN/g
trh: <0.36 MPN/g to >110,000 MPN/g
ORF8: <0.36 MPN/g to >110,000 MPN/g
vvhA: <0.36 MPN/g to >110,000 MPN/g

15. Reporting Results

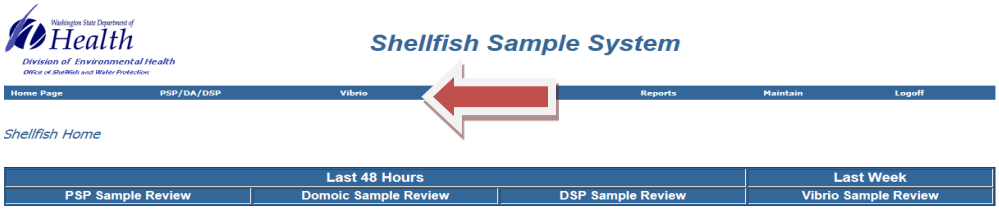
15.1 Environmental Health Applications (EHAPPS) database

Access to the database must be authorized. Lead Microbiologist or Supervisor will facilitate the authorization process.

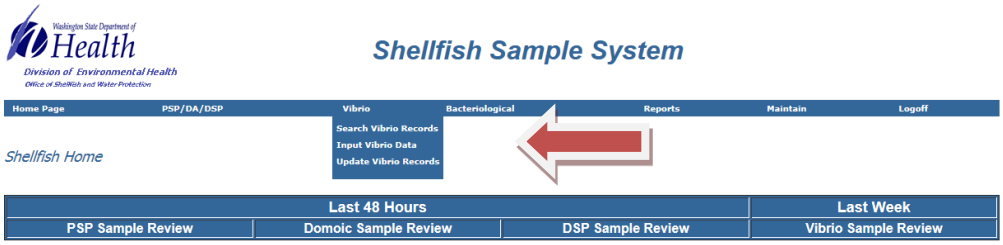
a. After entering web address, find column “Shellfish and Wastewater” and click “Shellfish Sample System” (red arrow)



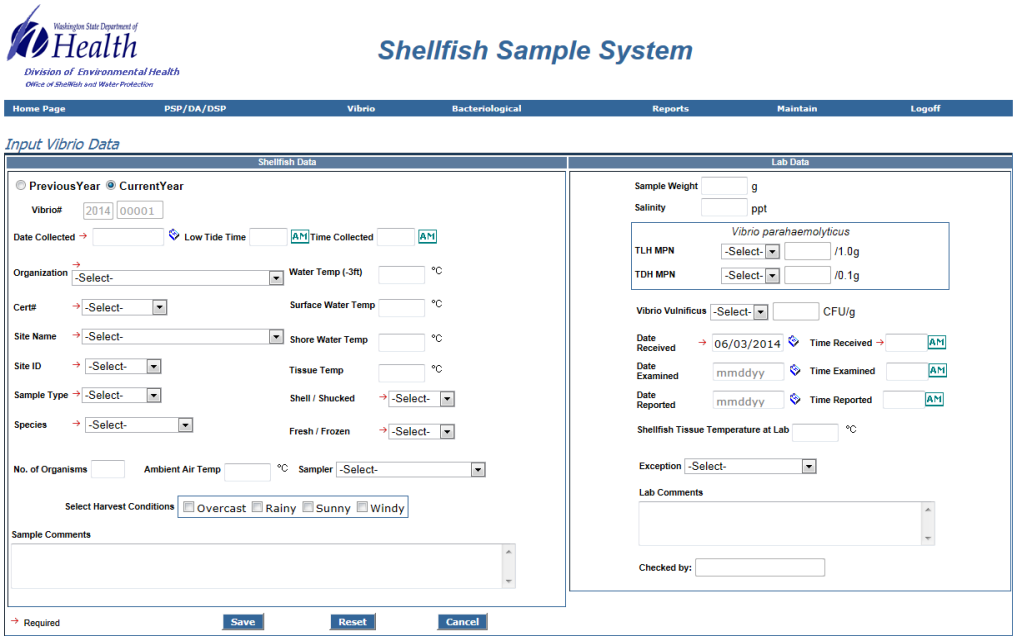
b. Along the top, hover mouse over “Vibrio” (red arrow)



c. Click “Input Vibrio Data” (red arrow)



d. The sample number will auto-populate. Enter all information and data provided from Vibrio Sample Submission form. Enter final test results when available.



15.2 Notification of Test Results

a. Environmental Health Applications (EHAPPS)

All test results will be entered into the Shellfish Sample System via EHAPPS. Results are reviewed and checked off by the Lead Microbiologist.

b. Email

Test results can be emailed to the Office of Shellfish and Water Protection (OSWP) after Lead Microbiologist approval and signature.

c. Phone

For STAT results (per request of OSWP), the Lead Microbiologist will contact the appropriate personnel at OSWP.

15.3 Archiving Results & Retention

a. Filing Results

The Vibrio Testing Worksheet and Sample Submission Form are to be filled in a filing cabinet located within the Food and Shellfish Bacteriology Laboratory.

All other documents (i.e. Mastermix worksheet, PCR Plate Map, MP96 Plate Map, Sample Tracking worksheet, etc.) are to be scanned and uploaded into the Scanned Testing Documents folder under the appropriate year on the PHL P: Drive server. All scanned documents for a given day can be saved under this folder as the date (MMDDYY).

For example:

P:\EHSPHL\PHL\MICRO\FOODLAB\Vibrio\2019 Vp Season\Scanned Testing Documents\060119

All uploaded testing documents will be reviewed by the Lead Microbiologist prior to discarding any hard copies.

b. Retention

Reports and results for samples tested will be archived according to the Department of Health Records Retention Schedule.

EHAPPS database is maintained by the Office of Shellfish and Water Protection.

16. 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.

17. 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 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.

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

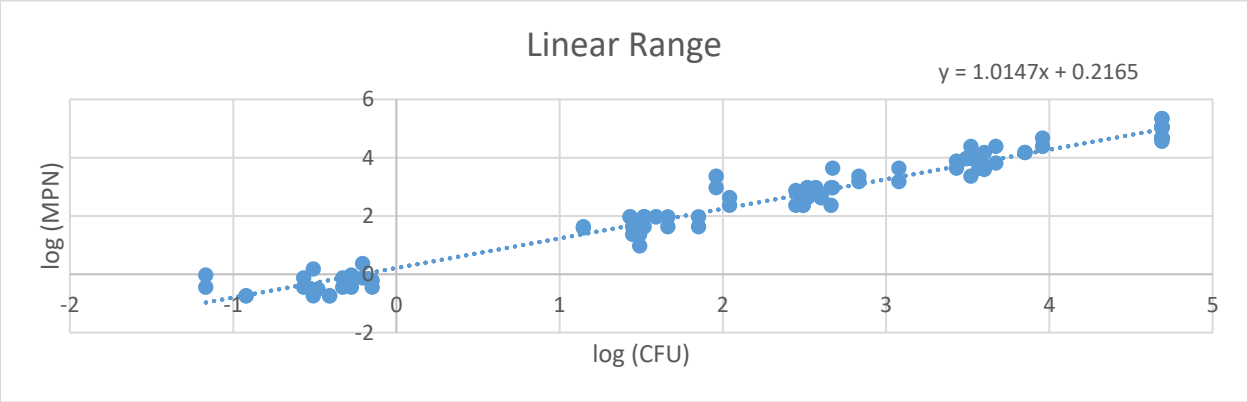
Purpose/Method

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. spledidus	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

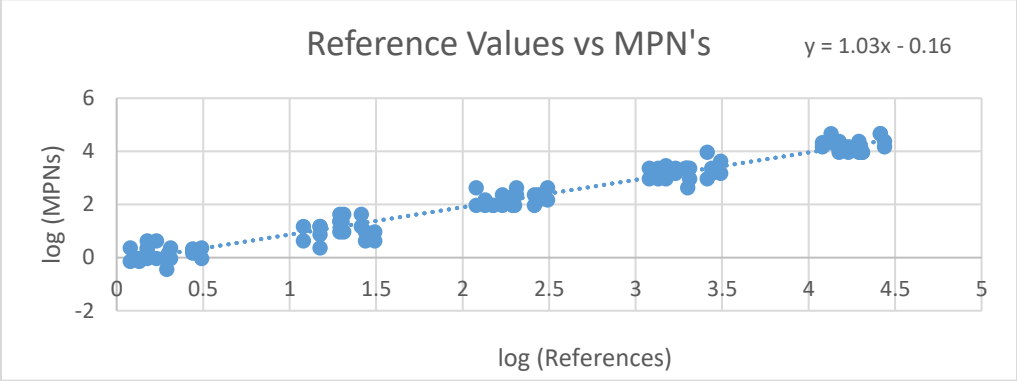
Purpose/Method

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					

Proposal No. 19-128

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Gina Olson
3. Affiliation	Washington State Dept of Health
4. Address Line 1	1610 NE 150th Street
5. Address Line 2	
6. City, State, Zip	Shoreline, WA 98155
7. Phone	206-418-5606
8. Fax	206-364-0072
9. Email	Gina.olson@doh.wa.gov
10. Proposal Subject	Laboratory Method for *Vibrio parahaemolyticus* and *Vibrio vulnificus* Enumeration and Detection Through MPN and Real-Time PCR
11. Specific NSSP Guide Reference	Section IV Guidance Documents Chapter II Growing Areas .14 Approved NSSP Laboratory Tests
12. Text of Proposal/ Requested Action	5. Approved Methods fir Vibrio Enumeration

	Vibrio Type:	**Application: PHP Sample Type: Shucked**	**Application: Reopening**
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
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	X
Direct Plating Method[8]	***Vibrio parahaemolyticus (V.p.)***	x	X

Footnotes:

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

	[2] 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. [3] 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. [4] 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.
13. 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.
14. 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.

Proposal No. 19-129

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Leonora Porter- Spokesperson
3. Affiliation	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
4. Address Line 1	205 N. Belle Mead Road
5. Address Line 2	Suite 1
6. City, State, Zip	East Setauket, NY 11733
7. Phone	(631) 444-0487
8. Fax	(631) 444-0472
9. Email	leonora.porter@dec.ny.gov
10. Proposal Subject	Micropipettor Verification
11. 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 Checklists, NSSP Laboratory Evaluation Checklists, 2. Shellfish Laboratory Evaluation Checklist for Mouse Bioassay (MBA) and Scotia Rapid Test for PSP.
12. Text of Proposal/ Requested Action	The requested action is to adopt the new text to be consistent across checklists for the NSSP MBS and Scotia Rapid Test (SRT) for PSP under Part III, Section 3.1, Screening by SRT item 3.1.7.
13. Public Health Significance	Quality Assurance and Standardization are integral to the validity of the NSSP laboratory. This includes verifying the measurement accuracy of pipetting instruments including micropipettors. There are no recognized references that state micropipettors must receive third party certifications. There is no indication as to what "Level" calibration should exist. The reference for this item is only **#2, Good Laboratory Practice**. Accuracy measurement assurance should be based on workload and use. Pipette calibration values on certificates obtained in a calibration laboratory (known as a controlled laboratory) do not accurately transfer to the NSSP laboratory and therefore do not provide assurance and defensibility. A pipette's measurement accuracy is influenced by its *physical uncertainty*, *environmental uncertainty* (i.e., temperature, vibration and humidity) and *operator use uncertainty*. These uncertainties will differ between laboratories. Pipette performance in the NSSP (non-controlled laboratories) is impacted by the temperature and viscosity of the fluid, the skill of the operator and choice of tip. Conducting in-house verifications for each operator, using a verified balance provides a better assessment of the actual measurement accuracy of what the pipet is delivering. When the uncertainty of measurement exceeds the stated laboratory established threshold, adjustments are made. As a component of a Laboratory's Quality Management System, the individual laboratory can institute legally defensible and measurement assurance practices appropriate for the laboratory's workload, testing and ambient conditions. Calibration Cost Information from one Pipet Manufacturer: 1. Calibration and Maintenance - Offers three "levels" of examination, with an

	assorted number of readings at 3 volumes, across different channel pipettors. Cost Range $30 - $225 per unit. 2. Calibration only (center channel only) - $30 - $180 if unit passed on the initial attempt. 3. Non-Operational pipette repair evaluation (no calibration and parts additional cost) starting at $28/unit.
14. Cost Information	N/A

Laboratory Evaluation Checklist – Mouse Bioassay and Scotia Rapid Test for Paralytic Shellfish Poisoning (PSP)

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 301-436-2672

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 "√"

Mouse Bioassay Assay (MBA) and Scotia Rapid Test (SRT)for Paralytic Shellfish Poisoning (PSP)			
PART I - Quality Assurance			
Code	**REF**	**Item Description**	
		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. 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 1or 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% bromthymol 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, 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 - 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 (*circle the 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 are allowed 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 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	**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 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**		**2.3.6 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 – Examination of Shellfish for PSP Toxins – 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~~ micropipettors are maintained and ~~calibrated~~ verified according to manufacturer's instructions and laboratory workload needs. Adjustment ~~R~~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:

1. Adams, W.N. and S.A Furfari. 1984. Evaluation of laboratory performance of the AOAC method for PSP toxin in shellfish. *J. Assoc. Off. Anal. Chem*. Vol 67, 6:1147-1148.
2. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
3. American Public Health Association. 1984. *Compendium for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
4. Consult freezer product literature.
5. APHA/WEF/AWWA. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA, Washington, D.C.
6. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
7. Fisher, J. 1985. Measurement of pH. *American Laboratory.* 16:54-60.
8. National Research Council. 1996. *Guide for the Care and Use of Laboratory Animals*. National Academy Press, Washington, D.C.
9. Good Laboratory Practice
10. U.S. Department of 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.
12. Consult pH electrode product literature.
13. Consult SRT manufacturer instruction manual / literature
14. Personal Communication with Dr. Sherwood Hall, USFDA.
15. Wilt, D.S. (ed). 1974. Proceedings of the 8th National Shellfish Sanitation Workshop. U.S. Food and Drug Administration, Washington, D.C.
16. Artel Lab Report eBook – your guide to liquid handling data quality. By Artel | Lab Report. 25 Bradley Drive, Westbrook, Maine 04092. Accessed June 5, 2019.

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:_____

Proposal No. 19-130

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. **X** Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Leonora Porter - Spokesperson
3. Affiliation	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
4. Address Line 1	205 N. Belle Mead Road
5. Address Line 2	Suite 1
6. City, State, Zip	East Setauket, NY 11733
7. Phone	(631) 444-0487
8. Fax	(631) 444-0472
9. Email	leonora.porter@dec.ny.gov
10. Proposal Subject	Microbiology Laboratory Evaluation Checklist- Standards Thermometer
11. 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 Checklists, 1. NSSP Laboratory Evaluation Checklist for Microbiology
12. Text of Proposal/ Requested Action	The requested action is to adopt modified standards thermometer language to correct checklist inconsistencies in Section 1.4 Laboratory Equipment item 1.4.21.
13. Public Health Significance	All standards thermometers allowed for in section 1.4.23, not just mercury-in-glass thermometers, should be calibrated and traceable to NIST at the points of use.
14. Cost Information	Cost of calibration.

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
☐	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	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 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 months of experience in laboratory sciences.
		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	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 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 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	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	**29**	☐	**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**	☐	**1.4.21**	**A ~~mercury-in-glass~~ 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13	☐	1.4.24	Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 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	**11, 30**	☐	**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	11	☐	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	16	☐	1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11	☐	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	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 in the range of 160 to 180°C.
K	9	☐	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	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 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 are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22**	**The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11	☐	1.7.9	Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**	☐	**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13**	**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.**

O	9	☐	1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2	☐	1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**	☐	**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**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	☐	2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**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	☐	2.2.9	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.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	2.3.4	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**	☐	**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**2.3.9**	**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, 31**	☐	**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**	☐	**2.5.8**	**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**	☐	**2.5.9**	**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**	☐	**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**2.5.11**	**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**	☐	**2.5.12**	**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 this 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 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	**2**	☐	**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**23**	☐	**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**2.9.5**	**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**	☐	**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26	☐	2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8**	**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**	☐	**2.9.9**	**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**	☐	**2.9.10**	**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**	☐	**2.9.11**	**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**	☐	**2.9.12**	**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 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**	☐	**2.9.13**	**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.**
				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	2,11	☐	3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2	Blades of shucking knives are not corroded.
O	9	☐	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	☐	3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9	☐	3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**	☐	**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9	☐	3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**	☐	**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	3.2.15	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.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**3.3.5**	**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	☐	3.3.6	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**	☐	**3.3.7**	**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	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	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.**
C	**2**	☐	**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	3.4.3	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**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9	☐	3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**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 may be performed in conjunction with the

				analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen 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 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**	☐	**3.8.8**	**Six 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 plates.
C	**1**	☐	**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**	☐	**3.8.13**	**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**	☐	**3.8.14**	**Plates are stacked no more than three high in the incubator.**
C	**2**	☐	**3.8.15**	**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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	30	☐	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 100 – 125 mL.
C	**27, 28**	☐	**3.10.2**	**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9	☐	3.10.3	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4**	**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5	The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6	The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**	☐	**3.10.8**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**	☐	**3.10.9**	**Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28	☐	3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28	☐	3.11.2	Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3	Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**	☐	**3.11.4**	**The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5	Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	3.11.6	Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28	☐	3.11.7	The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8	Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9	Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis		
K	2,11	☐	3.12.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2	The blades of shucking knives are not corroded.
O	9	☐	3.12.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.12.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	9	☐	3.12.5	The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.

O	9	☐	3.12.6	The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8**	**Shellfish are not shucked through the hinge.**
C	**9**	☐	**3.12.9**	**The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	3.12.10	A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	3.12.11	The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis		
C	**28**	☐	**3.13.1**	**E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2	Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3	Several host cell colonies are transferred to a tube of tempered, aerated 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**	☐	**3.13.4**	**After inoculation, the host cell growth broth culture is not shaken.**
C	**28**	☐	**3.13.5**	**A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6**	**The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7**	**The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**	☐	**3.13.8**	**Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**	☐	**3.13.9**	**The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10**	**The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**	☐	**3.13.11**	**The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13	Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14	The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15**	**2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**	☐	**3.13.16**	**The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17**	**The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18**	**Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19	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	☐	3.13.20	Growth broth is used as the negative control or blank.
K	27, 28	☐	3.13.21	Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	3.13.22	A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28	☐	3.13.23	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.

C	**27, 28**	☐	**3.13.24**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC		
C	**27**	☐	**3.14.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2**	**The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count 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	☐	3.14.3	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	9	☐	3.14.4	The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621*. PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC

24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8 (1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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. October 2015

Proposal No. 19-131

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Leonora Porter - Spokesperson
3. Affiliation	NELEOM – Northeast Laboratory Evaluation Officers and Managers
4. Address Line 1	205 N. Belle Mead Road
5. Address Line 2	Suite #1
6. City, State, Zip	East Setauket, New York, 11733
7. Phone	631-444-0487
8. Fax	631-444-0472
9. Email	leonora.porter@dec.ny.gov
10. Proposal Subject	NSSP Microbiology Laboratory Evaluation Checklist – Reagent Water Quality
11. 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 Checklists, 1. NSSP Laboratory Evaluation Checklist for Microbiology.
12. Text of Proposal/ Requested Action	The requested action is to adopt the modified text and update the reference in Section 1.7 Media Preparation for checklist item 1.7.6.
13. Public Health Significance	The suggested change addresses the importance of accurate information used in laboratory Quality Assurance Programs (QAPs) for recommended limits for the quality of reagent water used for microbiology testing by correcting the maximum acceptable limits for conductivity and resistivity testing based on the most current ***Standard Methods*** Edition. For 26 years, the incorrect units of measure for conductivity and resistivity have been printed in laboratory reference materials: ***Standard Methods for the Examination of Water and Wastewater***, 1992, 18th Edition; ***Standard Methods***, 2012, 22nd Edition; and ***Standard Methods***, 2017, 23rd Edition. The QA information is finally corrected in the ERRATA, dated 5/29/18 for ***Standard Methods*** 23rd Edition. The material states "In Section 9020, Table 9020:II (p. 9-14), the recommended Maximum Acceptable Limit for Conductivity Test should be "<2 µmhos/cm (µSiemens/cm) at 25°C." The incorrect "resistance" statement from the 18th Edition is removed in the 22nd and 23rd Editions of ***Standard Methods***. The resistivity (also called specific resistance) is the reciprocal of the conductivity, not resistance. A resistivity recommendation can be found in the Reagent Grade Water section.
14. Cost Information	N/A

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
☐	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	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 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 months of experience in laboratory sciences.
		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	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 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 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	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	**29**	☐	**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**	☐	**1.4.21**	**A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13	☐	1.4.24	Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 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	**11, 30**	☐	**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	11	☐	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	16	☐	1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11	☐	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	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 in the range of 160 to 180°C.
K	9	☐	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	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 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 are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22**	**The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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~~33	☐	**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)~~ for mixed-bed deionizers resistivity (measeured in-line) should be >10 megohm-cm at 25°C 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	11	☐	1.7.9	Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**	☐	**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13**	**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.
O	9	☐	1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2	☐	1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**	☐	**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**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	☐	2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**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	☐	2.2.9	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.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	2.3.4	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**	☐	**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**2.3.9**	**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, 31**	☐	**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**

C	**6**	☐	**2.5.8**	**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**	☐	**2.5.9**	**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**	☐	**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**2.5.11**	**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**	☐	**2.5.12**	**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 this 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 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	**2**	☐	**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**23**	☐	**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**2.9.5**	**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**	☐	**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26	☐	2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8**	**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**	☐	**2.9.9**	**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**	☐	**2.9.10**	**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**	☐	**2.9.11**	**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**	☐	**2.9.12**	**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 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**	☐	**2.9.13**	**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.**
			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	2,11	☐	3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2	Blades of shucking knives are not corroded.
O	9	☐	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	☐	3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9	☐	3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**	☐	**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9	☐	3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.

C	**9**	☐	**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	3.2.15	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.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**3.3.5**	**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	☐	3.3.6	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**	☐	**3.3.7**	**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	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	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.**
C	**2**	☐	**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	3.4.3	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**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9	☐	3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen 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 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**	☐	**3.8.8**	**Six 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 plates.
C	**1**	☐	**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**	☐	**3.8.13**	**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**	☐	**3.8.14**	**Plates are stacked no more than three high in the incubator.**

C	**2**	☐	**3.8.15**	**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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	30	☐	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 100 – 125 mL.
C	**27, 28**	☐	**3.10.2**	**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9	☐	3.10.3	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4**	**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5	The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6	The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**	☐	**3.10.8**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**	☐	**3.10.9**	**Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28	☐	3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28	☐	3.11.2	Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3	Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**	☐	**3.11.4**	**The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5	Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	3.11.6	Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28	☐	3.11.7	The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8	Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9	Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis		
K	2,11	☐	3.12.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2	The blades of shucking knives are not corroded.
O	9	☐	3.12.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.12.4	The faucet used for rinsing the shellfish does not contain an aerator.

K	9	☐	3.12.5	The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.12.6	The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8**	**Shellfish are not shucked through the hinge.**
C	**9**	☐	**3.12.9**	**The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	3.12.10	A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	3.12.11	The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis		
C	**28**	☐	**3.13.1**	**E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2	Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3	Several host cell colonies are transferred to a tube of tempered, aerated 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**	☐	**3.13.4**	**After inoculation, the host cell growth broth culture is not shaken.**
C	**28**	☐	**3.13.5**	**A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6**	**The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7**	**The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**	☐	**3.13.8**	**Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**	☐	**3.13.9**	**The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10**	**The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**	☐	**3.13.11**	**The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13	Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14	The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15**	**2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**	☐	**3.13.16**	**The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17**	**The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18**	**Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19	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	☐	3.13.20	Growth broth is used as the negative control or blank.
K	27, 28	☐	3.13.21	Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	3.13.22	A negative control plate is plated at the beginning and end of each set of samples analyzed.

K	27, 28	☐	3.13.23	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**3.13.24**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC		
C	**27**	☐	**3.14.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2**	**The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count 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	☐	3.14.3	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	9	☐	3.14.4	The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621*. PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.

23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8 (1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.
~~32.~~33. American Public Health Association (APHA). 2017. ***Standard Methods for the Examination of Water and Wastewater.*** 23rd Edition. APHA/AWWA/WEF. Errata (dated 5/29/2018) and Pg. 1-49.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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. October 2015

Proposal No. 19-132

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Leonora Porter, Spokesperson
3. Affiliation	NELEOM – Northeast Laboratory Evaluation Officers and Managers
4. Address Line 1	205 N. Belle Mead Road
5. Address Line 2	Suite #1
6. City, State, Zip	East Setauket, New York, 11733
7. Phone	631-444-0487
8. Fax	631-444-0472
9. Email	leonora.porter@dec.ny.gov
10. Proposal Subject	Microbiology Laboratory Evaluation Checklist - Working Thermometers
11. 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 Checklists, 1. NSSP Laboratory Evaluation Checklist for Microbiology
12. Text of Proposal/ Requested Action	The requested action is to adopt the modified text of the NSSP microbiology checklist, section 1.4 Laboratory Equipment, item 1.4.24:
13. Public Health Significance	The laboratory's goal is to ensure high-quality data using accepted scientific practices. The designated changes incorporate recommended best practices from a current recognized scientific publication. These types of acknowledged practices are used to develop a laboratory's Quality Assurance Program (QAP). The *verification* of working thermometers is now suitably referenced to support past and present practices in program laboratories and *recommends a rejection component (new)*. The newer/current reference material is cited to strengthen confidence in the acceptability of past practices for "checking" accuracy in working temperature monitoring devices. ***Standard Methods,*** 23rd Edition, states "Annually, or preferably semiannually, **verify** the accuracy of all working temperature-sensing devices (e.g., liquid-in-glass thermometers, thermocouples, and temperature-recording instruments) at the use temperature(s). To do this, compare each device's measurements to those of a certified NIST temperature-sensing device or one traceable to NIST and conforming to NIST specifications. Discard temperature-sensing devices that differ by >1ºC from the reference device."
14. Cost Information	N/A

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
☐	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	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 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 months of experience in laboratory sciences.
		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	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 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 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	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	**29**	☐	**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**	☐	**1.4.21**	**A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13, 33	☐	1.4.24	Incubator and water bath working thermometers are ~~checked~~ verified annually against the standards thermometer at the temperatures at which they are used. Discard working temperature-sensing devices that differ by >1ºC 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 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	**11, 30**	☐	**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	11	☐	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	16	☐	1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11	☐	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	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 in the range of 160 to 180°C.
K	9	☐	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	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 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 are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22**	**The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11	☐	1.7.9	Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**	☐	**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13**	**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.
O	9	☐	1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2	☐	1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**	☐	**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**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	☐	2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**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	☐	2.2.9	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.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	2.3.4	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**	☐	**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**2.3.9**	**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, 31**	☐	**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**

C	**6**	☐	**2.5.8**	**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**	☐	**2.5.9**	**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**	☐	**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**2.5.11**	**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**	☐	**2.5.12**	**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 this 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 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	**2**	☐	**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**23**	☐	**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**2.9.5**	**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**	☐	**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26	☐	2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8**	**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**	☐	**2.9.9**	**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**	☐	**2.9.10**	**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**	☐	**2.9.11**	**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**	☐	**2.9.12**	**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 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**	☐	**2.9.13**	**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.**
			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	2,11	☐	3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2	Blades of shucking knives are not corroded.
O	9	☐	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	☐	3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9	☐	3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**	☐	**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9	☐	3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.

C	**9**	☐	**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	3.2.15	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.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**3.3.5**	**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	☐	3.3.6	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**	☐	**3.3.7**	**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	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	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.**
C	**2**	☐	**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	3.4.3	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**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9	☐	3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen 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 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**	☐	**3.8.8**	**Six 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 plates.
C	**1**	☐	**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**	☐	**3.8.13**	**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**	☐	**3.8.14**	**Plates are stacked no more than three high in the incubator.**

C	**2**	☐	**3.8.15**	**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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	30	☐	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 100 – 125 mL.
C	**27, 28**	☐	**3.10.2**	**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9	☐	3.10.3	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4**	**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5	The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6	The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**	☐	**3.10.8**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**	☐	**3.10.9**	**Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28	☐	3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28	☐	3.11.2	Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3	Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**	☐	**3.11.4**	**The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5	Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	3.11.6	Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28	☐	3.11.7	The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8	Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9	Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis		
K	2,11	☐	3.12.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2	The blades of shucking knives are not corroded.
O	9	☐	3.12.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.12.4	The faucet used for rinsing the shellfish does not contain an aerator.

K	9	☐	3.12.5	The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.12.6	The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8**	**Shellfish are not shucked through the hinge.**
C	**9**	☐	**3.12.9**	**The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	3.12.10	A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	3.12.11	The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis		
C	**28**	☐	**3.13.1**	**E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2	Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3	Several host cell colonies are transferred to a tube of tempered, aerated 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**	☐	**3.13.4**	**After inoculation, the host cell growth broth culture is not shaken.**
C	**28**	☐	**3.13.5**	**A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6**	**The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7**	**The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**	☐	**3.13.8**	**Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**	☐	**3.13.9**	**The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10**	**The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**	☐	**3.13.11**	**The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13	Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14	The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15**	**2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**	☐	**3.13.16**	**The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17**	**The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18**	**Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19	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	☐	3.13.20	Growth broth is used as the negative control or blank.
K	27, 28	☐	3.13.21	Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	3.13.22	A negative control plate is plated at the beginning and end of each set of samples analyzed.

K	27, 28	☐	3.13.23	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**3.13.24**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC		
C	**27**	☐	**3.14.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2**	**The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count 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	☐	3.14.3	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	9	☐	3.14.4	The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621*. PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.

23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8 (1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.
~~32.~~33. #33. American Public Health Association (APHA). 2017. ***Standard Methods for the Examination of Water and Wastewater***, 23rd Edition, APHA/AWWA/WEF. Pg. 9-8.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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. October 2015

Proposal No. 19-133

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Leonora Porter - Spokesperson
3. Affiliation	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
4. Address Line 1	205 N. Belle Mead Road
5. Address Line 2	Suite 1
6. City, State, Zip	East Setauket, NY 11733
7. Phone	(631) 444-0487
8. Fax	(631) 444-0472
9. Email	leonora.porter@dec.ny.gov
10. Proposal Subject	Microbiology & PCR Laboratory Evaluation Checklists - Working Thermometers
11. 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 Checklists, NSSP Laboratory Evaluation Checklists
12. Text of Proposal/ Requested Action	The requested action is to adopt modified working thermometer language for these two NSSP laboratory evaluation checklists items. The modification is to remove the word "calibrated" and add thermometer accuracy requirements.
13. Public Health Significance	There are currently no NSSP accuracy criteria established for Liquid-in-Glass thermometers. This proposal establishes uncertainty requirements that should be considered prior to purchase since all thermometers and temperature recording devices are not created equally. Quality Assurance and Standardization are integral to the validity of the NSSP laboratory. For thermometers there are several factors that influence temperature readings; therefore, controlling thermometer accuracy will impact thermometer standardization across NSSP laboratories. A thermometer's accuracy is a product of its *manufacturing uncertainty*, *measurement uncertainty* and *environmental uncertainty* which all must be considered and evaluated by the purchaser. Only thermometers that are manufactured accurately and are found *fit for purpose* for the NSSP laboratory should be purchased. Some Liquid-in-Glass thermometers are manufactured with accuracies (> 0.2ºC) that are greater than the water bath temperature limit of ±0.2°C; these thermometers should not be purchased for the NSSP laboratory. As stated in Reference #4, NIST Monograph 150 "the accuracy attainable is principally limited by the characteristics of the thermometer itself." Therefore, a working thermometer's accuracy should be assessed prior to purchase. Calibration is performed post purchase. *Calibration quantifies <u>only</u> the temperature **measurement uncertainty** at the single temperature point assessed.* Calibration without also considering the ***manufacturing uncertainties*** of the thermometer is inaccurate: generating a false security for accuracy. Calibration values are only accurate at the environmental conditions found within the calibration laboratory; when total immersion thermometers are immersed to the

	test temperature being measured with the emergent stem at ambient temperature. In the NSSP laboratory, the emergent stem <u>is not</u> at ambient temperature. This creates ***environmental uncertainty*** which invalidates the calibration certificate and requires experience and knowledge in generating an accurate stem correction. An inaccurate stem correction compounds the degree of error in the final temperature reading. The current NSSP practice of calibrating an inappropriate thermometer against the undefined calibration standard (NIST, ASTM, Primary, Secondary, etc) and then using this thermometer incorrectly in the laboratory environment negates any assurance received by having a calibration certificate. This practice would not be legally defensible. NSSP Quality Assurance and Standardization would be better served to establish manufacturing accuracy requirements that only allow for the use of appropriate working thermometers. *These working thermometers will then be verified against a calibrated standards thermometer, that is traceable to NIST in section 1.4.24.* <u>Savings</u>: Calibration costs <u>per thermometer</u>: $125 for the first point and $60 for each additional point. Most lab are locked into local calibration facilities, within driving distance of their labs, if their thermometers are mercury. Postal hazard restrictions prohibit mercury thermometers being shipped in the mail.
14. Cost Information	none

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
☐	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	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 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 months of experience in laboratory sciences.
		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	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 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 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	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	**29, 33**	☐	**1.4.20**	**Working thermometers are either: ~~calibrated~~ mercury-in-glass thermometers, ~~calibrated~~ non-mercury-in-glass thermometers having the accuracy and tolerance of mercury, or ~~appropriately calibrated~~low drift electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs)with an accurscy of less than or equal to ≤ ±0.05°C.**
C	**11**	☐	**1.4.21**	**A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13	☐	1.4.24	Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 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	**11, 30**	☐	**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	11	☐	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	16	☐	1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11	☐	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	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 in the range of 160 to 180°C.
K	9	☐	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	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 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 are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22**	**The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11	☐	1.7.9	Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**	☐	**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13**	**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.
O	9	☐	1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2	☐	1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**	☐	**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**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	☐	2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**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	☐	2.2.9	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.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	2.3.4	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**	☐	**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**2.3.9**	**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, 31**	☐	**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**

C	**6**	☐	**2.5.8**	**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**	☐	**2.5.9**	**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**	☐	**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**2.5.11**	**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**	☐	**2.5.12**	**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 this 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 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	**2**	☐	**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**23**	☐	**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**2.9.5**	**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**	☐	**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26	☐	2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8**	**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**	☐	**2.9.9**	**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**	☐	**2.9.10**	**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**	☐	**2.9.11**	**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**	☐	**2.9.12**	**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 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**	☐	**2.9.13**	**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.**
			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	2,11	☐	3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2	Blades of shucking knives are not corroded.
O	9	☐	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	☐	3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9	☐	3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**	☐	**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9	☐	3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.

C	**9**	☐	**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	3.2.15	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.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**3.3.5**	**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	☐	3.3.6	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**	☐	**3.3.7**	**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	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	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.**
C	**2**	☐	**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	3.4.3	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**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9	☐	3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen 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 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**	☐	**3.8.8**	**Six 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 plates.
C	**1**	☐	**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**	☐	**3.8.13**	**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**	☐	**3.8.14**	**Plates are stacked no more than three high in the incubator.**

C	**2**	☐	**3.8.15**	**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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	30	☐	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 100 – 125 mL.
C	**27, 28**	☐	**3.10.2**	**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9	☐	3.10.3	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4**	**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5	The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6	The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**	☐	**3.10.8**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**	☐	**3.10.9**	**Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28	☐	3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28	☐	3.11.2	Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3	Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**	☐	**3.11.4**	**The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5	Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	3.11.6	Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28	☐	3.11.7	The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8	Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9	Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis		
K	2,11	☐	3.12.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2	The blades of shucking knives are not corroded.
O	9	☐	3.12.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.12.4	The faucet used for rinsing the shellfish does not contain an aerator.

K	9	☐	3.12.5	The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.12.6	The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8**	**Shellfish are not shucked through the hinge.**
C	**9**	☐	**3.12.9**	**The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	3.12.10	A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	3.12.11	The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis		
C	**28**	☐	**3.13.1**	**E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2	Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3	Several host cell colonies are transferred to a tube of tempered, aerated 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**	☐	**3.13.4**	**After inoculation, the host cell growth broth culture is not shaken.**
C	**28**	☐	**3.13.5**	**A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6**	**The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7**	**The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**	☐	**3.13.8**	**Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**	☐	**3.13.9**	**The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10**	**The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**	☐	**3.13.11**	**The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13	Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14	The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15**	**2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**	☐	**3.13.16**	**The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17**	**The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18**	**Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19	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	☐	3.13.20	Growth broth is used as the negative control or blank.
K	27, 28	☐	3.13.21	Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	3.13.22	A negative control plate is plated at the beginning and end of each set of samples analyzed.

K	27, 28	☐	3.13.23	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**3.13.24**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC		
C	**27**	☐	**3.14.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2**	**The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count 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	☐	3.14.3	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	9	☐	3.14.4	The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621*. PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.

23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8 (1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.
~~32.~~33. NIST Monograph 150 states “the accuracy attainable is principally limited by the characteristics of the thermometer itself.”

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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. October 2015

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

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

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast

PART I – Quality Assurance			
ITEM			
CODE	**REF**		
		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, 23**		**1.4.18 Working thermometers are either: ~~calibrated~~ mercury-in-glass thermometers, ~~calibrated~~ non-mercury-in-glass thermometers having the accuracy and tolerance of mercury, or ~~appropriately calibrated~~low drift electronic devices, including Resistance Temperature Devises (RTDs) and Platinum Resistance Devices (PTDs) with an accuracy of less than or equal to ≤ ±0.05°C.**
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 heterotropic 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'-GATGAGCGGTTGATGTCCAAA-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
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.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. U.S. Department of Commerce. 1976. *NBS Monograph 150*. U.S. Department of Commerce, Washington, D.C.
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.
8. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition. APHA, Washington, D.C.
9. Fisher, J. 1985. Measurement of pH. American Laboratory 16:54 – 60.
10. Association of Official Analytical Chemists (AOAC). 1999. AOAC *Methods Validation and Technical Programs – Criteria for Laboratories Performing Food Testing*. AOAC, Arlington, Va.
11. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA – 670/9-75-006. U.S. EPA, Cincinnati, Ohio.
12. Adams, W.N. 1974. NETSU. Personal Communication to Dr. Wallace Andrews, FDA.
13. U.S. Food and Drug Administration (FDA). 1995. *Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
14. Campbell, Mark, S. and Wright, Anita, C. Real-time PCR analysis of *Vibrio vulnificus* from oysters, Appl Environ Microbiol. 69, 12 (2003).
15. Wright, Anita, C., Garrido, V, Debuex, G, Farrell-Evans, M, Mudbidri, A, A. and Otwell, W, S. Appl Environ Microbiol. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. 73, 22 (2007).
16. Integrated DNA Technologies. Oligonucleotide Stability Study. 2014.
17. Section IV Guidance Documents, Naturally Occurring Pathogens, NSSP *Guide for the Control of Molluscan Shellfish*, 2009 Revision.
18. Nordstrom, J.L., M.C.L. Vickery, G.M. Blackstone, S.L. Murray, and A. DePaola. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic V. parahaemolyticus bacteria in oysters. Appl. Environ. Microbiol. 73(18):5840-5847.
19. Kinsey, T.P., K.A. Lydon, J.C. Bowers, J.L. Jones. 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): 1574-1580.
20. National Institute of Standards and Technology Special Publication 250-23, 128 pages (Sept. 1988) U.S. Government Printing office, Washington, D.C. Library of Congress Catalog Number: 88-6000580.
21. Integrated Solutions — Real-Time PCR Applications: Critical Factors for Successful Real-Time PCR. www.qiagen.com
22. FDA Reagent Stability Study, unpublished. 2013.
23. NIST Monograph 150 states "the accuracy attainable is principally limited by the characteristics of the thermometer itself."

LABORATORY:	**DATE of EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY of NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	

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**

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:_____

LABORATORY:

Page	Item	Observation

Proposal No. 19-134

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	J. Michael Hickey, Jeff Kennedy, Diane Regan
3. Affiliation	Massachusetts Division of Marine Fisheries
4. Address Line 1	84 82nd Street
5. Address Line 2	
6. City, State, Zip	Newburyport, MA 01950
7. Phone	978-465-3553
8. Fax	978-465-5947
9. Email	Michael.Hickey@mass.gov
10. Proposal Subject	Membrane Filtration Technique for Seawater using mEndo Agar LES Checklist
11. 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 Checklists , NSSP Laboratory Evaluation Checklists, NSSP Laboratory Evaluation Checklist for Microbiology
12. Text of Proposal/ Requested Action	The Requested Action is to adopt the attached checklist for the Membrane Filtration Technique for Seawater using mEndo Agar LES and to append the NSSP Laboratory Evaluation Checklist for Microbiology found at the end of section .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists to include this checklist.
13. Public Health Significance	The NSSP does not have a checklist for Total Coliform analysis on UV Seawater using the NSSP approved method of Membrane Filtration with mEndo Agar LES. Checklists provide quality assurance and method support for laboratories and for Laboratory Evaluation Officers to standardize and evaluate laboratories which use approved methods in support of the NSSP. The attached checklist for this NSSP approved method provides such standardization, quality assurance and background documentation for method procedures. As a laboratory evaluation tool with critical and key codes identified it will be used for determination of laboratory conformance and compliance.
14. Cost Information	none

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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 Seawater 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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	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 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 months of experience in laboratory sciences.
		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	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 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 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	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	**29**	☐	**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**	☐	**1.4.21**	**A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used*.)**
K	13	☐	1.4.24	Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 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	**11, 30**	☐	**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	11	☐	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	16	☐	1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11	☐	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	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 in the range of 160 to 180°C.
K	9	☐	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	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 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 are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22**	**The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11	☐	1.7.9	Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**	☐	**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13**	**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.**
O	9	☐	1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2	☐	1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**	☐	**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**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	☐	2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**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	☐	2.2.9	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.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	2.3.4	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**	☐	**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**2.3.9**	**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, 31**	☐	**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**	☐	**2.5.8**	**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**	☐	**2.5.9**	**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**	☐	**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**

C	**5**	☐	**2.5.11**	**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**	☐	**2.5.12**	**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 this 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 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	**2**	☐	**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**23**	☐	**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**2.9.5**	**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**	☐	**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26	☐	2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8**	**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**	☐	**2.9.9**	**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**	☐	**2.9.10**	**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**	☐	**2.9.11**	**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**	☐	**2.9.12**	**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 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**	☐	**2.9.13**	**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 Seawater by Membrane Filtration (MF) using mEndo Agar LES- Materials and Equipment
C	**9, 11, 19, 21**	☐	**2.11.1 The temperature of the air incubator is maintained at 35.0 +0.5°C under any loading capacity.**
K	**9, 11, 21**	☐	2.11.2 A high level of humidity is maintained in the incubator (at least 60% relative humidity).
C	**9, 11, 21**	☐	**2.11.3 Pre-sterilized plastic or sterile glass culture plates that are clear, flat bottomed, free of bubbles and scratches are used.**
C	**2**	☐	**2.11.4 The sterility of pre-sterilized culture plates is determined for each lot received. Results are recorded and the records maintained.**
K	9, 11, 19, 21	☐	2.11.5 Colonies are counted with the aid of magnification.
C	**11, 19,21**	☐	**2.11.6 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 coliform analysis.**
K	2	☐	2.11.7 Lot number, date of receipt and if provided the expiration date on the membrane filters are recorded and the records maintained.
C	**2**	☐	**2.11.8 When initiating monitoring by mEndo Agar LES 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 comparison testing implemented. The results are recorded and this record is maintained.**
C	2, 11	☐	**2.11.9 New lots of membrane filters are checked by comparing recovery of fecal coliform organisms against membrane filters from previously acceptable lots.**
K	2	☐	2.11.10 The sterility of each lot or autoclave batch of membrane filters is checked before use.
K	2	☐	2.11.11 Membrane filters which are beyond their expiration date are not used.
O	9, 11, 21	☐	2.11.12 Forceps tips are clean.
O	9, 11	☐	2.11.13 Forceps tips are smooth without pitting or corrugations to damage the filters being manipulated.
K	9, 11, 21	☐	2.11.14 Forceps are dipped in alcohol and flame sterilized between sample filters.
K	11	☐	2.11.15 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	9, 11, 19,21	☐	2.11.16 Membrane filtration units are made of stainless steel, glass or autoclavable plastic free of scratches, corrosion and leaks.
C	**9, 11**	☐	**2.11.17 Membrane filter assemblies are autoclave sterilized for 15 minutes at 121°C prior to the start of a filtration series. A new series occurs when there is a break of 30 minutes or more between the last filtration series.**
O	11,19,26	☐	2.11.18 A UV sterilization unit is used to disinfect filter assemblies between sample and filtration runs.
K	11	☐	2.11.19 The effectiveness of the UV sterilization unit is determined by biological testing monthly. Results are recorded and records maintained.
K	2	☐	2.11.20 Maintenance of the UV sterilization unit is performed as needed. This

			maintenance is documented and records maintained.
		2.12 Media Preparation and Storage - MF using mEndo Agar LES	
K	9, 11, 21	☐	2.12.1 Phosphate buffered water is used as the sample diluent and filter funnel rinse.
C	**9, 11, 21**	☐	**2.12.2 The phosphate buffered water is properly sterilized.**
C	**9, 11, 19, 21**	☐	**2.12.3 mEndo Agar LES is used.**
C	**11, 21**	☐	**2.12.4 The media is prepared under sterile conditions using presterilized glassware, sterile distilled water and presterilized stir bar.**
K	9, 11	☐	2.12.5 The media is prepared using 95% alcohol that is not denatured.
C	**9, 11, 21**	☐	**2.12.6 The media is brought just to boiling. It is never autoclaved.**
K	9, 11, 21	☐	2.12.7 The media is then tempered to 45 to 50°C.
K	9, 21	☐	2.12.8 A sufficient amount of medium (4-5 mL) is aseptically aliquotted to each culture plate.
O	9, 11, 21	☐	2.12.9 The prepared plates are stored at 4°C for no more than two (2) weeks in sealed plastic bags or containers to minimize evaporation. Exposure to light is minimized.
		2.13 Sample Analysis - MF using mEndo Agar LES	
C	**2**	☐	**2.13.1 Appropriate, properly diluted positive and negative productivity controls for mEndo Agar LES medium are used. The results are recorded and the records maintained.** **Positive productivity control _____** **Negative productivity control _____**
C	**9, 11, 21**	☐	**2.13.2 The sample is shaken vigorously (25 times in a 12 inch arc in 7 seconds) before filtration.**
K	11, 21	☐	2.13.3 The membrane is placed grid side up within the sterile filter apparatus.
C	**11**	☐	**2.13.4 The total sample volumes tested are not less than 100 mL.**
C	**9, 19, 21**	☐	**2.13.5 Sample volumes are filtered under vacuum.**
K	26	☐	2.13.6 The pressure of the vacuum pump does not exceed 15 psi.
C	**9, 11, 21, 26**	☐	**2.13.7 The sides of the filter funnel are rinsed at least twice with 20-30 mL of sterile phosphate buffered water after sample filtration.**
C	**9, 11, 21**	☐	**2.13.8 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	11	☐	2.13.9 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 water, filter funnels, forceps, membrane filters, media and culture plates.
C	**2, 11**	☐	**2.13.10 An appropriately diluted positive control process culture accompanies the samples throughout incubation. Results are recorded and the records maintained.** **Positive process control _____**
C	**9, 11, 19, 21**	☐	**2.13.11 Inoculated plates are incubated inverted at 35.0+0.5°C for 22 to 24 hours.**
		2.14 Computation of Results - MF using mEndo Agar LES	
C	**9, 11, 19, 21**	☐	**2.14.1 All metallic sheen colonies are counted and are considered to be total coliform.**

C	2	☐	2.14.2	When multiple aliquots of a sample are filtered, the laboratory has developed a procedure for assessing the contribution of all aliquots to the final total sample count.
C	9, 11, 21	☐	2.14.3	Results are reported as CFU/100 mL of sample.
			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	2,11	☐	3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2	Blades of shucking knives are not corroded.
O	9	☐	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	☐	3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9	☐	3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**	☐	**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9	☐	3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**	☐	**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	3.2.15	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.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN**

				series.
C	**9**	☐	**3.3.5**	**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	☐	3.3.6	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**	☐	**3.3.7**	**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	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	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.**
C	**2**	☐	**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained. Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	3.4.3	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**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9	☐	3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen 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 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**	☐	**3.8.8**	**Six 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 plates.
C	**1**	☐	**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**	☐	**3.8.13**	**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**	☐	**3.8.14**	**Plates are stacked no more than three high in the incubator.**
C	**2**	☐	**3.8.15**	**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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	30	☐	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 100 – 125 mL.
C	**27, 28**	☐	**3.10.2**	**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000**

				x g and maintain a temperature of 4°C.
K	9	☐	3.10.3	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4**	**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5	The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6	The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**	☐	**3.10.8**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**	☐	**3.10.9**	**Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28	☐	3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28	☐	3.11.2	Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3	Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**	☐	**3.11.4**	**The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5	Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	3.11.6	Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28	☐	3.11.7	The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8	Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9	Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis		
K	2,11	☐	3.12.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2	The blades of shucking knives are not corroded.
O	9	☐	3.12.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.12.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	9	☐	3.12.5	The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.12.6	The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8**	**Shellfish are not shucked through the hinge.**
C	**9**	☐	**3.12.9**	**The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	3.12.10	A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	3.12.11	The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis		
C	**28**	☐	**3.13.1**	**E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2	Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3	Several host cell colonies are transferred to a tube of tempered, aerated 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**	☐	**3.13.4**	**After inoculation, the host cell growth broth culture is not shaken.**
C	**28**	☐	**3.13.5**	**A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6**	**The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7**	**The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**	☐	**3.13.8**	**Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**	☐	**3.13.9**	**The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10**	**The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**	☐	**3.13.11**	**The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13	Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14	The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15**	**2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**	☐	**3.13.16**	**The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17**	**The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18**	**Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19	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	☐	3.13.20	Growth broth is used as the negative control or blank.
K	27, 28	☐	3.13.21	Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	3.13.22	A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28	☐	3.13.23	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**3.13.24**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC		
C	**27**	☐	**3.14.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2**	**The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count 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	☐	3.14.3	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	9	☐	3.14.4	The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.

3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621*. PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual.* U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8 (1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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:_____

Proposal No. 19-135

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Leonora Porter, Spokesperson
3. Affiliation	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
4. Address Line 1	205 N. Belle Mead Road
5. Address Line 2	Suite 1
6. City, State, Zip	East Setauket, NY 11733
7. Phone	(631) 444-0487
8. Fax	(631) 444-0472
9. Email	leonora.porter@dec.ny.gov
10. Proposal Subject	Microbiology Laboratory Evaluation Checklist - Sterilization
11. 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 Checklists, 1. NSSP Laboratory Evaluation Checklist for Microbiology
12. Text of Proposal/ Requested Action	The requested action is to adopt the modified text of the NSSP microbiology checklist, section 1.6 Sterilization and Decontamination, item 1.6.3:
13. Public Health Significance	The laboratory's goal is to ensure high-quality data using accepted scientific practices. The denoted information acknowledges recommended best practices used in recognized scientific publications to develop a laboratory's Quality Assurance Program (QAP) for sterilization practices at a wider range of temperature. The sterilization temperature range and the verification of working thermometers are now acceptably referenced to support past and present practices in program laboratories. The current reference material is cited to foster confidence in accepting the changes to an elevated sterilization temperature range and strengthen confidence in the acceptability of past practices for checking accuracy of working temperature monitoring devices. Most references for media sterilization simply state "121ºC for no less than 15 minutes." ***Difco***, a leading media manufacturer, states "A temperature range of 121-124ºC for 15 minutes is an accepted standard condition for sterilizing up to one liter of culture medium. The definition of "autoclave at 121ºC for 15 minutes" refers to the temperature of the contents of the container being held at 121ºC for 15 minutes, not to the temperature and time at which the autoclave has been set." ***Standard Methods,*** 23rd Edition, states "Annually, or preferably semiannually, **verify** the accuracy of all working temperature-sensing devices (e.g., liquid-in-glass thermometers, thermocouples, and temperature-recording instruments) at the use temperature(s). To do this, compare each device's measurements to those of a certified NIST temperature-sensing device or one traceable to NIST and conforming to NIST specifications. Discard temperature-sensing devices that differ by >1ºC from the reference device.….For general sterilization tasks, the recommended autoclave temperature range is 121 to 124ºC (at 200 kPa/29 PSI), although higher temperatures (≥121ºC) are acceptable for decontaminating laboratory material." *Each lab's QAP **must validate** temperature, time and pressure parameters for successful sterilization for media, reagents, supplies and spores using a verified working temperature monitoring device.*

14. Cost Information	No Cost. Minor adjustment during regularly scheduled sterilizer preventative maintenance service.

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
☐	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	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 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 months of experience in laboratory sciences.
		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	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 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 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	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	**29**	☐	**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**	☐	**1.4.21**	**A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22	Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13	☐	1.4.24	Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 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	~~11,~~ **30**, 33, 34	☐	**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.~~a sterilizing temperature of 121± 2°C as~~ Sterilization is determined for each load using a ~~calibrated~~verified 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	11	☐	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	16	☐	1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check _____ Method _____
K	11	☐	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	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 in the range of 160 to 180°C.
K	9	☐	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	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 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 are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22**	**The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11	☐	1.7.9	Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10	The volume and concentration of media in the tube are suitable for the amount of sample inoculated.
C	**11**	☐	**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13**	**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.
O	9	☐	1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2	☐	1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**	☐	**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**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	☐	2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**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	☐	2.2.9	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.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	2.3.4	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**	☐	**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**2.3.9**	**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, 31**	☐	**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing supports use of A-1medium without salicin. Study records are available.**
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**9**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5**

				tubes are recommended).
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**	☐	**2.5.8**	**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**	☐	**2.5.9**	**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**	☐	**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**2.5.11**	**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**	☐	**2.5.12**	**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 this 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 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	**2**	☐	**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
C	**23**	☐	**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.**
C	**23**	☐	**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**2.9.5**	**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**	☐	**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26	☐	2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8**	**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**	☐	**2.9.9**	**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**	☐	**2.9.10**	**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**	☐	**2.9.11**	**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**	☐	**2.9.12**	**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 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**	☐	**2.9.13**	**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.**
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	2,11	☐	3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2	Blades of shucking knives are not corroded.
O	9	☐	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	☐	3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9	☐	3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**	☐	**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9	☐	3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**	☐	**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	3.2.15	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.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**3.3.5**	**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	☐	3.3.6	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**	☐	**3.3.7**	**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	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	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.**
C	**2**	☐	**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control _____Negative productivity control _____**
K	9, 11	☐	3.4.3	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**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9	☐	3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen 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 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**	☐	**3.8.8**	**Six 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 plates.
C	**1**	☐	**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture _____ Negative control culture _____**
C	**3, 13**	☐	**3.8.13**	**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**	☐	**3.8.14**	**Plates are stacked no more than three high in the incubator.**
C	**2**	☐	**3.8.15**	**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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	30	☐	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 100 – 125 mL.
C	**27, 28**	☐	**3.10.2**	**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9	☐	3.10.3	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4**	**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5	The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6	The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7**	**The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**	☐	**3.10.8**	**The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**	☐	**3.10.9**	**Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation		
K	28	☐	3.11.1	Media preparation and sterilization is according to the validated method.
K	27, 28	☐	3.11.2	Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3	Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**	☐	**3.11.4**	**The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5	Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	3.11.6	Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28	☐	3.11.7	The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8	Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9	Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis		
K	2,11	☐	3.12.1	Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2	The blades of shucking knives are not corroded.
O	9	☐	3.12.3	The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.

O	2	☐	3.12.4	The faucet used for rinsing the shellfish does not contain an aerator.
K	9	☐	3.12.5	The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.12.6	The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8**	**Shellfish are not shucked through the hinge.**
C	**9**	☐	**3.12.9**	**The contents of shellfish (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	9	☐	3.12.10	A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19	☐	3.12.11	The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis		
C	**28**	☐	**3.13.1**	**E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2	Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3	Several host cell colonies are transferred to a tube of tempered, aerated 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**	☐	**3.13.4**	**After inoculation, the host cell growth broth culture is not shaken.**
C	**28**	☐	**3.13.5**	**A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6**	**The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7**	**The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**	☐	**3.13.8**	**Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**	☐	**3.13.9**	**The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10**	**The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**	☐	**3.13.11**	**The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12	The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13	Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14	The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15**	**2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**	☐	**3.13.16**	**The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17**	**The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18**	**Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19	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	☐	3.13.20	Growth broth is used as the negative control or blank.
K	27, 28	☐	3.13.21	Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.

K	2	☐	3.13.22	A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28	☐	3.13.23	The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**3.13.24**	**All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC		
C	**27**	☐	**3.14.1**	**Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2**	**The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count 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	☐	3.14.3	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	9	☐	3.14.4	The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621*. PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual.* U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.

22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8 (1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.
33. 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.
34. ***Difco Manual***, 11th Edition, 1998, Division of Becton Dickinson and Company, Sparks, Maryland, Pg. 13.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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. October 2015

Proposal No. 19-136

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	US Food and Drug Administration (FDA)
3. Affiliation	US Food and 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	240-402-2401
8. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	NSSP DSP Laboratory Evaluation Checklist
11. 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 Checklists
12. Text of Proposal/ Requested Action	The requested action is to adopt the laboratory evaluation checklist for Diarrhetic Shellfish Poisoning LC-MS/MS.
13. Public Health Significance	The Diarrhetic Shellfish Poisoning (DSP) LC-MS/MS checklist will provide the means of assessing the competence of the laboratory to perform the test method.
14. 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. 240-402-2151/2055/4960 FAX 301-436-2601

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:

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	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. 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. 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		1.4.10 An ultra-performance liquid chromatography system (UPLC) equipped with the following is used: a. mobile phase system delivering a pulse-free flow of 0.12 mL/min b. solvent degasser 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 filtered before use if the UPLC does not have a degasser.
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 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 polypropylene tube.
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 in the polypropylene tube is adjusted to 20 mL with 100% methanol.
K	3		2.2.8 The crude extract is hydrolyzed or stored in the freezer at < -20 °C.
			2.3 Sample Hydrolysis and Cleanup
K	3		2.3.1 A two (2) mL aliquot of the sample extract is transferred to a 16 × 100 mm glass tube with a phenolic PTFE lined screw cap using a positive displacement pipette or syringe.
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.
K	3		2.3.5 Nonpolar lipids are removed by adding seven (7) mL of hexanes to the hydrolyzed sample extract and vortexing to mix.
K	3		2.3.6 The sample extract/hexane mixture is partitioned by centrifuging for 10 minutes at 2000 x g.
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 certified glass vial using a 0.2 µm PTFE syringe tip filter.
K	2		2.3.8 The cleaned-up extract is loaded into the autosampler immediately for analysis.

<table>
<tr><td></td><td></td><td></td><td>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 before and after each set of samples.</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</td><td></td><td>2.4.5 Samples are stored in the sample compartment of the autosampler at ≤ 10°C during analysis..</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 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 Procedural Blanks (methanol carried through sample preparation process at the same time as the samples) should be analyzed before and after extracted samples.</td></tr>
</table>

			2.5 System Suitability
C	**3**		**2.5.1 Each calibration curve should be derived from at least six (6) calibration points and the linear regression of the combined curves should yield a correlation coefficient (R^2) ≥ 0.98. Results are recorded and records are maintained.**
C	**3**		**2.5.2 If a calibration curve yields a correlation coefficient ≤ 0.98, or if non□linearity is visually observed, or if the variation in the slopes between the first and second calibration curves exceeds 25%, a new calibration curve is prepared and samples are reanalyzed.**
C	**3**		**2.5.3. The retention time of analytes in all matrix solution should be within 3% that of the toxin standards.**
C	**3**		**2.5.4 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).** $$Rs = 2 \times (RT2 - RT1)/(W1 - W2)$$
K	2		2.5.5 Each chromatographic peak must be defined by at least 10 data points.
C	**3**		**2.5.6 A new calibration curve is performed, or one mid□point calibration standard is analyzed, at least every 10 samples to ensure that no retention time shifts or loss in signal intensity has occurred.**
K	2		2.5.7 Peak asymmetry must be <0.9 or >1.3.
C	**3**		**2.5.8 Reagent blanks (methanol) are analyzed after the high calibration standard and periodically after fortified samples to insure that analyte carryover is not occurring.**
C	**2**		**2.5.9 Repeated injections of calibrated standards/samples agree within ± 5% (as determined through the use of the coefficient of variation).**
C	**3**		**2.5.10 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 → 151.1 for DTX-1, 803.5 → 151.1 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**		**2.5.11 The ratio of the abundance of the quantitative ion transition to the confirmation 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.**
			2.6 Calculation of Sample Toxicity
C	**4, 15**		**2.6.1 The toxicity of the individual toxins is calculated as follows:** $$\frac{ug}{g}toxin = C \times \frac{V}{W}$$ **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**
C	**12**		**2.6.2 Any value at or above 16 ppm (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 to identify positive result 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.

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:

Proposal No. 19-137

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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	CPK 1, HFS - 325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-1401
8. Fax	301-436-2601
9. Email	Melissa.abbott@fda.hhs.gov
10. Proposal Subject	Checklist for the Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration (MF) using mEndo Agar LES
11. Specific NSSP Guide Reference	NSSP *Guide for the Control of Molluscan Shellfish*, 2017 Revision, "Guidance Documents", Chapter II. Growing Areas, .15 Evaluation of Laboratories by State Shellfish Laboratory Evaluation Officers Including Laboratory Evaluation Checklists, 1. NSSP Laboratory Evaluation Checklists for Microbiology.
12. Text of Proposal/ Requested Action	Incorporate Sections 2.11 through 2.14 for the Bacteriological Analysis of UV Treated Process Water Samples by Membrane Filtration using mEndo Agar LES into the NSSP Laboratory Evaluation Checklist for Microbiology.
13. Public Health Significance	Incorporation of the mEndo Agar LES membrane filtration method into the Microbiology Checklist will provide the means of assessing the competence of the laboratory to perform the test method.
14. Cost Information	NA

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
	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 Soft-shelled Clams and American Oysters [PART III]
	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]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
			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 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 months of experience in laboratory sciences.
		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	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 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 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	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 water bath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15 The thermometers used in the water bath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16 The water bath has adequate capacity for workload.**
K	9		1.4.17 The level of water in the water bath 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	**29-**		**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21 A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22 Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**		**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 Incubator and water bath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 1.1 mL 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 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	**11, 30**		**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	11		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	16		1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check_____Method _____
K	11		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	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 in the range of 160 to 180°C.

K	9		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	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 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 are properly sterilized. Method of sterilization _____
C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11		1.7.9 Media prepared from commercial dehydrated components sterilized according to the manufacturer's instructions.
K	9		1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.

C	**11**		**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**		**1.7.13**	**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.**
O	9		1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11		1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**		**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**		**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**

C	**6**		**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**		**2.2.6**	**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		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5 °C.
C	**2**		**2.2.8**	**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		2.2.9	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.**
C	**2**		**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11		2.3.4	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**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7**	**EC tubes are incubated in a circulating water bath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**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, 31**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing**

				supports use of A-1medium without salicin. Study records are available
C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**		**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc In 7 seconds) before inoculation.**
C	**9**		**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8**	**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**		**2.5.9**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and water bath incubation.* Results are recorded and the records maintained.** **Positive process control_____Negative process control _____**
C	**2,5**		**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating water bath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12**	**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 water bath 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 this 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 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	**2**		**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**23**		**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in7 seconds) before filtration.**
C	**23**		**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5**	**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**		**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26		2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8**	**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		2.9.9 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		2.9.10 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		2.9.11 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		2.9.12 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 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		2.9.13 After two (2) hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating water bath 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	11		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		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	11		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	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, 26, 33		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	11		2.11.15 Forceps tips are clean and smooth without pitting or corrugations.
		2.12 Media Preparation and Storage	
C	11, 33		2.12.1 mEndo Agar LES is used.
K	11, 33		2.12.2 mEndo Agar LES is prepared aseptically using pre-sterilized glassware, sterile reagent water and pre-sterilized stir bar.
K	11, 33		2.12.3 mEndo Agar LES is prepared using 95% alcohol that is not denatured.
C	11, 33		2.12.4 mEndo Agar LES is brought to near boiling; removed from the heat and tempered at 45-50°C before dispensing.
C	11, 33		2.12.5 mEndo Agar LES is never autoclaved.
K	11, 33		2.12.6 A sufficient amount of medium (4-5 mL) is aseptically aliquoted to each culture plate.
O	11, 33		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.
C	2		2.12.8 Appropriate, properly diluted positive and negative productivity controls for mEndo Agar LES medium are used. Results are recorded and the records maintained. Positive productivity control _____ Negative productivity control _____
K	11, 33		2.12.9 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		2.12.10 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	33		2.13.1 The sample is shaken vigorously (25 times in a 12-inch arc in 7 seconds) before filtration.
C	33		2.13.2 The membrane filter is placed grid side up within the sterile filter apparatus.
C	26, 33		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	11, 26, 33		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	11, 33		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	11, 33		2.13.7 Forceps are dipped in alcohol and flame sterilized between sample filters.

C	11, 33		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, 33		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	11, 33		2.13.10	Inoculated plates are incubated inverted at 35+/- 0.5°C for 22 to 24 hours.
K	2		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	11		2.14.1	Colonies are counted with the aid of magnification.
C	11, 23		2.14.2	All metallic sheen colonies are counted as total coliforms.
C	11, 33		2.14.3	Results are reported as total coliforms/100mL.
C	11, 33, 20		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	2,11		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2		3.2.2	Blades of shucking knives are not corroded.
O	9		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		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9		3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9		3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.

C	**9**		**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15	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.)***
C	**2**		**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9		3.3.3	Immediately (within2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4**	**No fewer than5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5**	**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		3.3.6	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**		**3.3.7**	**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		3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9	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.**
C	**2**		**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11		3.4.3	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**		**3.4.4**	**EC tubes are incubated in a circulating water bath at 44.5 ± 0.2°C**
K	9		3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	Fifteen 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 7 seconds) before plating.
C	**9**		**3.6.7**	**Not more than <u>one (1)</u> 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 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 Part 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/g 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 within2 minutes of blending.**
C	**2,3**		**3.8.8**	**Six 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 plates.
C	**1**		**3.8.11**	**Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**		**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture_____Negative control culture _____**
C	**3, 13**		**3.8.13**	**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**		**3.8.14**	**Plates are stacked no more than three high in the incubator.**
C	**2**		**3.8.15**	**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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30		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 100 – 125 mL.
C	**27, 28**		**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9		3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**		**3.10.4 Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1		3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1		3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**		**3.10.7 The balance used provides a sensitivity of at least 10 mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**		**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28		3.11.1 Media preparation and sterilization is according to the validated method.
CK	27, 28		**3.11.2.** 3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28		3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28		3.11.5Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28		3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28		3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28		3.11.9 Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11		3.12.1 Shucking knives, scrub brushes, and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.12.2 The blades of shucking knives are not corroded.
O	9		3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.12.7 Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.

C	**9**		**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile,tared blender jar or other sterile container.**
K	9		3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.12.11 The sample is weighed to the nearest 0.1 gram.
		3.143 MSC Sample Analysis	
C	**28**		**3.13.1 E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated 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**		**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**		**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**		**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4 °C.**
C	**27, 28**		**3.13.10 The supernatant is pipetted off, weighed, and the weight recorded.**
C	**27, 28**		**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28		3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**3.13.15 2.5 mL of sample supernatant is added to each tube of temperingsoft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**		**3.13.18 Ten (10) plates are used2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28		3.13.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are and records maintained. Positive control _____
K	27, 28		3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**3.13.24 All plates are incubated at 36 ± 1 °C for 18 ± 2 hours.**
		3.154 Computation of Results -MSC	
C	**27**		**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**

C	**28, 32**		**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten(10) plates, the count 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		3.14.3 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	9		3.14.4 The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report,* Reprint #*1621.* PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8(1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.

26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.
33. Total Coliform Method for Shellfish Dealer Process Water Using the Membrane Filtration Technique with mEndo Agar LES. Summary of Actions 2011,Proposal 11-111, page 230.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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:_____

Proposal No. 19-138

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	US Food and Drug Administration (FDA)
3. Affiliation	US Food and 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	240-402-2401
8. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	NSSP Microbiology Laboratory Evaluation Checklist
11. 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 Checklists
12. Text of Proposal/ Requested Action	The requested action is to adopt the modified text of four (4) NSSP microbiology checklist items in the Laboratory Equipment and Sterilization and Decontamination sections; said NSSP checklist items are 1.4.5, 1.4.21, 1.6.10, and 1.6.11.
13. Public Health Significance	The proposed modifications are to improve consistency in current NSSP microbiology checklist language and account for technology improvements to laboratory equipment.
14. Cost Information	N/A

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
			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 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 months of experience in laboratory sciences.
		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~~a minimum of two standard buffer solutions is used to calibrate the pH meter. If the calibration sequence of standard buffer solutions is not stipulated by the manufacturer, ~~T~~the first must be near the electrode isopotential point (pH 7)~~.~~ and ~~T~~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 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	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 water bath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15 The thermometers used in the water bath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16 The water bath has adequate capacity for workload.**
K	9		1.4.17 The level of water in the water bath 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	**29-**		**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21 A ~~mercury-in-glass~~ 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22 Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**		**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 Incubator and water bath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 1.1 mL 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 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	**11, 30**		**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	11		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	16		1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check_____Method _____
K	11		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	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 ~~in the range~~ of ≥160 ~~to 180~~°C.

K	9		1.6.11 A thermometer capable of determining temperatures ~~accurately in the range of~~ ≥160 ~~to 180~~°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 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 are properly sterilized. Method of sterilization _____
C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11		1.7.9 Media prepared from commercial dehydrated components sterilized according to the manufacturer's instructions.
K	9		1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.

C	**11**		**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**		**1.7.13**	**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.**
O	9		1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11		1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**		**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**		**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**

C	**6**		**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**		**2.2.6**	**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		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5 °C.
C	**2**		**2.2.8**	**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		2.2.9	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.**
C	**2**		**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11		2.3.4	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**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7**	**EC tubes are incubated in a circulating water bath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**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, 31**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing**

				supports use of A-1medium without salicin. Study records are available
C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**		**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc In 7 seconds) before inoculation.**
C	**9**		**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8**	**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**		**2.5.9**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and water bath incubation.* Results are recorded and the records maintained.** **Positive process control_____Negative process control _____**
C	**2,5**		**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating water bath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12**	**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 water bath 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 this 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 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	**2**		**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**23**		**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in7 seconds) before filtration.**
C	**23**		**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5**	**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**		**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26		2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8**	**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**		**2.9.9**	**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**		**2.9.10**	**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**		**2.9.11**	**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**		**2.9.12**	**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 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**		**2.9.13**	**After two (2) hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating water bath 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.**
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	2,11		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2		3.2.2	Blades of shucking knives are not corroded.
O	9		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		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9		3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9		3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**		**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15	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.)***
C	**2**		**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9		3.3.3	Immediately (within2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4**	**No fewer than5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5**	**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		3.3.6	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**		**3.3.7**	**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		3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9	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.**
C	**2**		**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**

K	9, 11		3.4.3	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**		**3.4.4**	**EC tubes are incubated in a circulating water bath at 44.5 ± 0.2°C**
K	9		3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	Fifteen 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 7 seconds) before plating.
C	**9**		**3.6.7**	**Not more than <u>one (1)</u> 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**		**3.8.8**	**Six 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 plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**		**3.8.12 Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared. Positive control culture_____Negative control culture _____**
C	**3, 13**		**3.8.13 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**		**3.8.14 Plates are stacked no more than three high in the incubator.**
C	**2**		**3.8.15 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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30		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 100 – 125 mL.
C	**27, 28**		**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9		3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**		**3.10.4 Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1		3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1		3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**		**3.10.7 The balance used provides a sensitivity of at least 10 mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**		**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28		3.11.1 Media preparation and sterilization is according to the validated method.
CK	27, 28		**3.11.2.** 3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28		3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28		3.11.5Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28		3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.

K	27, 28		3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28		3.11.9 Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11		3.12.1 Shucking knives, scrub brushes, and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.12.2 The blades of shucking knives are not corroded.
O	9		3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.12.7 Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**		**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile,tared blender jar or other sterile container.**
K	9		3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.12.11 The sample is weighed to the nearest 0.1 gram.
		3.143 MSC Sample Analysis	
C	**28**		**3.13.1 E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated 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**		**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**		**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**		**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4 °C.**
C	**27, 28**		**3.13.10 The supernatant is pipetted off, weighed, and the weight recorded.**
C	**27, 28**		**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28		3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**3.13.15 2.5 mL of sample supernatant is added to each tube of temperingsoft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28**		**3.13.18 Ten (10) plates are used2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28		3.13.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are and records maintained. Positive control _____
K	27, 28		3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**3.13.24 All plates are incubated at 36 ± 1 °C for 18 ± 2 hours.**
		3.154 Computation of Results -MSC	
C	**27**		**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**		**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten(10) plates, the count 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		3.14.3 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	9		3.14.4 The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report,* Reprint #*1621.* PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical LaboratoryStudy.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical*

Programs - Criteria for Laboratories Performing Food Testing. AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8(1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.

MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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:_____

Proposal No. 19-139

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	NSSP Laboratory Evaluation Officers Team
3. Affiliation	FDA LEO and State LEO Team- represented by Melissa Farrell
4. Address Line 1	5001 Campus Drive
5. Address Line 2	CPK1, HFS-325
6. City, State, Zip	College Park, MD 20740
7. Phone	240-402-2055
8. Fax	301-436-2601
9. Email	Melissa.Farrell@fda.hhs.gov
10. Proposal Subject	NSSP Microbiology Laboratory Evaluation Checklist
11. 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 Checklists
12. Text of Proposal/ Requested Action	The requested action is to adopt the modified text of NSSP microbiology checklist item 1.4.24 in the Laboratory Equipment section and 3.2.7 in the Preparation of Shellfish for Examination section and add an additional reference to item 3.2.7.
13. Public Health Significance	1.4.24: One of the most basic attributes of any thermometer is its accuracy, and because a thermometer is only as valuable as the temperature it measures, accuracy is of the utmost importance. Calibration defines the accuracy by quantifying and controlling uncertainties within the measurement process. The quality of data must be known and established beyond a reasonable doubt before it can be used logically in any application; thus, calibration is an integral part of the lab's Quality Assurance. When individuals record and maintain data, proof of calibration demonstrates that the measurements performed are consistent with the "true value." An intermediate check is an action that the user takes to verify that the measuring instrument continues to be suitable for its purpose. Currently, the NSSP requires laboratories to perform intermediate checks on incubator and water bath thermometers at the temperature at which they are used. This requirement does not include refrigerator or freezer thermometers; however, NSSP Microbiology checklist items 1.4.9 and 1.4.10 require laboratories to measure and record refrigerator temperature data. When properly performed, an ice point is recommended as a “fixed point” for calibration of liquid in glass thermometers as it provides a reliable reference temperature at 0 °C with an estimated measurement uncertainty of ± 0.002 °C for determining the thermometer’s accuracy at all calibration points. The reliability and high degree of accuracy achieved by performing a proper ice point is due to the ice-water mixture stabilizing at its own “triple point.” Due to the nature of an ice point, it is the most common calibration point used for intermediate checks. 3.2.7 and reference addition: This change corrects an oversight in the current checklist regarding the role of gloves when shucking.
14. Cost Information	N/A

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
			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 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 months of experience in laboratory sciences.
		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	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 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 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	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 water bath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15 The thermometers used in the water bath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16 The water bath has adequate capacity for workload.**
K	9		1.4.17 The level of water in the water bath 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	**29-**		**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21 A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22 Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**		**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 of ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 The accuracy of ~~Incubator and water bath~~ working thermometers is ~~are~~ 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	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 1.1 mL 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 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	**11, 30**		**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	11		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	16		1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check_____Method _____
K	11		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	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 in the range of 160 to 180°C.

K	9		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	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 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 are properly sterilized. Method of sterilization _____
C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11		1.7.9 Media prepared from commercial dehydrated components sterilized according to the manufacturer's instructions.
K	9		1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.

C	**11**		**1.7.11**	**Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**		**1.7.13**	**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.**
O	9		1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11		1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2		1.8.5	Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11		1.8.6	Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**		**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation.**
C	**9**		**2.2.4**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**

C	**6**		**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**		**2.2.6**	**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		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5 °C.
C	**2**		**2.2.8**	**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		2.2.9	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.**
C	**2**		**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11		2.3.4	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**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7**	**EC tubes are incubated in a circulating water bath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**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, 31**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing**

				supports use of A-1medium without salicin. Study records are available
C	**5**		**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**		**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc In 7 seconds) before inoculation.**
C	**9**		**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**		**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8**	**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**		**2.5.9**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and water bath incubation.* Results are recorded and the records maintained.** **Positive process control_____Negative process control _____**
C	**2,5**		**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating water bath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12**	**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 water bath 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 this 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 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	**2**		**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**23**		**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in7 seconds) before filtration.**
C	**23**		**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5**	**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**		**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26		2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8**	**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**		**2.9.9**	**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**		**2.9.10**	**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**		**2.9.11**	**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**		**2.9.12**	**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 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**		**2.9.13**	**After two (2) hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating water bath 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.**
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	2,11		3.2.1	Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2		3.2.2	Blades of shucking knives are not corroded.
O	9		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		3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	1, 9		3.2.7	Immediately prior to shucking, the hands ~~(or gloved hands)~~ of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol or clean gloves are donned.
C	**9**		**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**		**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9		3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**		**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15	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.)***
C	**2**		**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9		3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5**	**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		3.3.6	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**		**3.3.7**	**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		3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9	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.**
C	**2**		**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**

K	9, 11		3.4.3	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**		**3.4.4**	**EC tubes are incubated in a circulating water bath at 44.5 ± 0.2°C**
K	9		3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9		3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2		3.6.3	Fifteen 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 7 seconds) before plating.
C	**9**		**3.6.7**	**Not more than <u>one (1)</u> 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**		**3.8.8**	**Six 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 plates.
C	**1**		**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**		**3.8.12 Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared. Positive control culture_____Negative control culture _____**
C	**3, 13**		**3.8.13 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**		**3.8.14 Plates are stacked no more than three high in the incubator.**
C	**2**		**3.8.15 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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30		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 100 – 125 mL.
C	**27, 28**		**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9		3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**		**3.10.4 Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1		3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1		3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**		**3.10.7 The balance used provides a sensitivity of at least 10 mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**
C	**28**		**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28		3.11.1 Media preparation and sterilization is according to the validated method.
CK	27, 28		**3.11.2.** 3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28		3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28		3.11.5Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28		3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28		3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.

K	27, 28		3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28		3.11.9 Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11		3.12.1 Shucking knives, scrub brushes, and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.12.2 The blades of shucking knives are not corroded.
O	9		3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2		3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.12.7 Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**		**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile,tared blender jar or other sterile container.**
K	9		3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.12.11 The sample is weighed to the nearest 0.1 gram.
		3.143 MSC Sample Analysis	
C	**28**		**3.13.1 E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28		3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated 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**		**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**		**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**		**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**		**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture are weighed into centrifuge tubes.**
C	**28**		**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4 °C.**
C	**27, 28**		**3.13.10 The supernatant is pipetted off, weighed, and the weight recorded.**
C	**27, 28**		**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28		3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28		3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28		3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**		**3.13.15 2.5 mL of sample supernatant is added to each tube of temperingsoft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**		**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**

C	**28**		**3.13.18 Ten (10) plates are used2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28		3.13.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are and records maintained. Positive control _____
K	27, 28		3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28		3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**		**3.13.24 All plates are incubated at 36 ± 1 °C for 18 ± 2 hours.**
		3.154 Computation of Results -MSC	
C	**27**		**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**		**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten(10) plates, the count 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		3.14.3 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	9		3.14.4 The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621.* PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical*

Programs - Criteria for Laboratories Performing Food Testing. AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8(1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.

MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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:_____

Proposal No. 19-140

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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	240-402-24001
8. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	NSSP Microbiology Laboratory Evaluation Checklist
11. 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 Checklists
12. Text of Proposal/ Requested Action	The requested action is to adopt the modified text of the attached checklist for Bacteriological Examination of Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC), starting at section 3.10.
13. Public Health Significance	The proposed modifications are to provide clarification to bench analysts and LEOs for consistent performance and evaluation of the method for the NSSP.
14. Cost Information	N/A

PUBLIC HEALTH SERVICE
U.S. FOOD AND DRUG ADMINISTRATION
OFFICE OF FOOD SAFETY
SHELLFISH AND AQUACULTURE POLICY BRANCH
~~5100 PAINT BRANCH PARKWAY~~ 5001
CAMPUS DRIVE

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
	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 ~~Soft-shelled Clams and American Oysters~~ Shellfish Meats [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
			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 sciences.
		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	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 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 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	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 water bath is maintained at 44.5 ± 0.2°C under all loading conditions.**
C	**9**		**1.4.15 The thermometers used in the water bath are graduated in at least 0.1°C increments.**
C	**13**		**1.4.16 The water bath has adequate capacity for workload.**
K	9		1.4.17 The level of water in the water bath covers the level of liquid in the incubating tubes.
K	8, 11		1.4.18 Air incubator/water bath 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	~~29~~ 9		**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**		**1.4.21 A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9		1.4.22 Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	~~29~~ 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 of ~~at least~~ ≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 Incubator and water bath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 1.1 mL 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	**11, ~~30~~ 29**		**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	11		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	16		1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1		1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check_____Method _____
K	11		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	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 in the range of 160 to 180°C.

K	9		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	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 are properly sterilized. Method of sterilization _____
C	**2**		**1.6.22 The sterility of the hardwood applicator transfer sticks 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 ~~medium~~ 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 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	11		1.7.9 Media ~~prepared~~ from commercially dehydrated components are prepared sterilized according to the manufacturer's instructions.
K	9		1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.

C	**11**		**1.7.11**	**Total time of exposure of sugar containing broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**		**1.7.12**	**Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**		**1.7.13**	**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.**
O	9		1.7.14	Sterile phosphate buffered dilution water is used as the sample diluent.
K	11		1.7.15	The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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	**2**		**2.2.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.2.3**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in seven (7) seconds) before inoculation.**
C	**9**		**2.2.4**	**In a multiple dilution series of not less than three (3) tubes per dilution are used (five (5) tubes are recommended).**

C	**6**		**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least five (5) tubes are used).**
C	**6**		**2.2.6**	**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		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5 °C.
C	**2**		**2.2.8**	**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		2.2.9	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.**
C	**2**		**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11		2.3.4	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**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9		2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**		**2.3.7**	**EC tubes are incubated in a circulating water bath maintained at 44.5 ± 0.2°C.**
C	**9**		**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**		**2.3.9**	**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, ~~31~~ 30**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing**

				supports use of A-1medium 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	**2**		**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9**		**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc In seven (7) seconds) before inoculation.**
C	**9**		**2.5.6**	**In a multiple dilution series of not less than three(3) tubes per dilution are used (five(5) tubes are recommended).**
C	**6**		**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**		**2.5.8**	**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**		**2.5.9**	**Appropriately diluted process control cultures accompany the samples *throughout both resuscitation and water bath incubation.* Results are recorded and the records maintained.** **Positive process control_____Negative process control _____**
C	**2,5**		**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**		**2.5.11**	**After 3 ± 0.5 hours resuscitation at 35°C, inoculated tubes are incubated at 44.5 ± 0.2°C in a circulating water bath for the remainder of the 24 ± 2 hours.**
C	**5**		**2.5.12**	**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 water bath 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~~is~~ 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	**2**		**2.9.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**23**		**2.9.3**	**The sample is shaken vigorously (25 times in a 12″ arc in seven (7) seconds) before filtration.**
C	**23**		**2.9.4**	**The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**		**2.9.5**	**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**		**2.9.6**	**Sample volumes are filtered under vacuum.**
K	26		2.9.7	The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**		**2.9.8**	**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**		**2.9.9**	**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**		**2.9.10**	**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**		**2.9.11**	**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**		**2.9.12**	**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**		**2.9.13**	**After two (2) hours of resuscitation at 35°C, the watertight, tightly sealed containers are transferred to a circulating water bath 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.**
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, ~~11,~~ 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	9, 32		3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
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.
O	9		3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**		**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9		3.2.15	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.)***
C	**2**		**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9		3.3.3	Immediately (within two(2) minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**		**3.3.4**	**No fewer than five (5) tubes per dilution are used in a multiple dilution MPN series.**
C	**9**		**3.3.5**	**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		3.3.6	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**		**3.3.7**	**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		3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10		3.3.9	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.**
C	**2**		**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11		3.4.3	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**		**3.4.4**	**EC tubes are incubated in a circulating water bath at 44.5 ± 0.2°C**
K	9		3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**		**3.4.6**	**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 may be 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~~Fifteen~~ 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 one (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 Part 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	**1**		**3.8.12**	**Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared.** **Positive control culture_____Negative control culture _____**
C	**3, 13**		**3.8.13**	**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**		**3.8.14**	**Plates are stacked no more than three (3) high in the incubator.**
C	**2**		**3.8.15**	**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 ~~Soft-shelled Clams and American Oysters~~ Shelfish Meats for Male Specific Coliphage (MSC)		
		3.10 MSC Equipment and Supplies		
K	~~30~~ 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 100 ~~125~~ mL.
~~**C**~~	~~**27, 28**~~		~~**3.10.2**~~	~~**The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4 °C.**~~
K	~~9~~ 2		3.10.~~3~~2	The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
~~**C**~~	~~**27, 28**~~		~~**3.10.4**~~	~~**Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**~~
K	1		3.10.~~3~~5	The sterility of each batch/lot of pre-sterilized or reusable syringes, ~~and syringe~~ filters and/or filter units is determined. Results are recorded and records maintained.
~~K~~	~~1~~		~~3.10.6~~	~~The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.~~
C	~~**27, 28**~~**2**		**3.10.~~4~~7**	**The balance used provides a sensitivity of at least 10 mg (0.01 g.).**
C	**27, 28, 31**		**3.10.~~5~~8**	**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	~~28~~ ~~1~~		**3.10.~~9~~7 The ~~S~~sterility of ~~e~~ disposable 50 mL centrifuge tubes ~~are used and their sterility~~ 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.
~~C~~K	27, 28, 31		**3.11.2 Antibiotic solutions are filter sterilized using sterile 0.22 µm pore size filters.** ~~3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.~~
~~K~~	~~27, 28~~		~~3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.~~
~~C~~	~~27, 28~~		~~3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.~~
O	27, 28, 31		3.11.~~5~~3 Storage of the bottom agar under refrigeration does not exceed one (1) month.
K	~~27, 28~~2		3.11.~~6~~ 4Unsterilized soft agar is stored at ~~-20 °C~~≤ -15 °C for up to three (3) months.
K	27, 28, 31		3.11.~~7~~5 The soft agar is ~~removed from the freezer and~~ sterilized for 15 minutes at 121°C before use.
~~K~~	~~27, 28~~		~~3.11.8 Storage under refrigeration of prepared of growth broth broth in the refrigerator in loosely capped tubes/bottles does with loose fitting closures shall not exceed one (1) month and in screw capped tubes/bottles does not exceed 3 months.~~
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.~~9~~7 Bottom agar plates and growth broth 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~~2~~ Preparation of ~~the Soft-Shelled Clams and American Oysters~~Shellfish for MSC Analysis	
K	2,~~11~~32		3.13~~2~~.1 Shucking knives, scrub brushes, and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2		3.13~~2~~.2 The blades of shucking knives are not corroded.
O	9		3.13~~2~~.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~~2~~.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.13~~2~~.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9		3.13~~2~~.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9		3.13~~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.
C	**9**		**3.13~~2~~.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.13~~2~~.9 The contents of shellfish (liquor and meat) are shucked into a sterile,tared blender jar or other sterile container.**
K	9		3.13~~2~~.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.13~~2~~.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 of sterile growth broth, by volume, is**

			added.
C	28, 31		3.13.13 Samples are blended at high speed for 180 seconds.
		3.143 MSC Sample Analysis	
C	28, 31		3.13.1 E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.
K	27, 28, 31		3.13.2 Host cell growth broth is tempered at 36 ± 1 °C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28, 31		3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated 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		3.13.4 After inoculation, the host cell growth broth culture is not shaken.
C	28		3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.
C	28		3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.
C	28		3.13.7 The elution mixture is homogenized at high speed for 180 seconds.
C	28, 31		3.138 4.1 Immediately after blending, 33 grams of the homogenate.ized elution mixture areis weighed into a centrifuge tubes.
C	28, 31		3.14.213.9 8The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4 °C.
C	27, 28, 31		3.14.313.109 The supernatant is pipetted offtransferred to a new sterile tube, weighed, and the weight recorded.
C	27, 28, 31		3.14.413.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutesprior to analysis.
K	27, 28, 31		3.14.513.12 The autoclaved soft agar is tempered and held at 51 ± 1 °C throughout the period of sample analysis.
K	27, 28, 31		3.14.613.13 Two hundred 200 microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering tempered soft agar immediately prior to adding the sample supernatant.
K	27, 28, 31		3.14.713.14 The sample supernatant is shaken or vortexed before being added to the tempering tempered soft agar.
C	27, 28, 31		3.14.813.15 2.5 mL of sample supernatant is added to the each tube of tempering tempered soft agar.
C	27, 28, 31		3.14.93.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.
C	27, 28, 31		3.14.103.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.
C	28, 31		3.14.1113.18 Ten (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.
K	27, 28, 31		3.14.1213.19 Negative and positive control plates are prepared and accompany each set of samples analyzed. The results are and records maintained. Positive control _____
K	27, 28, 31		3.14.1313.20 Growth broth is used as the negative control or blank.
K	27, 28, 31		3.14.1413.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2		3.14.1513.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28, 31		3.14.1613.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	27, 28, 31		3.14.173.24 All plates are incubated at 36 ± 1 °C for 18 ± 2 hours.
		3.154 Computation of Results -MSC	

C	**27**		**3.154.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32, 31**		**3.154.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten (10) plates, the ~~count~~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		3.145.3 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	~~9~~ 2		3.154.4 The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria.*" 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.
4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report,* Reprint *#1621.* PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in

marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8(1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
~~29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.~~
~~30.~~29. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
~~31.~~30. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
~~32.~~31. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.
1. American Public Health Association. 2001. *Compendium of Methods for the Microbiological Examination of Foods.* APHA. Washington, D.C.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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:_____

Proposal No. 19-141

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	US Food and Drug Administration (FDA)
3. Affiliation	US Food and 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	240-402-2401
8. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	NSSP Receptor Binding Assay for Paralytic Shellfish Poisoning (PSP) Laboratory Evaluation Checklist
11. 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 Checklists
12. Text of Proposal/ Requested Action	The requested action is to adopt the laboratory evaluation checklist for the Receptor Binding Assay for Paralytic Shellfish Poisoning (PSP).
13. Public Health Significance	The Receptor Binding Assay for Paralytic Shellfish Poisoning (PSP) checklist will provide the means of assessing the competence of the laboratory to perform the test method.
14. Cost Information	N/A

Receptor Binding Assay for Paralytic Shellfish Poisoning (PSP)

PART I – Quality Assurance

ITEM			
CODE	**REF**		
		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 and verification of licensing must be provided.**
C	**15**		**1.2.6 Laboratory has a Nuclear Regulatory Commission (NRC) license for the use of tritiated saxitioxin 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 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 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.** **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 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		2.2.5 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 Five (5) grams of tissue +/- 0.1g is extracted using an equal amount 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, reference blank, and an inter-assay QC calibration standard 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.
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 If [^{3}H] STX working solution is checked for counts per minute (CPM) it should be consistent and 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			**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.

Proposal No. 19-142

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Shelley Lankford
3. Affiliation	WA DOH Public Health Laboratories
4. Address Line 1	1610 NE 150th St
5. Address Line 2	
6. City, State, Zip	Shoreline, WA 98155-7224
7. Phone	(206)418-5441
8. Fax	(206)367-1790
9. Email	Shelley.Lankford@DOH.WA.GOV
10. Proposal Subject	Add the use of a mechanical shaker to the water microbiology methods checklist in the sample preparation requirements section and include a reference.
11. 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 Checklists SHELLFISH LABORATORY EVALUATION CHECKLIST PART II - SEAWATER SAMPLES 2.2 Bacteriological Examination of Seawater by the APHA MPN 2.2.3 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation. 2.5 Bacteriological Examination of Seawater by the MA-1 Method 2.5.5 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds) before inoculation. 2.9 Sample Analyses - MF using mTEC Agar 2.9.3 The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds) before filtration.
12. Text of Proposal/ Requested Action	Adopt the text of update the shellfish laboratory evaluation microbiology checklist (attached) to include the use of a mechanical shaker for sample preparation and include a reference for the use in the checklist's lists of references.
13. Public Health Significance	This proposal does not have direct public health significance but directly impacts the health of laboratorians performing water microbiological testing by allowing the use of a mechanical shaker to reduce or alleviate repetitive motion injuries caused by hand shaking the water samples. Work related injuries in the laboratory due to poor ergonomics are increasing every year and are costly to the laboratory due to work related injury claims. FDA LEO's currently allow the use of this equipment but there is no mention of the use of the equipment, no guidance for use of the equipment nor any reference from a reliable source in the current microbiology checklist for allowing the use of a mechanical shaker for sample preparation purposes.
14. Cost Information	This proposal updates text in the NSSP Manual wherever found in the microbiology checklist if approved by the conference. Minimal costs will be incurred by the ISSC administration when the laboratory evaluation checklist development and updating occurs at the ISSC office as part of the biannual NSSP Manual update process.

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: **Conformity it noted by a "√"**

C- Critical K - Key O - Other NA- 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]
	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 Soft-shelled Clams and American Oysters [PART III]

PART 1 - QUALITY ASSURANCE				
CODE	**REF.**			**ITEM**
K	8, 11	**1.1 Quality Assurance (QA) Plan**		
		☐	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 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 months of experience in laboratory sciences.
		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	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 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 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	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	**29**		**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 Devises (RTDs) and Platinum Resistance Devices (PTDs).**
C	**11**	☐	**1.4.21 A mercury-in-glass 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 and 44.5°C (45.5°C for ETCP). These calibration records are maintained.**
K	9	☐	1.4.22 Standards thermometers are checked annually for accuracy by ice point determination. Results recorded and maintained. Date of most recent determination_____.
C	**29**	☐	**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 of≤ ±0.05°C are used as the laboratory standards thermometer. (*Circle the thermometer type used.*)**
K	13		1.4.24 Incubator and waterbath working thermometers are checked annually against the standards thermometer at the temperatures at which they are used. 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 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	**11, 30**		**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	11	☐	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	16		1.6.5	The autoclave standards thermometer is checked every five (5) years for accuracy at 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	1	☐	1.6.6	Working autoclave thermometers are checked against the autoclave standards thermometer at 121°C yearly. Date of last check_____Method _____
K	11	☐	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	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 in the range of 160 to 180°C.

K	9	☐	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	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 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 are properly sterilized. Method of sterilization _____
C	**2**	☐	**1.6.22 The sterility of the hardwood applicator transfer sticks 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 medium A-1 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 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. (C*ircle 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	11	☐	1.7.9 Media prepared from commercial dehydrated components are sterilized according to the manufacturer's instructions.
K	9	☐	1.7.10 The volume and concentration of media in the tube are suitable for the amount of sample inoculated.

C	**11**	☐	**1.7.11 Total time of exposure of sugar broths to autoclave temperatures does not exceed 45 minutes.**
C	**1**	☐	**1.7.12 Media sterility is determined for each load sterilized. Results are recorded and the records maintained.**
C	**1**	☐	**1.7.13 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.**
O	9	☐	1.7.14 Sterile phosphate buffered dilution water is used as the sample diluent.
K	11	☐	1.7.15 The pH of the prepared media is determined after sterilization to ensure that it is consistent with manufacturer's requirements. 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 7 days.
K	2		1.8.5 Storage under refrigeration of prepared culture media with loose fitting closures shall not exceed 1 month.
K	11	☐	1.8.6 Storage under refrigeration of prepared culture media with screw-cap closures does not exceed 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 collectors 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	**2**	☐	**2.2.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9, 33**	☐	**2.2.3 Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds** by hand or for 15 seconds when using a mechanical shaker**) before inoculation.**
C	**9**	☐	**2.2.4 In a multiple dilution series not less than 3 tubes per dilution are used (5**

				tubes are recommended).
C	**6**	☐	**2.2.5**	**In a single dilution series not less than 12 tubes are used (for depuration at least 5 tubes are used).**
C	**6**	☐	**2.2.6**	**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		2.2.7	Inoculated tubes are incubated in air at 35 ± 0.5°C.
C	**2**	☐	**2.2.8**	**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	☐	2.2.9	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.**
C	**2**	☐	**2.3.3**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11	☐	2.3.4	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**		**2.3.5**	**BGB tubes are incubated at 35 ± 0.5°C.**
K	9	☐	2.3.6	BGB tubes are read after 48 ± 3 hours of incubation.
C	**9**	☐	**2.3.7**	**EC tubes are incubated in a circulating waterbath maintained at 44.5 ± 0.2°C.**
C	**9**	☐	**2.3.8**	**EC tubes are read after 24 ± 2 hours of incubation.**
C	**9**	☐	**2.3.9**	**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, 31**		**2.5.2**	**A-1 medium without salicin is used in the analysis. Comparability testing**

				supports use of A-1medium without salicin. Study records are available.
C	**5**	☐	**2.5.3**	**A-1 medium sterilized for 10 minutes at 121°C.**
C	**2**	☐	**2.5.4**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**9, 33**	☐	**2.5.5**	**Sample and dilutions of sample are shaken vigorously (25 times in a 12" arc in 7 seconds** by hand or for 15 seconds when using a mechanical shaker**) before inoculation.**
C	**9**	☐	**2.5.6**	**In a multiple dilution series not less than 3 tubes per dilution are used (5 tubes are recommended).**
C	**6**	☐	**2.5.7**	**In a single dilution series at least 12 tubes are used.**
C	**6**	☐	**2.5.8**	**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**	☐	**2.5.9**	**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**	☐	**2.5.10**	**Inoculated tubes are placed in an air incubator at 35 ± 0.5°C for 3 ± 0.5 hours of resuscitation.**
C	**5**	☐	**2.5.11**	**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**	☐	**2.5.12**	**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 this 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 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	**2**		**2.9.2 The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
C	**23, 33**	☐	**2.9.3 The sample is shaken vigorously (25 times in a 12″ arc in 7 seconds** by hand or for 15 seconds when using a mechanical shaker**) before filtration.**
C	**23**	☐	**2.9.4 The membrane is placed grid side up within the sterile filter apparatus.**
C	**23, 25**	☐	**2.9.5 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**	☐	**2.9.6 Sample volumes are filtered under vacuum.**
K	26		2.9.7 The pressure of the vacuum pump does not exceed 15 psi.
C	**23, 26**	☐	**2.9.8 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**	☐	**2.9.9 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**	☐	**2.9.10 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**	☐	**2.9.11 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**	☐	**2.9.12 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 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**	☐	**2.9.13 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.**
		☐	**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	2,11	☐	3.2.1 Shucking knives, scrub brushes and blender jars are (autoclave) sterilized for 15 minutes prior to use.
O	2	☐	3.2.2 Blades of shucking knives are not corroded.

O	9	☐	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	☐	3.2.4	The faucet used for rinsing the shellstock does not contain an aerator.
K	9		3.2.5	Shellstock are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.2.6	Shellstock are allowed to drain in a clean container or on clean towels prior to opening.
K	9	☐	3.2.7	Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.2.8**	**Shellstock are not shucked directly through the hinge.**
C	**9**	☐	**3.2.9**	**Contents of shellstock (liquor and meat) are shucked into a sterile, tared blender jar or other sterile container.**
K	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.
O	9	☐	3.2.13	Sterile phosphate buffered dilution water is used as the sample diluent.
C	**9**		**3.2.14**	**Samples are blended at high speed for 60 to 120 seconds until homogenous.**
K	9	☐	3.2.15	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.)***
C	**2**	☐	**3.3.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9	☐	3.3.3	Immediately (within 2 minutes) after blending, the ground sample is diluted and inoculated into tubes of presumptive media.
C	**9**	☐	**3.3.4**	**No fewer than 5 tubes per dilution are used in a multiple dilution MPN series.**
C	**9**	☐	**3.3.5**	**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	☐	3.3.6	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**		**3.3.7**	**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	☐	3.3.8	Inoculated media are incubated at 35 ± 0.5°C.
K	10	☐	3.3.9	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.**
C	**2**		**3.4.2**	**The appropriate positive and negative productivity controls for the presumptive media are used. The results are recorded and the records maintained.** **Positive productivity control_____Negative productivity control _____**
K	9, 11	☐	3.4.3	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**	☐	**3.4.4**	**EC tubes are incubated in a circulating waterbath at 44.5 ± 0.2°C**
K	9		3.4.5	EC tubes are read for gas production after 24 ± 2 hours of incubation.
C	**9**	☐	**3.4.6**	**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 may be performed in conjunction with the analysis for fecal coliform organisms.
K	9	☐	3.6.2	In the standard plate count procedure at least four plates are used, duplicates of two dilutions. One of the dilutions should produce colonies of 30 to 300 per plate.
K	2	☐	3.6.3	Fifteen 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 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 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 Part 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/g 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 2 minutes of blending.**
C	**2,3**		**3.8.8 Six 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 plates.
C	**1**	☐	**3.8.11 Media and diluent sterility are determined with each use. Results are recorded and the records maintained.**
C	**1**	☐	**3.8.12 Media productivity is determined using media appropriate properly diluted pour plated positive and negative control cultures for each batch of Modified MacConkey agar prepared. Positive control culture_____Negative control culture _____**
C	**3, 13**	☐	**3.8.13 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**	☐	**3.8.14 Plates are stacked no more than three high in the incubator.**
C	**2**	☐	**3.8.15 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 Soft-shelled Clams and American Oysters for Male Specific Coliphage (MSC)	
		3.10 MSC Equipment and Supplies	
K	30	☐	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 100 – 125 mL.
C	**27, 28**	☐	**3.10.2 The refrigerated centrifuge used must have the capacity to accommodate the amount of shellfish sample required for the procedure, perform at 9000 x g and maintain a temperature of 4°C.**
K	9	☐	3.10.3 The level of water in the tempering bath covers the level of liquid and agar in the container or culture tubes.
C	**27, 28**	☐	**3.10.4 Sterile 0.22 µm pore size syringe filters and pre-sterilized plastic or sterile glass syringes are used to sterilize the antibiotic solutions.**
K	1	☐	3.10.5 The sterility of each lot of pre-sterilized syringes and syringe filters is determined. Results are recorded and records maintained.
K	1	☐	3.10.6 The sterility of each batch of reusable glass syringes is determined. Results are recorded and records maintained.
C	**27, 28**	☐	**3.10.7 The balance used provides a sensitivity of at least mg (0.01g.).**
C	**27, 28**		**3.10.8 The temperature of the incubator used is maintained at 36 ± 1°C.**

C	**28**	☐	**3.10.9 Sterile disposable 50 mL centrifuge tubes are used and their sterility is determined with each lot. Results are recorded and records maintained.**
		3.11 MSC Media Preparation	
K	28	☐	3.11.1 Media preparation and sterilization is according to the validated method.
K	27, 28		3.11.2 Bottom agar, double strength soft agar and growth broth are prepared from their individual components.
K	27, 28	☐	3.11.3 Soft agar is prepared double strength in volumes of 2.5 mL.
C	**27, 28**		**3.11.4 The streptomycin and ampicillin solutions are added to tempered bottom agar and vortex for 2 minutes on stir plate.**
O	27, 28	☐	3.11.5 Storage of the bottom agar under refrigeration does not exceed 1 month.
K	27, 28	☐	3.11.6 Unsterilized soft agar is stored at -20 °C -15C for up to 3 months.
K	27, 28		3.11.7 The soft agar is removed from the freezer and sterilized for 15 minutes at 121°C before use.
K	27, 28	☐	3.11.8 Storage of growth broth in the refrigerator in loosely capped tubes/bottles does not exceed 1 month and in screw capped tubes/bottles does not exceed 3 months.
K	27, 28	☐	3.11.9 Bottom agar plates are allowed to reach room temperature before use.
		3.12 Preparation of the Soft-Shelled Clams and American Oysters for MSC Analysis	
K	2,11	☐	3.12.1 Shucking knives, scrub brushes and blender jars are autoclave sterilized for 15 minutes prior to use.
O	2	☐	3.12.2 The blades of shucking knives are not corroded.
O	9		3.12.3 The hands of the analyst are thoroughly washed with soap and water immediately prior to cleaning the shells of debris.
O	2	☐	3.12.4 The faucet used for rinsing the shellfish does not contain an aerator.
K	9		3.12.5 The shellfish are scrubbed with a stiff, sterile brush and rinsed under tap water of drinking water quality.
O	9	☐	3.12.6 The shellfish are allowed to drain in a clean container or on clean towels unlayered prior to shucking.
K	9	☐	3.12.7 Immediately prior to shucking, the hands (or gloved hands) of the analyst are thoroughly washed with soap and water and rinsed in 70% alcohol.
C	**9**	☐	**3.12.8 Shellfish are not shucked through the hinge.**
C	**9**		**3.12.9 The contents of shellfish (liquor and meat) are shucked into a sterile,tared blender jar or other sterile container.**
K	9	☐	3.12.10 A representative sample of at least 12 shellfish is used for the analysis.
K	2, 19		3.12.11 The sample is weighed to the nearest 0.1 gram.
		3.13 MSC Sample Analysis	
C	**28**	☐	**3.13.1 E.coli *Famp* ATCC 700891 is the bacterial host strain used in this procedure.**
K	27, 28	☐	3.13.2 Host cell growth broth is tempered at 36 ± 1°C and vortexed (or shaken) to aerate prior to inoculation with host cells.
K	27, 28	☐	3.13.3 Several host cell colonies are transferred to a tube of tempered, aerated 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**	☐	**3.13.4 After inoculation, the host cell growth broth culture is not shaken.**
C	**28**	☐	**3.13.5 A 2:1 mixture of sterile growth broth to shellfish tissue is used for eluting the MSC.**
C	**28**	☐	**3.13.6 The elution mixture is prepared w/v by weighing the sample and adding two equal portions of sterile growth broth by volume to the shellfish tissue.**
C	**28**	☐	**3.13.7 The elution mixture is homogenized at high speed for 180 seconds.**
C	**28**		**3.13.8 Immediately after blending, 33 grams of the homogenized elution mixture**

			are weighed into centrifuge tubes.
C	**28**	☐	**3.13.9 The homogenized elution mixture is centrifuged for 15 minutes at 9000 x g at 4°C.**
C	**27, 28**	☐	**3.13.10 The supernatant is pipetted off, weighed and the weight recorded.**
C	**27, 28**	☐	**3.13.11 The supernatant is allowed to warm to room temperature about 20 to 30 minutes.**
K	27, 28	☐	3.13.12 The autoclaved soft agar is tempered and held at 51 ± 1°C throughout the period of sample analysis.
K	27, 28	☐	3.13.13 Two hundred microliters (0.2 mL) of log phase host strain *E coli* is added to the tempering soft agar immediately prior to adding the sample supernatant.
K	27, 28	☐	3.13.14 The sample supernatant is shaken or vortexed before being added to the tempering soft agar.
C	**27, 28**	☐	**3.13.15 2.5 mL of sample supernatant is added to each tube of tempering soft agar.**
C	**27, 28**		**3.13.16 The soft agar/sample supernatant/host cell mixture is gently rolled between the palms of the hands to mix.**
C	**27, 28**	☐	**3.13.17 The soft agar/sample supernatant/host cell mixture is overlaid onto bottom agar plates and swirled gently to distribute the mixture evenly over the plate.**
C	**28**	☐	**3.13.18 Ten (10) plates are used, 2.5 mL per plate for a total of 25 mL of supernatant analyzed per sample.**
K	27, 28	☐	3.13.19 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	☐	3.13.20 Growth broth is used as the negative control or blank.
K	27, 28		3.13.21 Type strain MS2 (ATCC 15597) male specific bacteriophage appropriately diluted to provide countable low levels of phage is used as the positive control.
K	2	☐	3.13.22 A negative control plate is plated at the beginning and end of each set of samples analyzed.
K	27, 28	☐	3.13.23 The positive control is plated after all the samples are inoculated and immediately prior to the final negative control.
C	**27, 28**	☐	**3.13.24 All plates are incubated at 36 ± 1°C for 18 ± 2 hours.**
		3.14 Computation of Results - MSC	
C	**27**	☐	**3.14.1 Circular zones of clearing or plaques of any diameter in the lawn of host bacteria are counted.**
C	**28, 32**	☐	**3.14.2 The working range of the method is 1 to 200 PFU per plate. When there are no plaques on all ten plates, the count 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	☐	3.14.3 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	9	☐	3.14.4 The MSC count is rounded off conventionally to give a whole number.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. "Interim Guides for the Depuration of the Northern Quahog, *Mercenaria mercenaria*." 1968. Northeast Marine Health Sciences Laboratory, North Kingstown, RI.

4. U.S. Department of Commerce. 1976. NBS *Monograph 150.* U.S. Department of Commerce, Washington, D.C.
5. Association of Official Analytical Chemists (AOAC). Current Edition. *Official Methods of Analyses of the Association of Official Analytical Chemists*. Official method 978.23. Chapter 17.305. AOAC Arlington, VA.
6. Wilt, D.S. (ed.). 1974. *Proceedings of the 8th National Shellfish Sanitation Workshop.* U.S. Food and Drug Administration, Washington, D.C.
7. U.S. Public Health Service (PHS). 1947. *Public Health Report*, Reprint #*1621.* PHS, Washington, D.C.
8. Association of Official Analytical Chemists (AOAC). 1991. *Quality Assurance Principles for Analytical Laboratories.* AOAC, Arlington, VA.
9. American Public Health Association (APHA). 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition. APHA, Washington, D.C.
10. Interstate Shellfish Sanitation Conference (ISSC). 1986. *Shellfish Sanitation Interpretation #SS-39.* ISSC, Columbia, S.C.
11. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Water and Wastewater*, 18th Edition. APHA/AWWA/WEF, Washington, D.C.
12. Title 21, Code of Federal Regulations, Part 58, *Good Laboratory Practice for Nonclinical Laboratory Study.* U.S. Government Printing, Washington, D.C.
13. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Dairy Products*, 16th Edition. APHA, Washington, D.C.
14. Fisher, J. 1985. Measurement of pH. *American Laboratory* 16:54-60.
15. Consult pH electrode product literature.
16. Association of Official Analytical Chemists (AOAC). 1999. *AOAC Methods Validation and Technical Programs - Criteria for Laboratories Performing Food Testing.* AOAC, Arlington, VA.
17. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA-670/9-75-006. U.S. EPA, Cincinnati, OH
18. Adams, W.N. 1974. NETSU. Personal communication to Dr. Wallace Andrews, FDA.
19. U.S. Food and Drug Administration (FDA).1995.*Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
20. U.S. Food and Drug Administration (FDA) and Interstate Shellfish Sanitation Conference (ISSC). 1997. *NSSP Guide to the Control of Molluscan Shellfish*. FDA/ISSC, Washington, D.C. and Columbia, S.C.
21. U.S. Environmental Protection Agency. 1978. *Microbiological Methods for Monitoring the Environment, Water and Wastes*. EPA/600/8/78/017. EPA, Washington, D.C.
22. Furfari, Santo. March 21, 1972. Personal Communication to Dan Hunt, FDA.
23. United States Environmental Protection Agency, *Improved Enumeration Methods for the Recreational Water Quality Indicators: Enterococci and Escherichia coli*. EPA/821/R-97-004, EPA, Washington, DC
24. Rippey, Scott, R, Adams, Willard, N, and Watkins, William, D. Enumeration of fecal coliforms and *E. coli* in marine and estuarine waters: an alternative to the APHA-MPN approach, Journal WPCF, 59, 8(1987).
25. FDA Manual of Interpretations, National Shellfish Sanitation Program *Guide for the Control of Molluscan Shellfish*, 2003 Revision, Interpretation Number 03-IV-@.02-102.
26. *Membrane filtration: A Users Guide and Reference Manual*, Thomas D. Brock, Science Tech Inc., Madison, WI, 1983.
27. Proceedings of the Male-specific Bacteriophage (MSC) Workshop, Gloucester, MA, March 9-12, 2004.
28. MSC Method and SLV write-up, Proposal 05-114 Spinney Creek Shellfish, Inc., September, 2009.
29. American Public Health Association. 1970. *Recommended Procedures for the Examination of Sea Water and Shellfish*, 4th Edition, APHA, New York, N.Y.
30. ASTM Manual on the Use of Thermocouples in Temperature Measurement, MNL-12 (ASTM, West Conshohocken, PA, 1993).
31. JOHN KAROLUS, MERCURIA CUMBO, SUSAN BOEHLER, and LAURA SAVINA. Modification of an Approved Medium for Fecal Coliform Detection in Seawater: A-1 Medium Minus Salicin. *Journal of Food Protection*: Vol. 66, No. 1, pp. 120–121.
32. MSC Method and SLV write-up, Proposal 13-120 Spinney Creek Shellfish, Inc., January, 2014.
33. Baird, R.B., Eaton, A. D., Rice, E.W, (2017). *Standard methods for the examination of water and wastewater.* 23rd ed. 2017. Washington, DC: American Public Health Association. p. 9-53.

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY OF NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	_____

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. October 2015

Proposal No. 19-143

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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	St. Petersburg, FL 33701
7. Phone	727-502-4891
8. Fax	
9. Email	leanne.flewelling@myfwc.com
10. Proposal Subject	MARBIONC Brevetoxin (Neurotoxic Shellfish Poisoning; NSP) ELISA Method Laboratory Evaluation Checklist
11. 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 Checklists
12. Text of Proposal/ Requested Action	The requested action is to adopt the text of the attached checklist for the MARBIONC Brevetoxin ELISA method 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.
13. Public Health Significance	The MARBIONC Brevetoxin ELISA method was approved for limited use at the 2017 ISSC meeting. Currently, there is no checklist adopted by the ISSC for this method. The attached checklist provides the quality assurance and method requirements that laboratory evaluation officers will use to evaluate laboratories implementing the MARBIONC Brevetoxin ELISA method 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.
14. 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. 240-402-2151/2055/4960 FAX 301-436-2601

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:

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	3, 6		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		**2. QA Plan is implemented.**
			1.2 Educational/Experience Requirements
C	State's Human Resources Department		**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. 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		**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		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. Adequate for workload and storage.
O	6		2. Clean and well lighted.
O	6		3. Adequate temperature control.
O	6		4. All work surfaces are nonporous and easily cleaned.
			1.4 Laboratory Equipment
O	4		1. The pH meter has a standard accuracy of 0.1 unit.
K	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		3. The pH meter is calibrated daily when in use. Results are recorded, and records are maintained.
K	6		4. Effect of temperature has been compensated for by an ATC probe, use of a triode or by manual adjustment.
K	6		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		6. Electrode acceptability is determined daily or with each use following either slope or millivolt procedure.

K	2, 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		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		9. Refrigerator temperature is maintained between 0 and 4 ºC.
K	6		10. Refrigerator temperature is monitored at least once daily. Results are recorded and records maintained.
K			11. Freezer temperature is maintained at -10 ºC or below.
K	6		12. Freezer temperature is monitored at least once daily. Results are recorded and records maintained.
C	9		**13. All in-service thermometers are properly calibrated and immersed.**
K	5		14. All glassware is clean.
C	**11**		**15. Absorbance Microplate reader equipped with filter for measurement at 450 nm is used.**
O			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		17. Autopipettors are calibrated for the appropriate volumes used and checked annually for accuracy. Results are recorded, and records are maintained.
O	11		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. All solvents and reagents used are ACS grade materials or better.**
O	6		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		3. Reagents are properly stored and labeled with the date of receipt, date opened or date prepared and expiration date.
C	**11**		**4. Brevetoxin-3 (BTX-3 or PbTx-3) provided with the MARBIONC ELISA kit is used as the reference standard.**
C	**11**		**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**		**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		7. Extraction solvent (80% methanol) is made by adding 800 ml of methanol to a 1L graduated cylinder and bringing the total volume to 1L with water.
K	11		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		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		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. Gelatin stock solution is aliquoted into smaller volumes (e.g. 15 ml centrifuge tubes) and refrigerated.
K	11		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		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**		13. **Stock and working standard solutions are stored -10 ºC or below.**
C	**5**		14. **All standards used are within expiration date (or 1 year if not provided).**
			1.6 Collection and Transportation of Samples
O	4, 1		**1.** Shellstock are collected in clean, waterproof, puncture resistant containers.
K	4, 1		**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		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		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**		**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**		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. The outside of the shell is thoroughly cleaned with fresh water.
O	4		3. Shellstock are opened by cutting the adductor muscles.
O	4		4. The inside surfaces of the shells are rinsed with fresh water to remove sand and other foreign materials.
O	4		5. Shellfish meats are removed from the shell by separating the adductor muscles and tissue connecting at the hinge.
C	4		6. **Damage to the body of the mollusk is minimized in the process of opening.**
O	4		7. Shucked shellfish are drained on a #10 mesh sieve or equivalent without layering for 5 minutes.

K	4		8. Pieces of shell and drainage are discarded.
C	2, 4		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		1. Sample homogenates are extracted as soon as possible (preferably the same day) or stored in the freezer.
C	**11**		**2. One (1) gram of homogenized sample is weighed into a 15 ml or 50 ml polypropylene centrifuge tube and subsequently extracted.**
C	**11**		**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**		**4. The homogenate/methanol mixture is centrifuged at a minimum of 3.000*xg* for 10 minutes.**
C	**11**		**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		6. Crude extracts are sealed tightly in glass vials and stored at -10 ºC or below until analyzed.
			2.3 Analysis
C	**11**		**1. Only high-binding flat-bottom plates no older than 1 year are used (e.g. Nunc Maxisorp Immunoplates).**
C	**2, 11**		**2. A standard calibration curve of seven concentrations (0.078-5 ng PbTx-3/ml) is included on each plate. Results are recorded, and records are maintained.**
C	**11**		**3. 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		4. Crude extracts are thoroughly mixed before withdrawing an aliquot for analysis.
C	**11**		**5. Crude extracts are diluted with PGT before analysis. The minimum dilution for shellfish extracts is 1:40 (25 ul + 975 ul PGT) (resulting in a sample dilution of 1:400).**
C	**11**		**6. For quantitative (actionable) results, serial dilutions (n=7) of each sample extract are assayed. Fewer dilutions are permissible for screening purposes only.**
C	**11**		**7. 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**		**8. 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 1 hour, the liquid is poured from the plate, and all wells are rinsed 3 times with 300 µl PBS-Tween.**
C	**11**		**9. Assay Step 3: Serial dilutions (n=7) of each crude sample extract and a standard calibration curve of seven concentrations (0.078-5.0 ng PbTx-**

			3/ml) are prepared in PGT.
C	**11**		**10. 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**		**11. 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**		**12. 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**		**13. 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		14. Plates are by covering covered with microplate sealing film during all incubation steps (Steps 1-6 above)
C	**11**		**15. Plates are protected from light by covering with aluminum foil during color development (Step 7 above).**
			2.4 Quality Control
C	**11**		**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. 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**		**1. Absorbance values are converted to % color inhibition:** **i. % 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. 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**		**3. The concentrations for sample dilutions falling within 30%-70% inhibition are interpolated from the standard curve.**
C	**11**		**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**		**5. If more than one dilution of a sample falls within 30%-70%, the mean of the two calculated concentrations is used.**
C	**8**		**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			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: _____

Proposal No. 19-144

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Thomas Howell
3. Affiliation	Spinney Creek Shellfish, Inc.
4. Address Line 1	27 Howell Lane
5. Address Line 2	
6. City, State, Zip	Eliot, ME 03903
7. Phone	207 451-8025
8. Fax	207 439-7643
9. Email	tlhowell@spinneycreek.com
10. 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.
11. 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
12. 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.
13. 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.
14. 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.

Proposal No. 19-145

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Guidance on cleansing studies
11. Specific NSSP Guide Reference	NSSP Section IV Chapter II .19 VI B.
12. Text of Proposal/ Requested Action	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, 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.
13. 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.
14. Cost Information	No additional cost. This is simply providing guidance for a requirement already in place.

Proposal No. 19-146

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. X Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	Leonora Porter - Spokesperson
3. Affiliation	Northeast Laboratory Evaluation Officers and Managers (NELEOM)
4. Address Line 1	205 N. Belle Mead Road
5. Address Line 2	Suite 1
6. City, State, Zip	East Setauket, NY 11733
7. Phone	(631) 444-0487
8. Fax	(631) 444-0472
9. Email	leonora.porter@dec.ny.gov
10. Proposal Subject	Micropipettor Verification
11. 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 Checklists, NSSP Laboratory Evaluation Checklists, 6. Shellfish Laboratory Evaluation Checklist for PCR Microbiology
12. Text of Proposal/ Requested Action	The requested action is to adopt the new text for the NSSP PCR Microbiology checklist, section 1.4 Laboratory Equipment item 1.4.24.
13. Public Health Significance	Quality Assurance and Standardization are integral to the validity of the NSSP laboratory. One QA component includes verifying the measurement accuracy of pipetting instruments including micropipettors. There are no recognized references that state micropipettors must receive third party certifications. There is no indication as to what "Level" calibration should exist. The reference for this item is only **#2, Good Laboratory Practice**. Accuracy measurement assurance should be based on workload and use, not calendar year. Pipette calibration values on certificates obtained in a calibration laboratory (known as a controlled laboratory) do not accurately transfer to the NSSP laboratory and therefore do not provide assurance and defensibility. A pipette's measurement accuracy is influenced by its *physical uncertainty*, *environmental uncertainty* (i.e., temperature, vibration and humidity) and *operator use uncertainty*. These uncertainties will differ between laboratories. Pipette performance in the NSSP (non-controlled laboratories) is impacted by the temperature and viscosity of the fluid, the skill of the operator and choice of tip. Conducting in-house verifications for each operator, using a verified balance provides a better assessment of the actual measurement accuracy of what the pipet is delivering. When the uncertainty of measurement exceeds the stated laboratory established threshold, adjustments are made. As a component of a Laboratory's Quality Management System, the individual laboratory can institute legally defensible and measurement assurance practices appropriate for the laboratory's workload, testing and ambient conditions. Savings: Calibration Cost Information from one Pipet Manufacturer: 1. Calibration and Maintenance - Offers three "levels" of examination, with an

	assorted number of readings at 3 volumes, across different channel pipettors. Cost Range $30 - $225 per unit. 2. Calibration only (center channel only) - $30 - $180 if unit passed on the initial attempt. Non-Operational pipette repair evaluation (no calibration and parts additional cost) starting at $28/unit.
14. 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- 402-2151/2055/4960 FAX 301-436-2601
CFSANDSSLEOS@FDA.HHS.GOV

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:**

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

Check the applicable analytical methods:

	MPN Real-time PCR method for *Vibrio vulnificus* detection in Oysters [PART III] SmartCycler II
	MPN Real-time PCR method for *Vibrio parahaemolyticus* detection in Oysters [PART III] SmartCycler II and AB 7500 Fast

PART I – Quality Assurance			
ITEM			
CODE	**REF**		
		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).**
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, 23		1.4.24 Micropipettors are ~~calibrated~~ verified annually at appropriate volumes used and checked for accuracy ~~quarterly~~as needed. Adjustment ~~R~~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 heterotropic 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'-GATGAGCGGTTGATGTCCAAA-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
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.

REFERENCES

1. American Public Health Association 1984. *Compendium of Methods for the Microbiological Examination of Foods*, 2nd Edition. APHA, Washington, D.C.
2. Good Laboratory Practice.
3. U.S. Department of Commerce. 1976. *NBS Monograph 150*. U.S. Department of Commerce, Washington, D.C.
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.
8. American Public Health Association (APHA). 1992. *Standard Methods for the Examination of Diary Products*, 16th Edition. APHA, Washington, D.C.
9. Fisher, J. 1985. Measurement of pH. American Laboratory 16:54 – 60.
10. Association of Official Analytical Chemists (AOAC). 1999. AOAC *Methods Validation and Technical Programs – Criteria for Laboratories Performing Food Testing*. AOAC, Arlington, Va.
11. U.S. Environmental Protection Agency (EPA). 1975. *Handbook for Evaluating Water Bacteriological Laboratories*. EPA – 670/9-75-006. U.S. EPA, Cincinnati, Ohio.
12. Adams, W.N. 1974. NETSU. Personal Communication to Dr. Wallace Andrews, FDA.
13. U.S. Food and Drug Administration (FDA). 1995. *Bacteriological Analytical Manual*. U.S. FDA, 8th Edition, AOAC, Arlington, VA.
14. Campbell, Mark, S. and Wright, Anita, C. Real-time PCR analysis of *Vibrio vulnificus* from oysters, Appl Environ Microbiol. 69, 12 (2003).
15. Wright, Anita, C., Garrido, V, Debuex, G, Farrell-Evans, M, Mudbidri, A, A. and Otwell, W, S. Appl Environ Microbiol. Evaluation of postharvest-processed oysters by using PCR-based most-probable-number enumeration of *Vibrio vulnificus* bacteria. 73, 22 (2007).
16. Integrated DNA Technologies. Oligonucleotide Stability Study. 2014.
17. Section IV Guidance Documents, Naturally Occurring Pathogens, NSSP *Guide for the Control of Molluscan Shellfish*, 2009 Revision.
18. Nordstrom, J.L., M.C.L. Vickery, G.M. Blackstone, S.L. Murray, and A. DePaola. 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic V. parahaemolyticus bacteria in oysters. Appl. Environ. Microbiol. 73(18):5840-5847.
19. Kinsey, T.P., K.A. Lydon, J.C. Bowers, J.L. Jones. 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): 1574-1580.
20. National Institute of Standards and Technology Special Publication 250-23, 128 pages (Sept. 1988) U.S. Government Printing office, Washington, D.C. Library of Congress Catalog Number: 88-6000580.
21. Integrated Solutions — Real-Time PCR Applications: Critical Factors for Successful Real-Time PCR. www.qiagen.com
22. FDA Reagent Stability Study, unpublished. 2013.
23. Artel Lab Report eBook – your guide to liquid handling data quality. By Artel | Lab Report. 25 Bradley Drive, Westbrook, Maine 04092. Accessed June 5, 2019

LABORATORY:	**DATE of EVALUATION:**

SHELLFISH LABORATORY EVALUATION CHECKLIST

SUMMARY of NONCONFORMITIES

Page	Item	Observation	Documentation Required

LABORATORY STATUS	
LABORATORY	**DATE**
LABORATORY REPRESENTATIVE:	
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, Key and Other (O) Nonconformities in Parts I-III	

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**

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:_____

LABORATORY:

Page	Item	Observation

Proposal No. 19-147

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	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. Fax	301-436-2601
9. Email	Melissa.Abbott@fda.hhs.gov
10. Proposal Subject	Relay contaminant reduction studies.
11. Specific NSSP Guide Reference	Section II. Model Ordinance Chapter V. Shellstock Relaying Section @.02 Contaminant Reduction B. (2)
12. Text of Proposal/ Requested Action	(2) Contaminant levels of poisonous or deleterious substances in shellstock do not exceed FDA ~~tolerance~~action levels, tolerances and/or guidance levels and/or levels that are deemed safe through risk evaluation; or
13. Public Health Significance	Action levels, tolerances and/or guidance levels have not been established for all poisonous or deleterious substances. When there is concern about contamination of shellstock by a poisonous or deleterious substance and no action level, tolerance, or guidance level for that substance, regulators must evaluate risk and establish a level of concern. Suggested change from "tolerance" to "action levels, tolerances, and/or guidance levels" is made to make the language consistent with the title of National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish, Section IV Guidance Documents, Chapter II Growing Areas, .08 Action Levels, Tolerances and Guidance Levels for Poisonous or Deleterious Substances in Seafood.
14. Cost Information	Possible increased cost of unknown magnitude related to time necessary to conduct risk evaluations.

Proposal No. 19-148

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Executive Office
3. Affiliation	Interstate Shellfish Sanitation Conference
4. Address Line 1	209 Dawson Road
5. Address Line 2	Suite 1
6. City, State, Zip	Columbia, SC 29223
7. Phone	(803) 788-7559
8. Fax	(803) 788-7576
9. Email	issc@issc.org
10. Proposal Subject	Correct language of MO to reflect current checklists
11. Specific NSSP Guide Reference	Section II Model Ordinance – Chapter I. Shellfish Sanitation Program for the Authority @.03 Evaluation of Shellfish Sanitation Program Elements B. Criteria for evaluation of shellfish sanitation program elements shall be as follows: 1. Laboratory
12. Text of Proposal/ Requested Action	Section II Model Ordinance – Chapter I. Shellfish Sanitation Program for the Authority @.03 Evaluation of Shellfish Sanitation Program Elements 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. (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. (a) 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 technical.y evaluation and will be assigned the designation of conforms, provisionally conforms or non-confomance. 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

~~(b) Conforms. In order to achieve or maintain conforming status under the NSSP, a laboratory must meet the following laboratory evaluation criteria:~~

~~(c) No critical nonconformities in the microbiological or marine biotoxin component under evaluation have been identified using the appropriate NSSP Shellfish Laboratory Evaluation Checklist; and~~

~~(d) (b) Not more than thirteen (13) key nonconformities in the microbiological component or six (6) in the marine biotoxin components have been identified using the appropriate NSSP Shellfish Laboratory Evaluation Checklist; and~~

~~(c) Not more than eighteen (18) critical, key, and other nonconformities in total in the microbiological component, twelve (12) critical, key and other nonconformities in total for the paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) components, or ten (10) critical, key and other~~

~~nonconformities in total for the neurotoxic shellfish poisoning (NSP) component have been identifiedusing the appropriate NSSP Shellfish Laboratory Evaluation Checklist. This number must not exceed the numerical limits established for either the critical or key criteria; and~~

~~(d) No repeat key nonconformities have been identified in the microbiological or marine biotoxin component under evaluation in consecutive evaluations using the appropriate NSSP Shellfish Laboratory Evaluation Checklist.~~

~~iii. Technical Evaluation: Provisionally Conforms. In order to be deemed provisionally conforming under the NSSP, a laboratory must meet the following laboratory evaluation criteria:~~

~~(a) Not more than three (3) critical nonconformities in the microbiological component, four (4) in the PSP and ASP components, or three (3) in the NSP component have been identified using the appropriate NSSP Shellfish Laboratory Evaluation Checklist; and~~

~~(b) Not more than thirteen (13) key nonconformities in the microbiological component or six (6) in the marine biotoxin component have been identified using the appropriate NSSP Shellfish Laboratory Evaluation Checklist; and~~

~~(c) Not more than eighteen (18) critical, key and other nonconformities in total in the microbiological component, or twelve (12) critical, key and other nonconformities in total in the PSP and ASP components or ten (10) critical, key and other nonconformities in total in the NSP component have been identified using the appropriate NSSP Shellfish Laboratory Evaluation umber must not exceed the numerical limits established for either the critical or key criteria; and~~

~~(d) Not more than one (1) repeat key nonconformity has been identified in the microbiological or marine biotoxin component under evaluation in consecutive evaluations using the appropriate NSSP Shellfish Laboratory Checklist.~~

~~iv. Technical Evaluation: Nonconformance. When~~

	~~a laboratory exceeds the following criteria, it will be determined to be in nonconformance:~~ (a) ~~More than three (3) critical nonconformities in the microbiological component or four (4) in the PSP and ASP components, or three (3) in the NSP component have been identified using the appropriate NSSP Shellfish Laboratory Checklist; or~~ ~~(b) More than thirteen (13) key nonconformities in the microbiological component or six (6) in the marine biotoxin component have been identified using the appropriate NSSP Shellfish Laboratory Evaluation Checklist;~~ ~~(c) More than eighteen (18) critical, key, and other nonconformities in total in the microbiological component, or more than twelve (12) critical, key and other nonconformities in total in the PSP and ASP components, or more than ten (10) critical, key, and other nonconformities in total in the NSP component have been identified using the appropriate NSSP Shellfish Laboratory Evaluation Checklist; or~~ ~~(d) One (1) or more repeat critical or two (2) or more repeat key nonconformities have been identified in consecutive evaluations in either the microbiological or marine biotoxin components using the appropriate NSSP Shellfish Laboratory Evaluation Checklist.~~
13. Public Health Significance	The goal of a laboratory evaluation is to monitor implementation of NSSP Quality Systems and Approved methods. Laboratory data is standardized as a result of this process and reciprocity of shellfish in the commercial market is protected and preserved through defensible practices and transparent requirements. As the laboratory program in the NSSP continues to develop and grow it is prudent to keep requirements in accessible documents with few deviations. Checklists are a cornerstone document for laboratories, referring to these documents ensures laboratories have access to requirements at all times. As laboratorians are the target audience, this is the most sensible place for the actual numbers of nonconformities to reside, and the reference to the checklists in the Model Ordinance ensures the checklists are part of the overarching document adopted by reference or into legislation. Multiple locations of numbers of permissible nonconformities only ensures updates will be missed. As existing structure is in place through the Lab Committee to handle checklists and edits therein, this seems the most reasonable solution.
14. Cost Information	No cost incurred by change. Practice is already in place.
15. Research Needs Information (Optional)	

a. Proposed specific research need/ problem to be addressed	none
b. Explain the relationship between proposed research need and program change recommended in the proposal	**There is no research need to implement proposal recommendation. This is a change requested to reflect language that exists in the MO. The language changes proposed have not been changed as new Checklists were introduced and the numbers of Critical key and other nonconformities are not constant. Therefore, it makes sense to refer to the checklist rather than continue to have to occasionally update arbitrary numbers in Chapter 1. This will save time and money in the future as more checklists are introduced. Checklists have a great deal of attention by the Lab Committee, in fact, they have a subcommittee dedicated entirely to their drafting or editing. Any questions would be answered here.**
c. Estimated cost	**none**
d. Proposed sources of funding	**N/A**
e. Time frame anticipated	**N/A**
For Research Guidance Committee Use Only	Relative priority rank in terms of resolving research need ☐ Immediate ☐ Required ☐ Valuable ☐ Important ☐ Other

Proposal No. 19-149

Proposal for Task Force Consideration at the ISSC 2019 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative
2. Submitter	ISSC Executive Office
3. Affiliation	Interstate Shellfish Sanitation Conference
4. Address Line 1	209 Dawson Road
5. Address Line 2	Suite 1
6. City, State, Zip	Columbia, SC 29223
7. Phone	(803) 788-7559
8. Fax	(803) 788-7576
9. Email	issc@issc.org
10. Proposal Subject	Biotoxin Guidance
11. Specific NSSP Guide Reference	Section II. Chapter IV Shellstock Growing Areas
12. Text of Proposal/ Requested Action	In conjunction with the adoption of Proposal 13-116 at the 2017 ISSC Biennial Meeting, the voting delegates recommended the Biotoxin Committee develop a guidance document to include guidance for end product testing programs in closed state waters. In addition to proposing guidance, the committee will be making recommendations to modify the monitoring requirements of Chapter IV @.04 Marine Biotoxin Control. These proposed changes are under development. The purpose of this proposal is to advise the ISSC membership that the Biotoxin Committee will be making recommendations to modify Chapter IV @.04 as part of their committee charge from Proposal 13-116
13. Public Health Significance	The proposed changes should clarify and simplify biotoxin monitoring.
14. Cost Information	

Proposal No. 19-150

Proposal for Task Force Consideration at the ISSC 2017 Biennial Meeting	1. a. ☒ Growing Area b. ☐ Harvesting/Handling/Distribution c. ☐ Administrative

2. Submitter	Brooke Roman
3. Affiliation	Neogen Corporation
4. Address Line 1	620 Lesher Place
5. Address Line 2	
6. City, State, Zip	Lansing, MI 48912
7. Phone	1-800-234-5333
8. Fax	1-517-372-2006
9. Email	broman@neogen.com
10. Proposal Subject	Neogen's 'Reveal 2.0 for PSP' for detection of PSP
11. Specific NSSP Guide Reference	Section IV. Guidance Documents, Chapter II. Growing Areas, .11 Approved NSSP Laboratory Tests
12. 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.
13. 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
14. Cost Information	Approximately $20 per test. Reader based assay – approximate cost of reader is $2,700.00 USD.