Proposal No. **17-107**

Submitter	Leanne J. Flewelling Florida Fish and Wildlife Conservation Commission leanne.flewelling@myfwc.com
Proposal Subject	ISSC Method Application and Single Lab Validation of an Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in hard clams, sunray venus clams, and oysters.
Specific NSSP Guide Reference	Section IV. Guidance Documents Chapter II. Growing Areas. 14 Approved NSSP Laboratory Tests
Text of Proposal/ Requested Action	This submission proposes that the MARBIONC brevetoxin ELISA be approved for limited use in NSP testing such that samples with negative results by ELISA (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) would pass, while samples with positive results by ELISA (greater than these levels) would require additional testing by an Approved Method. Samples passing by ELISA would enable the same management actions as samples passing by NSP mouse bioassay (i.e., Growing Area closing or re-opening, controlled relay, and end product testing of controlled harvest as permitted within a State Authority's marine biotoxin contingency program). Samples failing by ELISA would either require additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method. ELISA could also be used as a screening method to initiate precautionary closures. Requested changes: Section IV. Guidance Documents Chapter II. Growing Areas. 14 Approved NSSP Laboratory Tests 4. Approved Limited Use Methods for Marine Biotoxin Testing Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP) Add columns for Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP) and for Application: Controlled Harvest end product testing Add MARBIONC brevetoxin ELISA to table for all applications except Dockside Testing with the following footnote: MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters: a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area. b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.80 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method. See attached proposed revisions to Table 4. Approved Limited Use Methods for Marine Biotoxin Testing

Public Health Significance

Brevetoxins produced by K. brevis are toxic to humans. Filter-feeding bivalves accumulate brevetoxins during blooms, and ingestion of contaminated shellfish can cause NSP in humans. Symptoms of NSP typically begin three to six hours after ingestion and may include nausea, diarrhea, tingling of lips or tongue, muscle ache, lack of coordination, temperature reversal, and vertigo. In severe cases, a feeling of constriction in the throat may occur. Individuals with NSP may require hospitalization but usually recover within days. To prevent NSP, shellfish harvesting areas are closed when K. brevis concentrations exceed 5,000 cells/L and are re-opened once K. brevis levels decrease and testing demonstrates that shellfish are no longer toxic. However, the APHA mouse bioassay - the only approved method for NSP testing - has many drawbacks, and the delays caused by the time required to analyze samples (two days) and low sample throughput compound economic losses. To mitigate economic harm to the shellfish industry and ensure the continued protection of public health, rapid alternative methods for NSP testing are needed.

Cost Information

Kit reagents are sold in bulk. The cost of reagents is currently $2,400 for 15 plates and $1,000 for 5 plates. The cost of additional consumables and reagents not included is approximately $20 per plate. Therefore cost per sample is $36-44 for full quantitation (5 samples per plate) and less than $6 per sample for qualitative screening (40 samples per plate).

Action By 2017 Laboratory Committee

Recommended adoption of Proposal 17-107 as submitted.

Action By 2017 Task Force I

Recommended adoption of Proposal 17-107 as amended:

This submission proposes that the MARBIONC brevetoxin ELISA be approved for limited use in NSP testing such that samples with negative results by ELISA (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) would pass, while samples with positive results by ELISA (greater than these levels) would require additional testing by an Approved Method. Samples passing by ELISA would enable the same management actions as samples passing by NSP mouse bioassay (i.e., Growing Area closing or re-opening, controlled relay, and end product testing of controlled harvest as permitted within a State Authority's marine biotoxin contingency program). Samples failing by ELISA would either require additional testing by an Approved Method to support management actions~~or could support the same management actions as samples failing by an Approved Method~~. ELISA could also be used as a screening method to initiate precautionary closures. 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.

Requested changes:

Section IV. Guidance Documents Chapter II. Growing Areas. 14 Approved NSSP Laboratory Tests

4. Approved Limited Use Methods for Marine Biotoxin Testing Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP)

Add columns for Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP) and for

Application: Controlled Harvest end product testing

Add MARBIONC brevetoxin ELISA to table for all applications except Dockside Testing with the following footnote:

MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters:
a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.
b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.80 ppm in oysters) requires additional testing by an Approved Method or to support management actions~~could support the same management actions as samples failing by an Approved Method.~~

See attached proposed revisions to Table 4. Approved Limited Use Methods for Marine Biotoxin Testing

Action by 2017 General Assembly: Adopted the recommendation of Task Force I on Proposal 17-107.

4. Approved Limited Use Methods for Marine Biotoxin Testing

	Biotoxin Type: Amnesic Shellfish Poisoning (ASP)	Biotoxin Type: Paralytic Shellfish Poisoning (PSP)	Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP)	Application: Growing Area Survey & Classification Sample Type: Shellfish	Application: Dockside Testing Program Sample Type: Shellfish	Application: Controlled Relaying Sample Type: Shellfish	Application: Controlled Harvest end product testing Sample Type: Shellfish
Abraxis Shipboard ELISA[3]		X			X		
JRT[2]		X		X	X	X	
HPLC[1]	X			X		X	
Reveal 2.0 ASP[4]	X			X	X	X	
RBA[5]		X		X	X	X	
MARBIONC Brevetoxin ELISA[6]			X	X		X	X

Footnotes:

[1]M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Biosciences, Technical Report #64, National Research Council Canada #33001. This method may also be used direct without cleanup.

[2]Jellett Rapid Test for PSP, Jellett Rapid Testing Ltd.

a. Method can be used to determine when to perform a mouse bioassay in a previously closed area.
b. A negative result can be substituted for a mouse bioassay to maintain an area in the open status.
c. A positive result shall be used for a precautionary closure.

[3]Saxitoxin (PSP) ELISA Kit. Method can be used in conjunction with rapid extraction method using 70% isopropanol (rubbing alcohol): 5% acetic acid (white vinegar) 2.5:1. ISSC Summary of Actions, Proposal 05-111 (page 15) and 09-107 (page 140).

[4]Reveal 2.0 ASP. Neogen Corporation. Screening Method for Qualitative Determination of Domoic Acid Shellfish. ISSC 2013 Summary of Actions Proposal 13-112.

[5]Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination. Dr. Fran Van Dolah. Method for Clams and Scallops for the Purpose of Screening and Precautionary Closure for PSP. ISSC 2013 Summary of Actions Proposal 13-114

[6]MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters:

a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.
b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.80 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method.

ISSC 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	Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish
Name of the Method Developer	The ELISA Kit was developed by UNCW and is sold through MARBIONC. The method was optimized and submitted for use with molluscan shellfish by Leanne Flewelling, Florida Fish and Wildlife Conservation Commission.
Developer Contact Information	Florida Fish and Wildlife Conservation Commission 100 8th Avenue SE St. Petersburg, FL 33701 (727) 502-4891 leanne.flewelling@myfwc.com

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		Blooms of the dinoflagellate *Karenia brevis* threaten the productive Gulf of Mexico shellfish industry. Brevetoxins produced by *K. brevis* are toxic to humans and can result in Neurotoxic Shellfish Poisoning (NSP) if contaminated shellfish are eaten. To prevent NSP, shellfish harvesting areas (SHAs) are closed when *K. brevis* concentrations exceed 5,000 cells/L and are re-opened once *K. brevis* levels decrease and testing demonstrates that shellfish are no longer toxic. This biotoxin plan successfully prevents occurrences of NSP from lawfully harvested shellfish, but NSP closures come at a steep economic cost to the shellfish industry. The APHA mouse bioassay - the only NSSP approved method for regulatory NSP testing - has many drawbacks. The delays caused by the time required to analyze samples (two full days) and very low sample throughput delay re-openings and add to economic losses. The assay is nonspecific, imprecise, and not calibrated against known levels of brevetoxins. It is costly in terms of labor and supplies, and the use of live animals is both undesirable and increasingly unacceptable. To mitigate economic harm to the shellfish industry and ensure the continued protection of public health, rapid alternative methods for NSP testing are needed. Among the many chemical and biological methods developed for brevetoxin detection, enzyme-linked immunosorbent assays (ELISAs) have performed well. The method proposed here was the first commercially-available brevetoxin ELISA to be offered. The assay uses goat anti-brevetoxin antibodies developed by Trainer and Baden (1991) and is based on the indirect competitive assay developed in 2002 by Naar et al. (2002). The kit is marketed by MARBIONC Development Group (MDG), which is based at the University of North

		Carolina at Wilmington. This assay is widely and routinely used to monitor brevetoxins in Florida's marine systems and to diagnose human, marine mammal, and other animal exposure to brevetoxins. This method is much faster than the mouse bioassay, more user-friendly, more sensitive, more specific to brevetoxins, less expensive, and does not involve the use of live animals.
2. What is the intended purpose of the method?		The proposed use for the MARBIONC ELISA is as a Limited Use Method for determination of NSP toxin levels in hard clams, sunray venus clams, and oysters. Applications include Growing Area Survey & Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing as permitted within a State Authority's marine biotoxin contingency program. We propose that the ELISA be approved for limited use in NSP testing such that samples with negative results by ELISA (≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters, at or below the estimated equivalent to one-half the 20 MU/100 g guidance level) would pass, while samples with positive results by ELISA (greater than these levels) would require additional testing by an Approved Method (currently, the NSP mouse bioassay). Samples passing by ELISA would enable the same management actions as samples passing by NSP mouse bioassay including: Growing Area Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing. Samples failing by ELISA would either require additional testing by NSP mouse bioassay or could support the same management actions as samples failing by NSP mouse bioassay. ELISA could also be used as a screening method to initiate precautionary closures.
3. Is there an acknowledged need for this method in the NSSP?		Yes, the ISSC Laboratory Committee has specified the need for qualitative or semi-quantitative (screening) and quantitative/confirmatory methods of analysis for all toxins and for each commercially-harvested bivalve species.
4. What type of method? i.e. chemical, molecular, culture, etc.		ELISA is a biological method that uses biological components (antibodies) to detect toxins. Detection relies on structural recognition of a region of the toxin molecule shared by PbTx-2-type brevetoxins (the most abundant forms) and provides an overall estimate of toxin content.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish.
Method Scope		This ELISA is a high-throughput, sensitive, accurate, quantitative assay for NSP toxins in shellfish. The method is being submitted for consideration as an NSSP Approved Limited Use Method for the purposes of screening for NSP toxins in hard clams, sunray venus clams, and oysters.
References		Original method reference: Naar J, Bourdelais A, Tomas C, Kubanek J, Whitney PL, Flewelling LJ, Steidinger KA, Lancaster J, Baden DG. 2002. A competitive ELISA to detect brevetoxins from

		Karenia brevis (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ Health Perspect 110(2):179-185. Antibody development reference: Trainer VL, Baden DG. 1991. An enzyme immunoassay for the detection of Florida red tide brevetoxins. Toxicon 29(11):1387-1394. Epitope identification reference: Melinek R, Rein KS, Schultz DR, Baden DG. 1994. Brevetoxin PbTx-2 immunology: differential epitope recognition by antibodies from two goats. Toxicon 32(8):883-90. Other relevant publications: Dickey RW, Plakas SM, Jester ELE, El Said KR, Johannessen JN, Flewelling LJ, Scott P, Hammond DG, Dolah FMV, Leighfield TA, Dachraoui M-YB, Ramsdell JS, Pierce RH, Henry MS, Poli MA, Walker C, Kurtz J, Naar J, Baden DG, Musser SM, White KD, Truman P, Miller A, Hawryluk TP, Wekell MM, Stirling D, Quilliam MA, Lee JK. 2004. Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. p. 300-302. Plakas SM, Wang Z, El-Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44:677-685. Plakas SM, Jester EL, El Said KR, Granade HR, Abraham A, Dickey RW, Scott PS, Flewelling LJ, Henry M, Blum P, Pierce R. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (Crassostrea virginica). Toxicon 52(1):32-8. Abraham A, El Said KR, Wang Y, Jester EL, Plakas SM, Flewelling LJ, Henry MS, Pierce RH. 2015. Biomarkers of brevetoxin exposure and composite toxin levels in hard clam (Mercenaria sp.) exposed to *Karenia brevis* blooms. Toxicon 96:82-88.
Principle		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. 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 horse radish peroxidase (HRP)-linked secondary antibodies, and addition of an HRP substrate (3,3'5,5'-Tetramethylbenzidine), which yields a blue color that changes to yellow (Amax = 450nm) upon addition of a sulfuric acid stop solution. The intensity of this color is inversely proportional to the amount of brevetoxin present in the well during incubation. Using this method, one ELISA plate can be used to quantitatively assay five shellfish samples. For quick screening, more samples can be run on one plate

		(up to 40).
Any Proprietary Aspects		Methods of production of key kit reagents (brevetoxin-BSA conjugate and anti-brevetoxin antibodies) are proprietary (MDG).
Equipment Required		Equipment required: Balance capable of measuring to 0.1g Number 10 sieve Laboratory blender Vortex mixer Centrifuge capable of 3,000x*g,* with rotor for 15 mL Microplate reader with filter for measurement at 450 nm Multichannel pipettor (50-200 µL) Individual pipettors (10-1000 µL) Orbital microplate shaker Refrigerator/freezer Consumables required: Disposable glass test tubes Disposable plastic dilution tubes (96-well cluster format) 15-ml and 50-ml polypropylene centrifuge tubes Nunc flat-bottom polystyrene 96-well Maxisorp Immunoplates (- substitution NOT recommended) Microplate sealing film Assorted pipet tips Solution basins Aluminum foil
Reagents Required		Included in MARBIONC ELISA Kit: • Reagent A: BSA-linked PbTx-3 • Reagent C: Goat anti-brevetoxin Ab • Reagent D: HRP-linked anti-goat secondary Ab • Brevetoxin standard (PbTx-3) Reagents required but not included: • Methanol • Reagent B: Superblock Blocking Buffer • Phosphate Buffered Saline, pH 7.4 • Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 • Gelatin • 3,3'5,5'-Tetramethylbenzidine (TMB) • Sulfuric acid stop solution (H_2SO_4, 0.5M) • Nanopure water (or equivalent quality water)
Sample Collection, Preservation and Storage Requirements		At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection, shellfish should be placed in dry storage between 0 and 10°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 sucked 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 (see protocol in Appendix A). Samples must be processed within 24 hours of shucking.
Safety Requirements		General chemical safety requirements (e.g., personal protective equipment including gloves, safety glasses,

		and laboratory coat) must be followed.
Clear and Easy to Follow Step-by-Step Procedure		See protocol detailed in Appendix A.
Quality Control Steps Specific for this Method		Acceptance of assay results is dependent on meeting the following criteria: Absorbance of reference wells (Amax) must be ≥ 0.6. %CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (20-70% inhibition) must be < 20%. 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; 20-70% inhibition) must be <20%. %CV of calculated concentrations of different sample dilutions within the linear range of the assay must be <20%.
C. Validation Criteria		
1. Accuracy / Trueness		Accuracy /trueness was determined by calculating the closeness of agreement between the test results and targeted value. Calculated % accuracy/trueness: Oysters: 96.27% Hard Clams: 98.39% Sunray Venus Clams: 95.12% Data and details in Appendix B
2. Measurement Uncertainty		Two-sided, 95% confidence intervals for the difference in concentrations between the reference and the spiked samples: Oysters: -0.0057 – 0.1137 Hard Clams: 0.0603 – 0.1898 Sunray Venus Clams: 0.0783 – 0.2487 Data and details in Appendix B
3. Precision Characteristics (repeatability and reproducibility)		Repeatability was assessed using duplicate determinations of 10 samples spiked with PbTx-3 to three levels (0.4, 1, and 4 ppm). %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams. Data and details in Appendix C
4. Recovery		The recovery of the method was consistent over the range of concentrations examined to determine Precision. The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams. Data and details in Appendix C
5. Specificity		Potentially interfering substances examined in this study included three types of microalgae (two types commonly used as food for hatchery raised bivalves and a non-brevetoxin producing *Karenia* species) as well as okadaic acid (a potentially co-occurring polyether dinoflagellate toxin). Two-sided t-tests indicated no significant difference in brevetoxin measurements in the presence or absence of these substances. Data and details in Appendix D
6. Working and Linear Ranges		The overall or dynamic linear range of this method results from a combination of the linear range of the assay standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate. The linear range of the ELISA standard curve varied slightly among two lots of kit reagents examined. One lot yielded a range of 0.21-1.04 ng PbTx-3/mL and a second lot yielded a range of 0.30-1.38 ng PbTx-3/mL. The overall or dynamic linear range of the method as

		described for this proposal (in PbTx-3 equivalents) is from 0.12 ppm to 26.62 ppm for the June 2014 kit lot and up to 35.33 ppm for the June 2016 kit lot. Data and details in Appendix E
7. Limit of Detection		The calculated assay LOD is 0.1 ng/mL. At the lowest sample dilution of 1:400, the LOD for brevetoxin in shellfish is 0.04 ppm. Data and details in Appendix E
8. Limit of Quantitation / Sensitivity		The calculated assay LOQ is 0.3 ng/mL. At the lowest sample dilution of 1:400, the LOQ for brevetoxin in shellfish is 0.12 ppm. Data and details in Appendix E
9. Ruggedness		Results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min). Significant differences were observed only with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). 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 of 1.0 ± 30%. Data and details in Appendix F
10. Matrix Effects		Brevetoxin-free samples (10 samples per species) for this study were obtained from shellfish harvest areas along Florida's Gulf coast that infrequently experience *K.. brevis* blooms during periods when *K. brevis* was verified to be absent. Farmed hard clams and sunray venus clams were sourced from Cedar Key, FL and were provided by a Shellfish Aquaculture Extension Agent and as well as local clam farmers. Hard clams were collected from 10 different locations over four days. Sunray venus clams were collected from two locations over six days. Wild oysters were collected by Florida Department of Agriculture and Consumer Services staff from five sites in Apalachicola Bay over nine days. At the lowest dilution (1:400), all samples tested <LOD and no matrix effects were observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Comparative data for 501 samples (173 oyster, 277 hard clam, and 51 sunray venus clam) are presented in Appendix G. For several reasons discussed in Appendix G, comparing NSP mouse bioassay and ELISA data is not straightforward, and analytical NSP methods of any type are unlikely to ever completely agree with mouse bioassay results. There was a very wide range of concentrations measured by ELISA in samples testing <20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay. In samples testing < 20MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. Where quantitative results were obtained by both mouse bioassay and ELISA (i.e., in samples testing ≥ 20 MU/100 g), significant positive correlations were observed. Using linear regression, the 20 MU/100 g equivalent by ELISA was predicted to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams (in PbTx-3 equivalents). Across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that "failed" by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.
D. Other Information		
1. Cost of the Method		Kit reagents are sold in bulk. The cost of reagents is currently $2,400 for 15 plates and $1,000 for 5 plates. The cost of additional consumables and reagents not included is approximately $20 per plate. Therefore cost per sample is $36-44 for full quantitation (5 samples per plate) and less than $6 per sample for qualitative screening (40 samples per plate).
2. Special Technical Skills Required to Perform the Method		General laboratory skills are required: reagent preparation, pipetting, basic equipment operation, data analysis using curve-fitting software, basic calculations.
3. Special Equipment Required and Associated Cost		Microplate reader with filter for measurement at 450 nm. Costs range, but basic readers start at approximately $5,000, and a used plate reader can be purchased for less than $1,000.
4. Abbreviations and Acronyms Defined		Ab Antibody BSA Bovine Serum Albumin ELISA Enzyme-linked Immunosorbent Assay HRP Horse radish peroxidase MDG MARBIONC Development Group NSP Neurotoxic Shellfish Poisoning PBS Phosphate Buffered Saline PBS-Tween Phosphate Buffered Saline with Tween 20 (0.05%) PbTx Brevetoxin PGT Phosphate Buffered Saline with gelatin (5%) Tween 20 (0.05%) TMB 3,3'5,5'-Tetramethylbenzidine
5. Details of Turn Around Times (time involved to complete the method)		The ELISA takes approximately 6 hours to complete, and one practiced analyst can comfortably process up to 4 plates per day.
6. Provide Brief Overview of the Quality Systems Used in the Lab		The Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research

		Institute's HAB Biotoxin Laboratory maintains and follows a Quality Assurance Program to ensure the precision, accuracy and reliability of all toxin analyses and for the production of scientifically sound, legally defensible data. Thorough documentation and standardization of laboratory processes, procedures and activities are required. The Laboratory Manager, Laboratory Safety Officer, Laboratory Secondary Staff and field staff are responsible for implementing QA/QC procedures outlined in the manual. Key practices include the use of Standard Operating Procedures, standard methods, training, quality control, and database record keeping and tracking. All QA practices are consistent with Good Laboratory Practices and all applicable safety, environmental and legal regulations and guidelines. **From the manufacturer (MARBIONC):** Each time new kit reagents are made from stocks, QC ELISAs are run and compared to previous assays. A standard ELISA set is retained to compare all new kits back to. New reagent stocks are given lot numbers. When new reagents are made (e.g. purified antibodies or PbTx-BSA conjugate), the ELISAs are designed with the new reagents to maintain continuity with previous kit lots. Kits are manufactured in a controlled environment to maintain cleanliness and avoid any cross contamination. Kits and kit components are validated. Kit and kit components are serialized to maintain traceability. Higher-level Good Manufacturing Processes are in process and as new reagents are produced, they will conform to requirements to allow for overall implementation of quality systems. Supply: MARBIONC Development Group, LLC has a future vision and is currently working to maintain an adequate supply of reagents. Sufficient supplies are on hand to cover current and projected increased demand for the foreseeable future (approximately 10-15 yrs). MARBIONC is committed to providing the kits for research and commercial use and has also committed to provide resources for the resupply of kit components in advance of the time when such components may be required.
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.

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:
New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method: This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data:

A total of 526 samples were tested using both ELISA and the NSP mouse bioassay (Table G1). Results of individual samples are contained in Table G2. Although additional data exists (both published and unpublished) comparing this ELISA with NSP mouse bioassay results, extraction methods have been modified over time. The data presented here includes only samples that were extracted for ELISA using 80% methanol with no additional clean-up. Almost all of the samples (495 of 526, 94%) were extracted and assayed in duplicate, and the mean is reported in the table. The mean %CV of duplicate analyses was 6.2%.

Table G1. Summary of comparative data using both NSP mouse bioassay and ELISA.

Shellfish Matrix	Total Samples	Mouse Bioassay < 20 MU/100g	Mouse Bioassay ≥ 20 MU/100g
Oysters	197	135 (69%)	62 (31%)
Hard Clams	277	238 (86%)	39 (14%)
Sunray Venus Clams	52	22 (42%)	30 (58%)

Table G2. Sample information and results of NSP mouse bioassay and ELISA

Sample ID	Shellfish Matrix	Harvest Area	Sample Date	MU/100g	ELISA (ppm)
HABB070327-017	oyster	Pine Island Sound	3/26/2007	<20	6.60
HABB070403-002	oyster	Pine Island Sound	4/2/2007	<20	5.26
HABB071115-001	oyster	St. Johns	11/14/2007	33.75	7.26
HABB071115-002	oyster	St. Johns	11/14/2007	38.63	16.31
HABB071128-004	oyster	St. Johns	11/27/2007	27.37	6.53
HABB071212-003	oyster	St. Johns	12/11/2007	<20	3.40
HABB080214-001	oyster	Alabama	2/8/2008	<20	0.52
HABB091117-001	oyster	Pine Island Sound	11/16/2009	<20	0.66
HABB091202-001	oyster	Pine Island Sound	12/1/2009	<20	0.42
HABB091202-002	oyster	Pine Island Sound	12/1/2009	<20	0.29
HABB100105-001	oyster	Pine Island Sound	1/4/2010	36.38	9.44
HABB100112-003	oyster	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-004	oyster	Pine Island Sound	1/11/2010	26.04	6.07
HABB100113-001	oyster	Gasparilla Sound	1/12/2010	<20	1.21
HABB100113-002	oyster	Gasparilla Sound	1/12/2010	<20	1.66
HABB100120-001	oyster	Pine Island Sound	1/19/2010	<20	<LOD
HABB100120-002	oyster	Pine Island Sound	1/19/2010	<20	2.34
HABB100224-001	oyster	Pine Island Sound	2/23/2010	<20	1.83
HABB100224-002	oyster	Pine Island Sound	2/23/2010	<20	1.01
HABB111026-003	oyster	Pine Island Sound	10/25/2011	<20	<LOD
HABB111026-004	oyster	Pine Island Sound	10/25/2011	<20	1.99
HABB111103-001	oyster	Gasparilla Sound	11/2/2011	33.31	9.57
HABB111103-002	oyster	Gasparilla Sound	11/2/2011	28.19	6.50
HABB111109-001	oyster	Pine Island Sound	11/8/2011	<20	0.53
HABB111109-002	oyster	Pine Island Sound	11/8/2011	32.93	10.09
HABB111115-001	oyster	Gasparilla Sound	11/14/2011	<20	4.80
HABB111115-002	oyster	Gasparilla Sound	11/14/2011	<20	2.98
HABB111122-002	oyster	Lemon Bay	11/21/2011	<20	7.76
HABB111213-001	oyster	Pine Island Sound	12/12/2011	<20	2.04
HABB111213-002	oyster	Pine Island Sound	12/12/2011	<20	1.71
HABB111220-001	oyster	Pine Island Sound	12/19/2011	<20	10.83
HABB111220-002	oyster	Pine Island Sound	12/19/2011	<20	3.85
HABB120124-003	oyster	Pine Island Sound	1/23/2012	<20	3.94
HABB120124-004	oyster	Pine Island Sound	1/23/2012	<20	1.31
HABB120131-001	oyster	Ten Thousand Islands	1/30/2012	37.70	14.01
HABB120214-001	oyster	Ten Thousand Islands	2/13/2012	22.80	6.19
HABB120214-002	oyster	Pine Island Sound	2/13/2012	<20	8.25
HABB120214-003	oyster	Pine Island Sound	2/13/2012	<20	1.79
HABB120221-001	oyster	Ten Thousand Islands	2/20/2012	27.43	6.72
HABB120228-001	oyster	Ten Thousand Islands	2/27/2012	<20	4.42
HABB121113-002	oyster	Lower Tampa Bay	11/6/2012	34.08	4.32
HABB130212-004	oyster	Lower Tampa Bay	11/14/2012	34.99	22.43
HABB130205-003	oyster	Lower Tampa Bay	2/4/2013	<20	3.28
HABB130409-001	oyster	Gasparilla Sound	4/8/2013	31.56	8.17
HABB130409-002	oyster	Gasparilla Sound	4/8/2013	29.65	15.40
HABB130501-001	oyster	Gasparilla Sound	4/30/2013	32.21	5.07
HABB130501-002	oyster	Gasparilla Sound	4/30/2013	24.07	3.26

HABB130501-003	oyster	Ten Thousand Islands	4/30/2013	<20	0.77
HABB130508-002	oyster	Gasparilla Sound	5/7/2013	<20	4.91
HABB130508-003	oyster	Gasparilla Sound	5/7/2013	<20	3.00
HABB130508-005	oyster	Lemon Bay	5/7/2013	<20	3.92
HABB130515-001	oyster	Pine Island Sound	5/14/2013	<20	3.17
HABB130515-002	oyster	Pine Island Sound	5/14/2013	<20	3.24
HABB130604-002	oyster	Sarasota Bay	6/3/2013	<20	2.43
HABB131210-001	oyster	Gasparilla Sound	12/9/2013	<20	4.52
HABB131210-002	oyster	Gasparilla Sound	12/9/2013	<20	0.79
HABB131210-003	oyster	Pine Island Sound	12/9/2013	<20	1.99
HABB131217-001	oyster	Pine Island Sound	12/16/2013	<20	2.03
HABB131217-002	oyster	Pine Island Sound	12/16/2013	<20	1.51
HABB131217-003	oyster	Matlacha	12/16/2013	<20	0.18
HABB131218-009	oyster	Lemon Bay	12/17/2013	<20	1.63
HABB141021-001	oyster	Suwannee Sound	10/20/2014	<20	4.62
HABB141021-002	oyster	Suwannee Sound	10/20/2014	<20	5.02
HABB141021-003	oyster	Suwannee Sound	10/20/2014	<20	3.34
HABB141022-002	oyster	Horseshoe Beach	10/21/2014	27.89	5.02
HABB141022-003	oyster	Horseshoe Beach	10/21/2014	<20	<LOD
HABB141028-001	oyster	Horseshoe Beach	10/27/2014	<20	4.44
HABB141028-002	oyster	Horseshoe Beach	10/27/2014	<20	5.20
HABB141028-003	oyster	Horseshoe Beach	10/27/2014	22.56	5.73
HABB141104-001	oyster	Horseshoe Beach	11/3/2014	<20	3.53
HABB141118-001	oyster	Gasparilla Sound	11/17/2014	<20	1.07
HABB141118-002	oyster	Gasparilla Sound	11/17/2014	<20	0.45
HABB141124-004	oyster	Pine Island Sound	11/23/2014	<20	2.57
HABB141209-001	oyster	Pine Island Sound	12/8/2014	<20	0.91
HABB141209-002	oyster	Pine Island Sound	12/8/2014	<20	2.49
HABB141216-001	oyster	Ten Thousand Islands	12/15/2014	<20	1.13
HABB151014-002	oyster	Indian Lagoon	10/13/2015	<20	0.84
HABB151119-001	oyster	East Bay	10/29/2015	94.60	25.50
HABB151103-001	oyster	Indian Lagoon	11/2/2015	<20	1.99
HABB151103-002	oyster	Pine Island Sound	11/2/2015	<20	0.98
HABB151103-003	oyster	Pine Island Sound	11/2/2015	<20	<LOD
HABB151110-001	oyster	Gasparilla Sound	11/9/2015	<20	1.34
HABB151110-002	oyster	Gasparilla Sound	11/9/2015	<20	3.87
HABB151117-001	oyster	East Bay	11/16/2015	34.05	7.08
HABB151117-002	oyster	North Bay	11/16/2015	<20	1.59
HABB151124-001	oyster	East Bay	11/23/2015	25.03	5.77
HABB151202-001	oyster	East Bay	12/1/2015	34.84	7.44
HABB151208-001	oyster	West Bay	12/7/2015	33.07	3.57
HABB151208-002	oyster	East Bay	12/7/2015	28.14	5.09
HABB151208-003	oyster	East Bay	12/7/2015	35.47	13.95
HABB151216-001	oyster	East Bay	12/15/2015	33.37	5.04
HABB151216-002	oyster	West Bay	12/15/2015	30.10	5.55
HABB151217-001	oyster	Gasparilla Sound	12/16/2015	<20	2.27
HABB151217-002	oyster	Gasparilla Sound	12/16/2015	26.79	4.73
HABB151217-003	oyster	Pine Island Sound	12/16/2015	31.47	3.96
HABB151217-004	oyster	Pine Island Sound	12/16/2015	20.21	3.56
HABB151222-001	oyster	Gasparilla Sound	12/21/2015	<20	4.31
HABB151222-002	oyster	Gasparilla Sound	12/21/2015	<20	1.77
HABB160105-001	oyster	Pine Island Sound	1/4/2016	<20	2.28

HABB160105-002	oyster	Pine Island Sound	1/4/2016	<20	2.17
HABB160105-003	oyster	Apalachicola Bay	1/4/2016	<20	3.27
HABB160105-004	oyster	Apalachicola Bay	1/4/2016	<20	2.52
HABB160106-001	oyster	East Bay	1/5/2016	30.63	2.45
HABB160106-002	oyster	North Bay	1/5/2016	17.07	7.91
HABB160112-001	oyster	West Bay	1/11/2016	22.35	3.28
HABB160112-002	oyster	North Bay	1/11/2016	23.94	7.28
HABB160112-003	oyster	West Bay	1/11/2016	35.43	12.59
HABB160113-001	oyster	Pensacola Bay	1/12/2016	<20	2.13
HABB160114-001	oyster	Apalachicola Bay	1/12/2016	<20	1.88
HABB160114-002	oyster	Indian Lagoon	1/12/2016	21.84	10.53
HABB160120-001	oyster	East Bay	1/19/2016	<20	2.02
HABB160120-002	oyster	North Bay	1/19/2016	<20	6.41
HABB160120-003	oyster	Mississippi	1/19/2016	<20	0.16
HABB160120-004	oyster	Mississippi	1/19/2016	<20	0.33
HABB160120-005	oyster	Mississippi	1/19/2016	<20	0.23
HABB160120-006	oyster	Mississippi	1/19/2016	<20	0.41
HABB160120-007	oyster	Mississippi	1/19/2016	<20	1.22
HABB160120-008	oyster	Mississippi	1/19/2016	<20	0.88
HABB160121-001	oyster	Indian Lagoon	1/20/2016	22.20	9.84
HABB160126-001	oyster	West Bay	1/25/2016	30.18	9.37
HABB160126-002	oyster	West Bay	1/25/2016	16.69	2.82
HABB160127-001	oyster	Alabama	1/25/2016	<20	3.17
HABB160127-002	oyster	Alabama	1/25/2016	<20	2.23
HABB160127-003	oyster	Alabama	1/25/2016	<20	3.11
HABB160127-004	oyster	Alabama	1/25/2016	<20	0.36
HABB160127-005	oyster	Alabama	1/25/2016	<20	0.42
HABB160128-001	oyster	East Bay	1/27/2016	<20	3.00
HABB160202-001	oyster	West Bay	2/1/2016	29.32	5.96
HABB160203-001	oyster	St. Joseph Bay	2/2/2016	28.40	14.20
HABB160203-002	oyster	Louisiana	2/2/2016	<20	0.29
HABB160203-003	oyster	Louisiana	2/2/2016	<20	0.77
HABB160203-004	oyster	Louisiana	2/2/2016	<20	0.84
HABB160203-005	oyster	Louisiana	2/2/2016	<20	1.08
HABB160203-006	oyster	Louisiana	2/2/2016	<20	0.33
HABB160203-007	oyster	Louisiana	2/2/2016	<20	0.29
HABB160204-001	oyster	Indian Lagoon	2/2/2016	<20	4.22
HABB160211-001	oyster	West Bay	2/10/2016	<20	5.56
HABB160223-001	oyster	Pine Island Sound	2/22/2016	31.66	6.77
HABB160223-005	oyster	St. Joseph Bay	2/22/2016	<20	12.37
HABB160224-001	oyster	Pine Island Sound	2/23/2016	<20	0.94
HABB160301-001	oyster	Alabama	2/29/2016	<20	1.72
HABB160302-001	oyster	Pine Island Sound	3/1/2016	<20	4.02
HABB160303-002	oyster	Gasparilla Sound	3/2/2016	19.81	5.07
HABB160308-001	oyster	Lower Tampa Bay	3/7/2016	23.53	10.51
HABB160309-001	oyster	Choctawhatchee Bay	3/8/2016	<20	0.60
HABB160317-001	oyster	Pine Island Sound	3/16/2016	25.90	3.87
HABB160317-002	oyster	Pine Island Sound	3/16/2016	<20	3.03
HABB160322-001	oyster	Lower Tampa Bay	3/22/2016	<20	4.33
HABB160328-002	oyster	Lower Tampa Bay	3/28/2016	<20	4.87
HABB160330-001	oyster	Pine Island Sound	3/29/2016	26.26	4.88
HABB160330-002	oyster	Pine Island Sound	3/29/2016	<20	2.19

HABB160407-002	oyster	Lower Tampa Bay	4/6/2016	<20	3.99
HABB160407-004	oyster	Pine Island Sound	4/7/2016	<20	3.00
HABB160411-013	oyster	Lower Tampa Bay	4/11/2016	<20	3.83
HABB160418-002	oyster	Lower Tampa Bay	4/18/2016	<20	2.76
HABB160421-002	oyster	Pine Island Sound	4/20/2016	23.66	3.01
HABB160421-003	oyster	Pine Island Sound	4/20/2016	<20	1.71
HABB160427-001	oyster	Pine Island Sound	4/26/2016	<20	3.37
HABB160427-002	oyster	Pine Island Sound	4/26/2016	<20	1.71
HABB160502-001	oyster	Boca Ceiga Bay	5/2/2016	21.65	4.59
HABB160505-001	oyster	Gasparilla Sound	5/4/2016	<20	2.70
HABB160505-002	oyster	Gasparilla Sound	5/4/2016	<20	1.67
HABB160510-001	oyster	Boca Ceiga Bay	5/10/2016	16.23	4.11
HABB161011-002	oyster	Lower Tampa Bay	10/10/2016	<20	0.74
HABB161018-002	oyster	Lower Tampa Bay	10/17/2016	<20	1.57
HABB161114-002	oyster	Lower Tampa Bay	11/14/2016	156.08	47.60
HABB170104-003	oyster	Pine Island Sound	1/3/2017	30.23	9.64
HABB170105-001	oyster	Lower Tampa Bay	1/4/2017	<20	2.31
HABB170110-001	oyster	Lower Tampa Bay	1/9/2017	<20	0.84
HABB170110-004	oyster	Gasparilla Sound	1/9/2017	28.32	8.43
HABB170111-001	oyster	Ten Thousand Islands	1/10/2017	19.63	3.14
HABB170111-002	oyster	Matlacha Pass	1/10/2017	<20	1.58
HABB170111-003	oyster	Pine Island Sound	1/10/2017	30.71	7.37
HABB170118-002	oyster	Gasparilla Sound	1/17/2017	29.46	6.65
HABB170119-003	oyster	Pine Island Sound	1/18/2017	33.87	5.64
HABB170119-004	oyster	Myakka River	1/18/2017	31.00	4.56
HABB170125-001	oyster	Gasparilla Sound	1/24/2017	<20	4.06
HABB170125-003	oyster	Pine Island Sound	1/24/2017	<20	4.31
HABB170131-002	oyster	Gasparilla Sound	1/30/2017	36.73	9.68
HABB170201-002	oyster	Myakka River	1/31/2017	22.45	3.56
HABB170207-002	oyster	Gasparilla Sound	2/6/2017	31.32	8.12
HABB170213-002	oyster	Lower Tampa Bay	2/13/2017	<20	1.47
HABB170214-004	oyster	Pine Island Sound	2/13/2017	<20	2.01
HABB170221-001	oyster	Myakka River	2/20/2017	<20	2.08
HABB170222-001	oyster	Gasparilla Sound	2/21/2017	42.30	10.51
HABB170307-002	oyster	Gasparilla Sound	3/6/2017	29.03	5.11
HABB170314-002	oyster	Gasparilla Sound	3/13/2017	<20	2.55
HABB170315-002	oyster	Lower Tampa Bay	3/14/2017	<20	2.21
HABB170322-002	oyster	Gasparilla Sound	3/21/2017	<20	2.49
HABB170405-001	oyster	Boca Ceiga Bay	4/4/2017	31.35	6.80
HABB170410-005	oyster	Gasparilla Sound	4/10/2017	<20	1.23
HABB170412-001	oyster	Pine Island Sound	4/11/2017	25.73	3.56
HABB170418-001	oyster	Pine Island Sound	4/17/2017	19.01	2.35
HABB170419-001	oyster	Lower Tampa Bay	4/18/2017	<20	5.89
HABB170419-002	oyster	Lower Tampa Bay	4/18/2017	<20	3.72
HABB170425-001	oyster	Gasparilla Sound	4/24/2017	25.81	4.13
HABB170425-002	oyster	Gasparilla Sound	4/24/2017	34.91	8.27
HABB080108-001	hard clam	Volusia County	1/7/2008	<20	0.97
HABB080108-002	hard clam	Volusia County	1/7/2008	<20	0.77
HABB080108-003	hard clam	Mosquito Lagoon	1/7/2008	52.8	4.2
HABB080109-003	hard clam	North Indian River	1/8/2008	<20	2.69
HABB080109-004	hard clam	Indian River Body F	1/8/2008	<20	0.14
HABB080115-001	hard clam	Mosquito Lagoon	1/14/2008	46.26	4

HABB080115-002	hard clam	Indian River Body A	1/14/2008	<20	1.18
HABB080115-003	hard clam	Indian River Body A	1/14/2008	38.66	4.44
HABB080123-022	hard clam	St. Lucie County	1/22/2008	<20	0.93
HABB080123-023	hard clam	Mosquito Lagoon	1/22/2008	<20	3.05
HABB080123-024	hard clam	Indian River Body A	1/22/2008	<20	2.35
HABB080123-025	hard clam	Indian River Body B	1/22/2008	<20	1.16
HABB090519-001	hard clam	Indian River Body F	5/18/2009	<20	<LOD
HABB091109-001	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-002	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-003	hard clam	Pine Island Sound	11/9/2009	<20	<LOD
HABB091109-004	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB100105-002	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-003	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-004	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-005	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100112-001	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-002	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100118-001	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-002	hard clam	Pine Island Sound	1/18/2010	<20	0.06
HABB100118-003	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-004	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB111011-001	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-002	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-003	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-004	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111018-001	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-002	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-003	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-004	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111024-001	hard clam	Pine Island Sound	10/23/2011	<20	<LOD
HABB111122-001	hard clam	Gasparilla Sound	11/21/2011	<20	4.13
HABB111206-001	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111206-002	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111213-003	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB111213-004	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB120104-001	hard clam	Pine Island Sound	1/4/2012	<20	0.63
HABB120104-002	hard clam	Pine Island Sound	1/4/2012	<20	0.66
HABB120109-001	hard clam	Pine Island Sound	1/9/2012	<20	0.63
HABB120109-002	hard clam	Pine Island Sound	1/9/2012	<20	0.48
HABB120117-001	hard clam	Pine Island Sound	1/16/2012	<20	0.24
HABB120117-002	hard clam	Pine Island Sound	1/16/2012	<20	0.23
HABB120124-001	hard clam	Pine Island Sound	1/23/2012	<20	0.14
HABB120124-002	hard clam	Pine Island Sound	1/23/2012	<20	0.13
HABB120131-003	hard clam	Ten Thousand Islands	1/25/2012	<20	1.39
HABB120131-004	hard clam	Ten Thousand Islands	1/25/2012	<20	1.49
HABB121002-001	hard clam	Gasparilla Sound	10/1/2012	37.63	12.68
HABB121002-002	hard clam	Gasparilla Sound	10/1/2012	<20	0.25
HABB121003-001	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121003-002	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121009-001	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-002	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-003	hard clam	Pine Island Sound	10/8/2012	<20	<LOD

HABB121009-004	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-005	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-006	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121016-001	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-002	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-003	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-004	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-005	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-006	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121017-001	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121017-002	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121023-005	hard clam	Pine Island Sound	10/22/2012	<20	0.28
HABB121023-006	hard clam	Pine Island Sound	10/22/2012	<20	0.26
HABB121023-007	hard clam	Pine Island Sound	10/22/2012	<20	0.18
HABB121023-008	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-009	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-010	hard clam	Pine Island Sound	10/22/2012	<20	0.22
HABB121024-001	hard clam	Lower Tampa Bay	10/23/2012	<20	0.92
HABB121024-002	hard clam	Lower Tampa Bay	10/23/2012	<20	1.05
HABB121024-003	hard clam	Lower Tampa Bay	10/23/2012	<20	0.7
HABB121024-004	hard clam	Lower Tampa Bay	10/23/2012	<20	0.66
Habb121024-005	hard clam	Pine Island Sound	10/23/2012	<20	0.18
HABB121024-006	hard clam	Pine Island Sound	10/23/2012	<20	0.23
HABB121030-001	hard clam	Lower Tampa Bay	10/29/2012	<20	0.5
HABB121030-002	hard clam	Lower Tampa Bay	10/29/2012	<20	0.34
HABB121030-003	hard clam	Pine Island Sound	10/29/2012	<20	1.2
HABB121030-004	hard clam	Pine Island Sound	10/29/2012	<20	0.88
HABB121113-001	hard clam	Lower Tampa Bay	11/6/2012	<20	1.78
HABB130212-003	hard clam	Lower Tampa Bay	11/14/2012	<20	<LOD
HABB121120-001	hard clam	Pine Island Sound	11/19/2012	<20	2.16
HABB121127-001	hard clam	Sarasota Bay	11/26/2012	<20	0.7
HABB121127-002	hard clam	Pine Island Sound	11/26/2012	<20	0.88
HABB121127-003	hard clam	Pine Island Sound	11/26/2012	<20	2.01
HABB121127-004	hard clam	Pine Island Sound	11/26/2012	<20	1.82
HABB121211-001	hard clam	Pine Island Sound	12/10/2012	<20	0.63
HABB121211-002	hard clam	Pine Island Sound	12/10/2012	<20	0.52
HABB121211-003	hard clam	Pine Island Sound	12/10/2012	<20	1.01
HABB121211-004	hard clam	Pine Island Sound	12/10/2012	<20	1.31
HABB121218-001	hard clam	Pine Island Sound	12/17/2012	<20	1.19
HABB121218-002	hard clam	Pine Island Sound	12/17/2012	<20	5.6
HABB121218-003	hard clam	Pine Island Sound	12/17/2012	<20	0.86
HABB121218-004	hard clam	Pine Island Sound	12/17/2012	<20	0.99
HABB121218-005	hard clam	Pine Island Sound	12/17/2012	<20	0.58
HABB121218-006	hard clam	Pine Island Sound	12/17/2012	<20	0.5
HABB121218-007	hard clam	Lower Tampa Bay	12/18/2012	<20	2.01
HABB121218-008	hard clam	Lower Tampa Bay	12/18/2012	<20	2.34
HABB121227-026	hard clam	Lower Tampa Bay	12/26/2012	23.59	3
HABB121227-027	hard clam	Lower Tampa Bay	12/26/2012	22.19	2.34
HABB121227-028	hard clam	Pine Island Sound	12/26/2012	<20	0.45
HABB121227-029	hard clam	Pine Island Sound	12/26/2012	<20	0.44
HABB130103-001	hard clam	Pine Island Sound	1/2/2013	<20	0.74
HABB130103-002	hard clam	Pine Island Sound	1/2/2013	<20	0.82

HABB130103-003	hard clam	Pine Island Sound	1/2/2013	22.09	2.18
HABB130103-004	hard clam	Pine Island Sound	1/2/2013	21.64	2.45
HABB130103-005	hard clam	Pine Island Sound	1/2/2013	<20	0.66
HABB130103-006	hard clam	Pine Island Sound	1/2/2013	<20	0.87
HABB130108-001	hard clam	Pine Island Sound	1/7/2013	<20	0.72
HABB130108-002	hard clam	Pine Island Sound	1/7/2013	<20	0.85
HABB130108-003	hard clam	Pine Island Sound	1/7/2013	<20	1.09
HABB130108-004	hard clam	Pine Island Sound	1/7/2013	<20	0.83
HABB130109-001	hard clam	Lower Tampa Bay	1/8/2013	20.2	4.38
HABB130109-002	hard clam	Lower Tampa Bay	1/8/2013	<20	1.96
HABB130109-003	hard clam	Lower Tampa Bay	1/8/2013	<20	1.51
HABB130115-003	hard clam	Pine Island Sound	1/14/2013	<20	1.07
HABB130115-004	hard clam	Pine Island Sound	1/14/2013	<20	1.74
HABB130122-001	hard clam	Lower Tampa Bay	1/22/2013	<20	1.57
HABB130122-002	hard clam	Lower Tampa Bay	1/22/2013	<20	1.54
HABB130130-001	hard clam	Lower Tampa Bay	1/28/2013	<20	1.8
HABB130130-002	hard clam	Lower Tampa Bay	1/28/2013	<20	1.82
HABB130205-001	hard clam	Lower Tampa Bay	2/4/2013	<20	1.41
HABB130205-002	hard clam	Lower Tampa Bay	2/4/2013	<20	1.44
HABB130212-001	hard clam	Pine Island Sound	2/11/2013	21.01	4.16
HABB130212-005	hard clam	Pine Island Sound	2/11/2013	29.23	5.68
HABB130226-002	hard clam	Pine Island Sound	2/24/2013	49.23	8.44
HABB130226-003	hard clam	Pine Island Sound	2/24/2013	44.71	8.37
HABB130226-004	hard clam	Pine Island Sound	2/24/2013	84.59	16.18
HABB130226-005	hard clam	Pine Island Sound	2/24/2013	39.34	9.89
HABB130226-006	hard clam	Pine Island Sound	2/24/2013	38.23	4.83
HABB130226-007	hard clam	Pine Island Sound	2/24/2013	27.18	4.82
HABB130226-008	hard clam	Pine Island Sound	2/24/2013	68.19	7.04
HABB130226-009	hard clam	Pine Island Sound	2/24/2013	<20	2.55
HABB130226-010	hard clam	Pine Island Sound	2/24/2013	44.16	6.33
HABB151007-002	hard clam	Pine Island Sound	2/25/2013	92.65	9.84
HABB130306-005	hard clam	Pine Island Sound	3/4/2013	<20	4.57
HABB130319-006	hard clam	Pine Island Sound	3/8/2013	<20	2.81
HABB130312-004	hard clam	Pine Island Sound	3/11/2013	205.34	37.33
HABB130312-005	hard clam	Pine Island Sound	3/11/2013	24.95	3.87
HABB130312-006	hard clam	Pine Island Sound	3/11/2013	<20	2.51
HABB130312-007	hard clam	Pine Island Sound	3/11/2013	<20	2.39
HABB130313-007	hard clam	Pine Island Sound	3/11/2013	36.89	3.26
HABB130313-008	hard clam	Pine Island Sound	3/11/2013	<20	1.73
HABB130313-001	hard clam	Pine Island Sound	3/12/2013	<20	1.93
HABB130313-002	hard clam	Pine Island Sound	3/12/2013	<20	2.46
HABB130313-003	hard clam	Pine Island Sound	3/12/2013	<20	2.47
HABB130313-004	hard clam	Pine Island Sound	3/12/2013	<20	2.35
HABB130319-007	hard clam	Pine Island Sound	3/13/2013	<20	2.24
HABB130319-004	hard clam	Pine Island Sound	3/18/2013	<20	2.14
HABB130319-005	hard clam	Pine Island Sound	3/18/2013	<20	4.2
HABB130319-012	hard clam	Pine Island Sound	3/18/2013	22.55	2.79
HABB140725-001	hard clam	Ten Thousand Islands	3/20/2013	<20	3.89
HABB130326-003	hard clam	Pine Island Sound	3/25/2013	<20	1.58
HABB130326-004	hard clam	Pine Island Sound	3/25/2013	<20	1.39
HABB130326-005	hard clam	Pine Island Sound	3/25/2013	<20	1.71
HABB130326-006	hard clam	Pine Island Sound	3/25/2013	<20	1.65

HABB130326-009	hard clam	Pine Island Sound	3/25/2013	<20	1.57
HABB130326-010	hard clam	Pine Island Sound	3/25/2013	<20	1.62
HABB130326-011	hard clam	Pine Island Sound	3/25/2013	<20	1.47
HABB130326-012	hard clam	Pine Island Sound	3/25/2013	<20	1.42
HABB130326-013	hard clam	Gasparilla Sound	3/25/2013	84.16	16.89
HABB130326-014	hard clam	Pine Island Sound	3/25/2013	75.9	16.4
HABB130403-002	hard clam	Pine Island Sound	3/29/2013	<20	2.23
HABB130402-001	hard clam	Pine Island Sound	4/1/2013	<20	2.05
HABB130402-002	hard clam	Pine Island Sound	4/1/2013	<20	1.98
HABB130402-003	hard clam	Pine Island Sound	4/1/2013	25.2	3.5
HABB130402-004	hard clam	Pine Island Sound	4/1/2013	24.3	2.92
HABB130402-005	hard clam	Pine Island Sound	4/1/2013	<20	1.4
HABB130402-006	hard clam	Pine Island Sound	4/1/2013	<20	1.27
HABB130402-007	hard clam	Pine Island Sound	4/1/2013	<20	1.55
HABB130402-008	hard clam	Pine Island Sound	4/1/2013	<20	3.51
HABB130402-009	hard clam	Pine Island Sound	4/1/2013	<20	3.27
HABB130409-003	hard clam	Pine Island Sound	4/8/2013	<20	0.97
HABB130409-004	hard clam	Pine Island Sound	4/8/2013	<20	1.17
HABB130409-008	hard clam	Pine Island Sound	4/8/2013	<20	1.81
HABB130409-009	hard clam	Pine Island Sound	4/8/2013	<20	1.09
HABB130409-010	hard clam	Pine Island Sound	4/8/2013	<20	0.85
HABB130409-011	hard clam	Pine Island Sound	4/8/2013	<20	3.82
HABB130409-012	hard clam	Pine Island Sound	4/8/2013	<20	4.12
HABB130409-013	hard clam	Pine Island Sound	4/8/2013	<20	3.81
HABB130409-014	hard clam	Pine Island Sound	4/8/2013	35.6	4.29
HABB130409-015	hard clam	Pine Island Sound	4/8/2013	<20	1.69
HABB130409-016	hard clam	Pine Island Sound	4/8/2013	<20	1.52
HABB130410-001	hard clam	Pine Island Sound	4/9/2013	<20	1.82
HABB130410-002	hard clam	Pine Island Sound	4/9/2013	<20	1.91
HABB130410-003	hard clam	Pine Island Sound	4/9/2013	<20	1.69
HABB130416-006	hard clam	Pine Island Sound	4/15/2013	<20	0.83
HABB130416-007	hard clam	Pine Island Sound	4/15/2013	<20	0.81
HABB130417-001	hard clam	Pine Island Sound	4/16/2013	<20	1.09
HABB130417-002	hard clam	Pine Island Sound	4/16/2013	<20	1.24
HABB130417-004	hard clam	Pine Island Sound	4/16/2013	<20	1.37
HABB130417-005	hard clam	Pine Island Sound	4/16/2013	<20	1.28
HABB130423-001	hard clam	Pine Island Sound	4/22/2013	<20	1.02
HABB130423-002	hard clam	Pine Island Sound	4/22/2013	<20	1.06
HABB130423-003	hard clam	Pine Island Sound	4/22/2013	<20	0.98
HABB130424-001	hard clam	Pine Island Sound	4/24/2013	<20	0.93
HABB130424-002	hard clam	Pine Island Sound	4/24/2013	<20	1
HABB130424-003	hard clam	Pine Island Sound	4/24/2013	<20	0.86
HABB130508-004	hard clam	Lemon Bay	5/7/2013	<20	17.33
HABB131113-001	hard clam	Pine Island Sound	11/12/2013	<20	0.5
HABB131113-002	hard clam	Pine Island Sound	11/12/2013	<20	0.32
HABB131113-003	hard clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-006	hard clam	Pine Island Sound	11/12/2013	<20	0.41
HABB131113-007	hard clam	Pine Island Sound	11/12/2013	<20	0.38
HABB131113-008	hard clam	Pine Island Sound	11/12/2013	<20	0.44
HABB131119-001	hard clam	Pine Island Sound	11/18/2013	<20	1.96
HABB131119-002	hard clam	Pine Island Sound	11/18/2013	<20	1.71
HABB131119-003	hard clam	Pine Island Sound	11/18/2013	<20	1.78

HABB131126-001	hard clam	Pine Island Sound	11/25/2013	<20	0.3
HABB131126-002	hard clam	Pine Island Sound	11/25/2013	<20	0.29
HABB131126-003	hard clam	Pine Island Sound	11/25/2013	<20	0.28
HABB131126-004	hard clam	Pine Island Sound	11/25/2013	<20	0.59
HABB131126-005	hard clam	Pine Island Sound	11/25/2013	<20	0.69
HABB131126-006	hard clam	Pine Island Sound	11/25/2013	<20	0.68
HABB131203-001	hard clam	Pine Island Sound	12/2/2013	<20	0.23
HABB131203-002	hard clam	Pine Island Sound	12/2/2013	<20	0.24
HABB131203-003	hard clam	Pine Island Sound	12/2/2013	<20	0.21
HABB131203-004	hard clam	Pine Island Sound	12/2/2013	<20	0.31
HABB131203-005	hard clam	Pine Island Sound	12/2/2013	<20	0.33
HABB131203-006	hard clam	Pine Island Sound	12/2/2013	<20	0.38
HABB131210-004	hard clam	Pine Island Sound	12/9/2013	<20	0.35
HABB131210-005	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131210-006	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131211-012	hard clam	Gasparilla Sound	12/10/2013	<20	0.84
HABB131218-010	hard clam	Gasparilla Sound	12/17/2013	36.91	8.96
HABB141014-001	hard clam	Cedar Key	10/13/2014	<20	0.33
HABB141014-002	hard clam	Cedar Key	10/13/2014	<20	0.31
HABB141014-003	hard clam	Cedar Key	10/13/2014	<20	0.42
HABB141113-002	hard clam	Pine Island Sound	11/12/2014	<20	0.34
HABB141113-003	hard clam	Pine Island Sound	11/12/2014	<20	0.44
HABB141113-004	hard clam	Pine Island Sound	11/12/2014	<20	0.69
HABB141113-005	hard clam	Pine Island Sound	11/12/2014	<20	0.7
HABB141113-006	hard clam	Pine Island Sound	11/12/2014	<20	0.66
HABB141113-007	hard clam	Pine Island Sound	11/12/2014	<20	0.62
HABB141119-001	hard clam	Pine Island Sound	11/18/2014	<20	0.15
HABB141119-002	hard clam	Pine Island Sound	11/18/2014	<20	0.13
HABB141119-003	hard clam	Pine Island Sound	11/18/2014	<20	0.2
HABB141119-004	hard clam	Pine Island Sound	11/18/2014	<20	0.18
HABB141119-005	hard clam	Pine Island Sound	11/18/2014	<20	0.23
HABB141119-006	hard clam	Pine Island Sound	11/18/2014	<20	0.25
HABB141124-001	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-002	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-003	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB160202-002	hard clam	Pine Island Sound	2/1/2016	<20	0.92
HABB160209-017	hard clam	Gasparilla Sound	2/8/2016	76.77	10.82
HABB160209-018	hard clam	Gasparilla Sound	2/8/2016	42.61	9.68
HABB160209-019	hard clam	Gasparilla Sound	2/8/2016	85.99	10
HABB160223-003	hard clam	Pine Island Sound	2/22/2016	<20	0.44
HABB160301-002	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-003	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-004	hard clam	Pine Island Sound	2/29/2016	<20	0.33
HABB160301-005	hard clam	Pine Island Sound	2/29/2016	<20	0.37
HABB160302-002	hard clam	Pine Island Sound	3/1/2016	<20	0.6
HABB160302-003	hard clam	Pine Island Sound	3/1/2016	<20	0.65
HABB160308-002	hard clam	Lower Tampa Bay	3/7/2016	40.05	6.21
HABB160322-002	hard clam	Lower Tampa Bay	3/22/2016	25	5.12
HABB160328-001	hard clam	Lower Tampa Bay	3/28/2016	35.83	4.9
HABB160407-001	hard clam	Lower Tampa Bay	4/6/2016	29.59	4.36
HABB160407-003	hard clam	Pine Island Sound	4/7/2016	<20	0.5
HABB160411-012	hard clam	Lower Tampa Bay	4/11/2016	<20	1.36

HABB160418-001	hard clam	Lower Tampa Bay	4/18/2016	<20	1.76
HABB160601-001	hard clam	Lemon Bay	5/31/2016	<20	0.43
HABB161011-001	hard clam	Lower Tampa Bay	10/10/2016	<20	1.16
HABB161013-001	hard clam	Gasparilla Sound	10/12/2016	<20	0.54
HABB161018-001	hard clam	Lower Tampa Bay	10/17/2016	<20	2.07
HABB170104-001	Hard clam	Pine Island Sound	1/3/2017	<20	1.66
HABB170104-002	Hard clam	Pine Island Sound	1/3/2017	<20	1
HABB170105-002	Hard clam	Lower Tampa Bay	1/4/2017	35.96	2.22
HABB170110-002	hard clam	Lower Tampa Bay	1/9/2017	<20	1.58
HABB170110-003	hard clam	Gasparilla Sound	1/9/2017	20.26	2.35
HABB131125-020	hard clam	Composite		<20	3.9
HABB130115-001	sunray venus clam	Pine Island Sound	1/14/2013	<20	1.85
HABB130212-002	sunray venus clam	Pine Island Sound	2/11/2013	34.13	12.04
HABB130212-005	sunray venus clam	Pine Island Sound	2/11/2013	39.09	19.74
HABB130226-001	sunray venus clam	Pine Island Sound	2/24/2013	42.41	15.41
HABB130226-011	sunray venus clam	Pine Island Sound	2/24/2013	<20	5.58
HABB130228-001	sunray venus clam	Pine Island Sound	2/25/2013	32.17	9.93
HABB130227-001	sunray venus clam	Pine Island Sound	2/26/2013	42.9	13.01
HABB130227-002	sunray venus clam	Pine Island Sound	2/26/2013	34.97	19.09
HABB130228-003	sunray venus clam	Pine Island Sound	2/27/2013	27.54	17.94
HABB130319-009	sunray venus clam	Pine Island Sound	3/8/2013	<20	3.13
HABB130312-001	sunray venus clam	Pine Island Sound	3/11/2013	27.65	6.59
HABB130312-002	sunray venus clam	Pine Island Sound	3/11/2013	26.33	7.39
HABB130312-003	sunray venus clam	Pine Island Sound	3/11/2013	28.7	5.16
HABB130312-009	sunray venus clam	Pine Island Sound	3/11/2013	<20	5.38
HABB150921-001	sunray venus clam	Pine Island Sound	3/11/2013	31.33	5.3
HABB130319-010	sunray venus clam	Pine Island Sound	3/13/2013	<20	3.1
HABB130319-001	sunray venus clam	Pine Island Sound	3/18/2013	22.05	4.48
HABB130319-002	sunray venus clam	Pine Island Sound	3/18/2013	20.67	4.28
HABB130319-003	sunray venus clam	Pine Island Sound	3/18/2013	27.85	7.69
HABB130319-011	sunray venus clam	Pine Island Sound	3/18/2013	25.87	5.43
HABB130326-001	sunray venus clam	Pine Island Sound	3/25/2013	23.16	3.48
HABB130326-002	sunray venus clam	Pine Island Sound	3/25/2013	22.36	3.4
HABB130326-007	sunray venus clam	Pine Island Sound	3/25/2013	24.4	4.44
HABB130326-008	sunray venus clam	Pine Island Sound	3/25/2013	22.5	3.35
HABB130409-006	sunray venus clam	Pine Island Sound	4/8/2013	22.84	2.53
HABB130409-020	sunray venus clam	Pine Island Sound	4/8/2013	<20	2.16
HABB130409-021	sunray venus clam	Pine Island Sound	4/8/2013	23.91	2.69
HABB130410-004	sunray venus clam	Pine Island Sound	4/9/2013	<20	2.18
HABB130410-005	sunray venus clam	Pine Island Sound	4/9/2013	<20	1.84
HABB130416-002	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.47
HABB130416-003	sunray venus clam	Pine Island Sound	4/15/2013	<20	0.99
HABB130416-004	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.48
HABB130417-006	sunray venus clam	Pine Island Sound	4/16/2013	<20	1.62
HABB130604-003	sunray venus clam	Pine Island Sound	6/3/2013	<20	0.56
HABB131113-004	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-005	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.24
HABB131125-019	sunray venus clam	Alligator Harbor	11/22/2013	25.88	4.11
HABB151120-001	sunray venus clam	Sarasota Bay	11/18/2015	33.21	11.05
HABB151120-002	sunray venus clam	Sarasota Bay	11/18/2015	33.58	12.11
HABB151207-001	sunray venus clam	Sarasota Bay	12/7/2015	53.21	14.47
HABB160111-002	Sunray venus clam	Lower Tampa Bay	12/15/2015	33.34	6.37

HABB160111-001	sunray venus clam	Sarasota Bay	1/6/2016	<20	2.77
HABB160202-003	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160202-004	sunray venus clam	Pine Island Sound	2/1/2016	<20	2.74
HABB160202-005	sunray venus clam	Pine Island Sound	2/1/2016	19.77	2.14
HABB160202-006	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160223-004	sunray venus clam	Pine Island Sound	2/22/2016	27.66	2.16
HABB160330-004	sunray venus clam	Lower Tampa Bay	3/16/2016	36.48	3.38
HABB160330-005	sunray venus clam	Lower Tampa Bay	3/16/2016	33.04	3.41
HABB161213-019	sunray venus clam	MML lab exposure	9/6/2016	<20	2.63
HABB161213-021	sunray venus clam	MML lab exposure	12/7/2016	20.66	4.04
HABB161213-022	sunray venus clam	MML exp control	12/7/2016	<20	<LOD

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:

Value for the test of symmetry for the distribution of the data generated by the officially recognized method

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method

Variance of the data generated from the officially recognized analytical method

Variance of the data generated from the proposed alternative analytical method

Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____

Is there a significant difference between the analytical methods Y/N

Comparative data for NSP mouse bioassays and ELISAs cannot be evaluated as described above. Please see below for additional discussion and comparisons permitted by the data presented above.

Brevetoxins in bivalves

At least nine brevetoxin congeners have been isolated from *K. brevis*[1]. PbTx-1 and PbTx-2 are presumed to be the parent toxins from which all other brevetoxins are derived via substitutions on the terminal ring. Consequently, brevetoxins are grouped into two types according to their backbone structure. Brevetoxin A-type (PbTx-1-type) toxins possess a 10-ring backbone, and brevetoxin B-type (PbTx-2-type) toxins possess an 11-ring backbone (Fig. G1). Although brevetoxin A-type toxins are more potent, the brevetoxin B-type toxins are much more abundant[2]. Polar derivatives identified in both culture and bloom materials have further increased the number of known brevetoxin structures[3,4].

In bivalves, the more reactive forms of brevetoxin are rapidly transformed into brevetoxin metabolites[3,5] that are generally the products of reduction, oxidation, and conjugation to other molecules including taurine, cysteine, cysteine sulfoxide, amino acids and fatty acids[5-7]. Literally dozens of metabolites have been identified in shellfish. Most modifications to brevetoxins occur at the side chain on the terminal ether ring that differentiates the brevetoxin congeners, resulting in an assortment of conjugates with either an A-type or B-type of backbone. Brevetoxin metabolites are known to contribute to NSP toxicity [3,6-8], but their individual potency varies. Toxicity information is available for only a small subset of the dozens of characterized metabolites. Some common shellfish metabolites are less potent than parent brevetoxins, while a few have demonstrated higher toxicities [7,9,10]. Different rates of tissue uptake and elimination of brevetoxin metabolites have also been described and may factor into their variable potencies[11].

The complexity of brevetoxins and their metabolic products is the primary reasons that so little progress has been made on moving away from the NSP mouse bioassay. Of the many chemical and biological methods evaluated for measuring brevetoxins in bivalves, those that recognize molecular structure (i.e., ELISAs and liquid chromatography-mass spectroscopy [LC-MS]) have outperformed activity-based assays (i.e., receptor-binding and cytotoxicity assay), demonstrating less variability and better agreement with mouse bioassays[7,12,13].

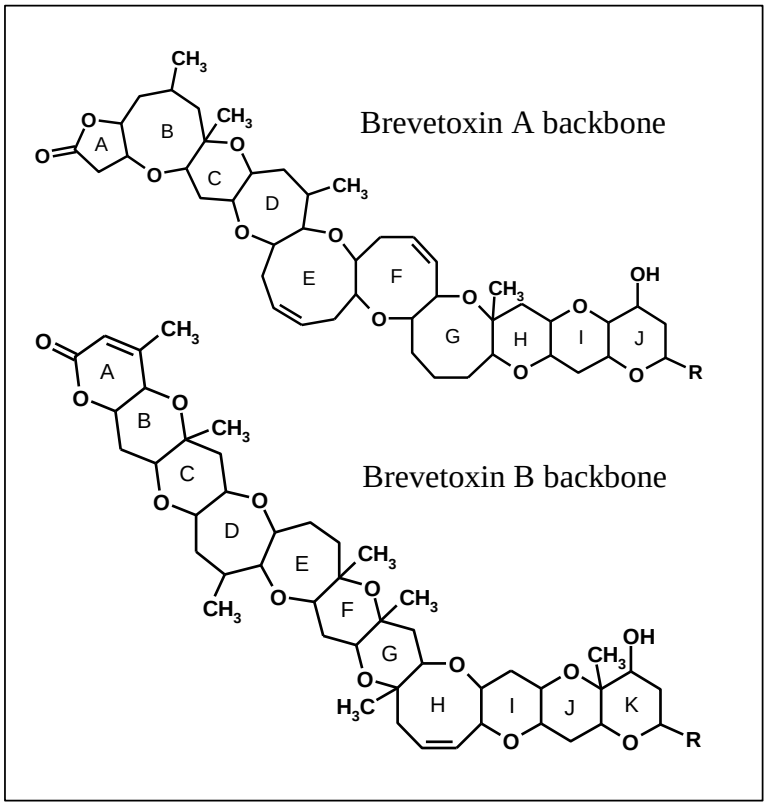

Figure G1. Brevetoxin backbone structures.

An LC-MS method has been developed by the FDA Gulf Coast Seafood Lab and will be submitted to the ISSC for consideration as an alternative to the mouse bioassay. LC-MS can provide confirmation of toxins detected by other assays, and sample throughput is higher compared to the mouse bioassay. However, the large number of brevetoxin metabolites in bivalves will necessitate a targeted approach. For routine analysis as a part of monitoring and management, it is not practical to attempt to identify and quantify them all. Nor is this even possible, given the lack of available standards for almost all metabolites. In the Gulf of Mexico, the most important commercial species are eastern oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*). In oysters, the brevetoxin profile is dominated by the cysteine metabolites S-desoxy-BTX-B2 and BTX-B2[3,5,12]. These were also the major metabolites identified in hard clams, along

with BTX-B1, a taurine conjugate[14,15]. Sunray venus clams (*Macrocallista nimbosa*), a relatively new aquaculture product gaining popularity in Florida, have been less well-studied, but analyses thus far indicate that this species metabolizes brevetoxins similarly to hard clams (Fig. G2), with the cysteine and taurine conjugates representing the major metabolites (Fig. G3).

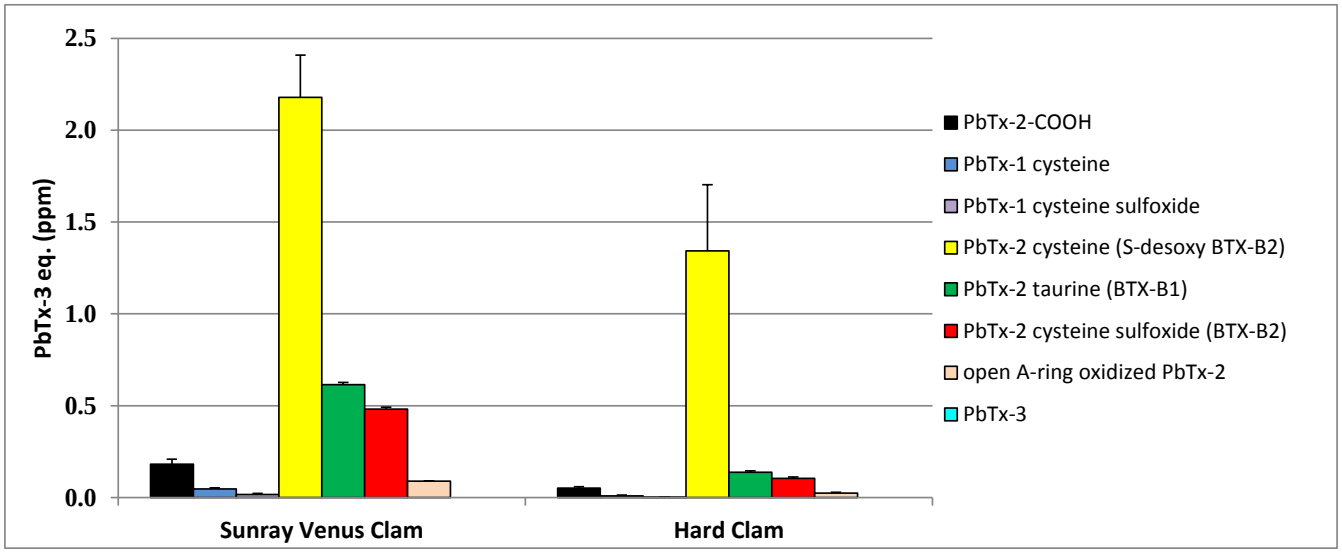

Figure G2. Brevetoxin metabolites identified by LC-MS in laboratory-exposed sunray venus and hard clams. (Error bars=standard deviation, n=3. Unpublished data provided by Dr. R. Pierce, Mote Marine Laboratory.)

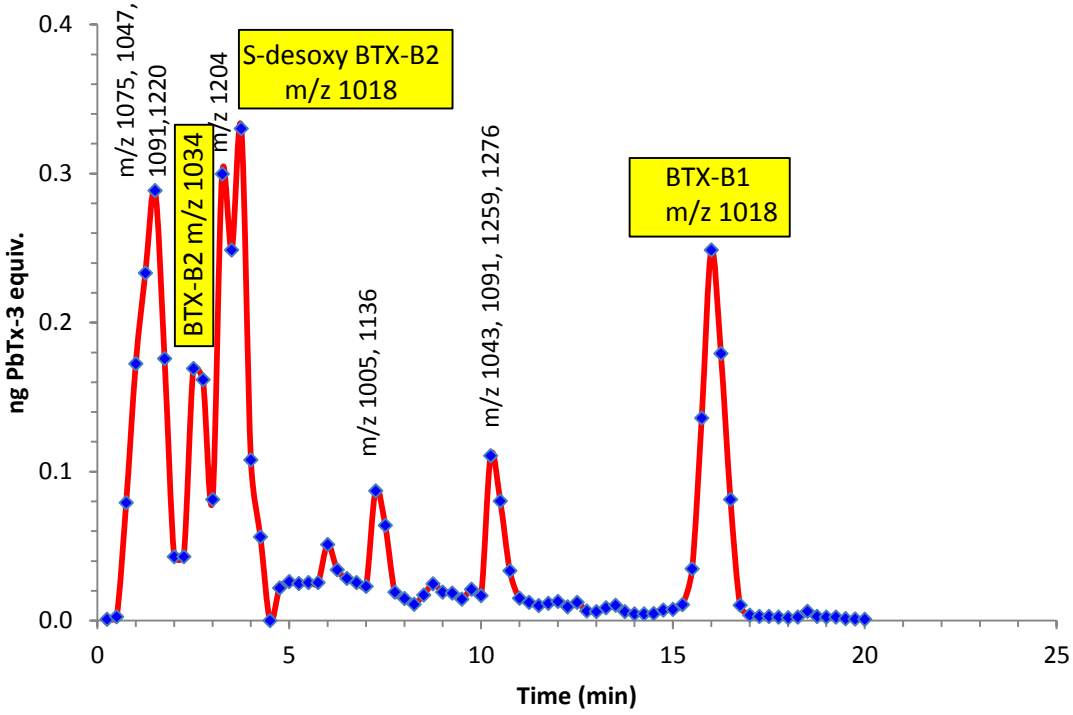

Figure G3. Chromatograms of brevetoxin metabolites in sunray venus clams based on ELISA of LC-fractionated shellfish extracts. (Unpublished data provided by Dr. A. Abraham, USFDA.)

Oral toxicity to mammals has not been assessed for any of the brevetoxin conjugates. Nevertheless, the cysteine and taurine metabolites were found to be excellent biomarkers of composite B-type brevetoxins as determined by ELISA for these species[12,14]. Based on these studies, the FDA's LC-MS protocol targets these three metabolites as biomarkers for NSP toxicity in oysters and clams.

LC-MS analyses require expensive instrumentation and highly technical expertise and are further limited by the time required for each sample to run. Where high throughput is required, the speed and cost-effectiveness of ELISA makes it a more attractive screening method.

MARBIONC Brevetoxin Competitive ELISA

The MARBIONC ELISA kit used in this method validation is the same kit that was used in the method comparisons and bivalve studies cited above. The method is based on the activity of anti-brevetoxin goat polyclonal antibodies, which were produced using a PbTx-3-KLH (keyhole limpet hemocyanin) conjugate[16]. The recognition epitope is believed to include the last four rings (excluding the side chain) of the brevetoxin B type toxins[17,18] (Fig. G4). This specific region is maintained in all brevetoxin B type toxins including in the secondary metabolites identified thus far. However, cross-reactivity of these antibodies have only been assessed for a few metabolites.

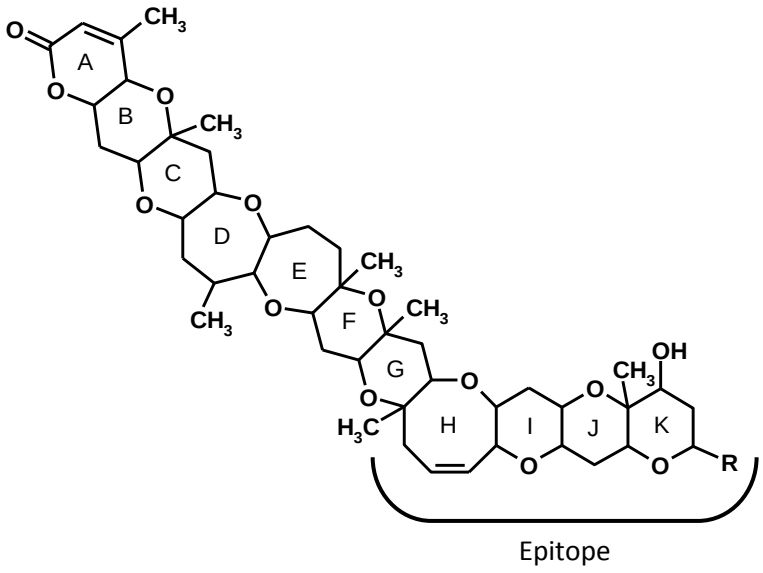

Figure G4. Brevetoxin B backbone with recognition epitope of anti-brevetoxin goat polyclonal antibodies

When this ELISA method was originally published, similar cross-reactivities were reported for PbTx-2, PbTx-3 and PbTx-9, which all share the B-type backbone[18] (Fig G5). MARBIONC reports cross-reactivities of 100% for PbTx-3, 97% for PbTx-2, 105% for oxidized-PbTx-2, and 7% for PbTx-1 at 10 ng/mL (Fig. G6).

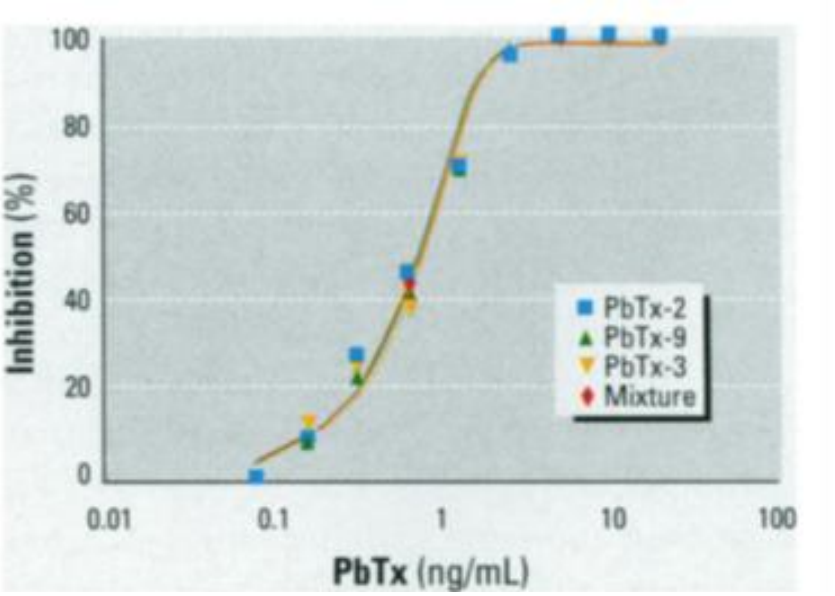

Figure G5. Figure taken from Naar et al.[18]. Anti-brevetoxin antibody cross-reaction with PbTx-2, PbTx-3, PbTx-9, and a mixture of the three toxins.

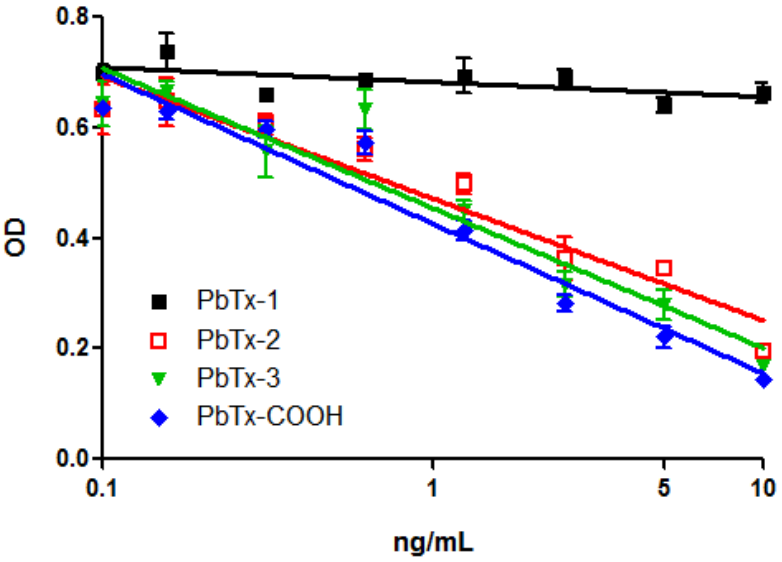

Figure G6. Figure provided by MARBIONC demonstrating degrees of anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2, PbTx-3, and oxidized-PbTx-2 (PbTx-COOH).

Competitive curves generated by L. Flewelling (FWC) are consistent with this, with calculated cross-reactivities (at 50% inhibition) of 97% for PbTx-2 and 2.4% for PbTx-1, relative to PbTx-3 (100%) (Fig. G7).

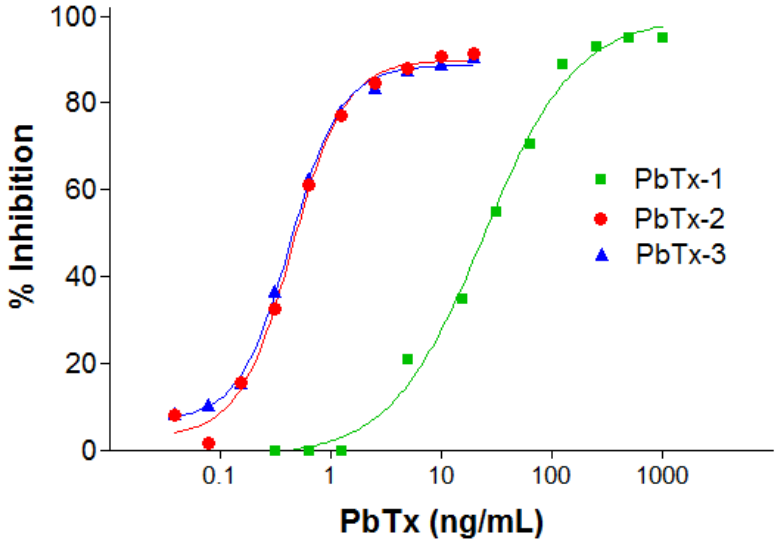

Figure G7. Anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2 and PbTx-3 (FWC data).

We also assessed the cross-reactivity of two shellfish metabolites (Fig. G8). The cross-reactivity of the cysteine conjugate S-desoxy BTX-B2 (provided by the FDA Gulf Coast Seafood Laboratory) was found to be 133% relative to PbTx-3. Cross reactivity of the brevetoxin lipid conjugate N-palmitoyl BTX-B2 (or BTX-B4, described in Bottein et al.[19] and provided by NOAA Center for Coastal Environmental Health and Biomolecular Research) was much lower (2.5%).

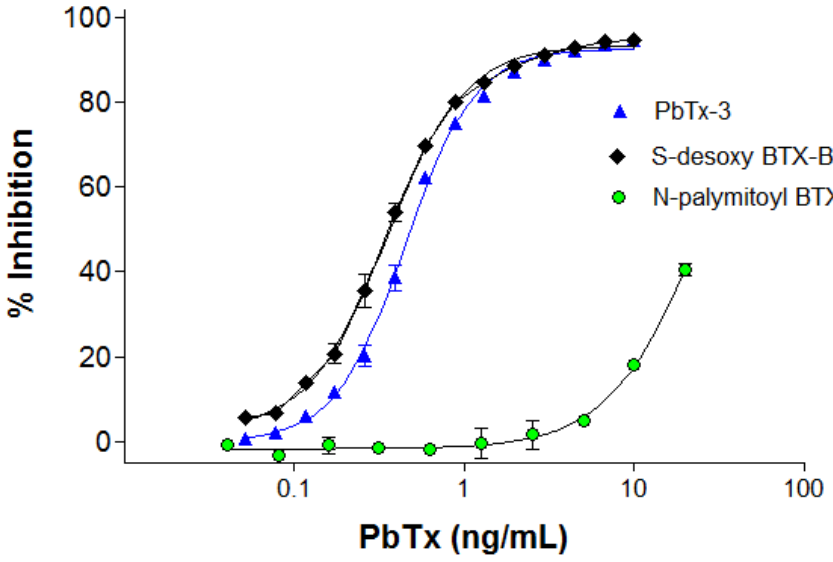

Figure G8. Anti-brevetoxin antibody cross-reaction with B-type brevetoxin metabolites S-desoxy BTX-B2 and N-palymitoyl BTX-B2 compared to PbTx-3 (FWC data).

The relatively low cross-reactivity of the antibodies with PbTx-1 (and presumably PbTx-1-derived conjugates) and with N-palmitoyl BTX-B2 indicates that ELISA results can underestimate of the total amount of brevetoxin and brevetoxin metabolites present in a sample. PbTx-1 is more potent than PbTx-2; however, the parent toxins PbTx-1 and -2 are not found in shellfish, and PbTx-2 type toxins consistently dominate the toxin profile in both *Karenia brevis* cells[20-22] and shellfish[22], typically accounting for 75% or more of the total toxins present. Additionally, although lipid conjugates of brevetoxin are thought to contribute substantially to NSP toxicity, these are derivatives of (and co-occur with) the more abundant amino acid metabolites that dominate the profile of toxic oysters and clams. The ELISA readily detects these forms, which have been identified as excellent biomarkers of NSP toxicity in oysters and clams. Therefore, the limited cross-reactivity of the ELISA with PbTx-1 and with N-palmitoyl BTX-B2 does not diminish the potential for the ELISA to perform successfully within a management program as proposed here.

In recent years, other brevetoxin ELISA kits have been introduced to the market, but prior to implementation into shellfish monitoring each kit would require individual evaluation of antibody cross-reactivity with dominant brevetoxin metabolites and comparisons with currently approved methods.

ELISA vs Mouse Bioassay

Currently, the only approved method for NSP testing is the APHA mouse bioassay[23]. The method is based on the bioassay developed by McFarren et al.[24] more than 50 years ago using toxic shellfish collected during an NSP outbreak in 1963. One mouse unit (MU) is the amount of crude lipid extract that will kill, on average, 50% of 20-g test mice in 15.5 hours. It is important to note that this method has never been validated, and the guidance limit used today (20 MU per 100g) is not based on any toxicological studies, but rather was described as the level of sensitivity of the test for 20g mice observed for 6 hours, which was deemed to be the longest reasonable observation time for the sake of accuracy and expediency. This guidance limit has proven to be effective, as no cases of NSP from legally harvested shellfish have been documented in Florida since the monitoring program began in the 1970's.

Comparing NSP mouse bioassay and ELISA data is not straightforward. The assays measure NSP toxins in very different ways. The mouse bioassay assesses toxicity by measuring the response of mice injected with a crude lipid extract of shellfish. This extract, prepared by repeated partitioning of acidified shellfish homogenate with diethyl ether, contains only a subset of the toxins present[7,25,26]. The method is semi-quantitative, yielding numerical results only at values ≥20 MU per 100g. Conversely, the ELISA is much more sensitive and yields continuous data to much lower concentrations, quantifying (relative to PbTx-3) a more comprehensive collection of brevetoxins and metabolites (regardless of potency) using antibodies that recognize a portion of the brevetoxin B-type backbone structure. Given that the NSP mouse bioassay measures only a subset of the toxins present, is semi-quantitative, has never been appropriately validated, and is not calibrated against known brevetoxin concentrations, a robust agreement of numerical results is unlikely to be achieved by any method.

At present, there is no validated brevetoxin equivalent of 'mouse units' in shellfish. Early work by Baden and Mende [27] established the toxicity of purified PbTx-2 and -3 dissolved in saline to mice intraperitoneally and calculated an LD50 (amount of toxin that kills half of the mice in 24 hours) of 0.2 mg/kg (similar for both toxins). This dose was used to derive a PbTx-2 "equivalent" of 4 µg per 20g-mouse and has since been extended to estimate the brevetoxin concentration in shellfish with a measured toxicity of 20 MU per 100g as 0.8 mg PbTx-2 equivalents per kg shellfish [13,16]. This number appears in several guidance documents; however**,** the extensive metabolism of brevetoxins in shellfish was unknown when the estimated equivalence was first proposed. We now know that shellfish exposed to *K. brevis* blooms contain a mixture of toxins with a multiplicity of potencies. In many cases the metabolites are less toxic, but in some cases they are more toxic. For these reasons, the use of this equivalent for brevetoxins in shellfish is inappropriate and has been acknowledged to be of little use for practical application[7].

Because a biomarker or indicator of toxicity approach is currently necessary for NSP, future NSP guidance limits may vary with the method used and may also vary across shellfish species. An appropriate non-mouse unit guidance limit for brevetoxins in shellfish will provide a level of protection for human health equal to that provided by the existing federal NSP guidance limit of 20 MU per 100 g shellfish. We know from existing data derived from naturally incurred eastern oysters and hard clams that such a limit *as measured using the MARBIONC ELISA with PbTx-3 as a standard* would exceed 0.8 mg per kg shellfish for these species.

Comparison of NSP Mouse Bioassay and ELISA results

Where quantitative results were obtained by both mouse bioassay and ELISA, Spearman rank correlation analysis was used to assess the correlation of brevetoxin concentrations measured by both methods for each shellfish matrix (Table G3). Significant correlations were observed in all cases.

Table G3. Spearman rank correlation coefficients (and p-values) for brevetoxin concentrations measured by NSP bioassay and ELISA

	Spearman rank correlation coefficient	p-value
oysters	0.5590	< 0.0001
hard clams	0.7866	< 0.0001
sunray venus clams	0.6859	< 0.0001

(From this portion on, changes to address early LMC comments are underway, and an updated Appendix G will be submitted.)

Given the differences between the assays and what they measure, strong agreement between numerical results was not expected. Nevertheless, the data were analyzed using linear regression analysis to estimate predicted concentrations by ELISA for samples testing at 20 MU per 100g (Fig. G9). Removal of the outlying (high) mouse bioassay results for oysters (>50MU) and clams (>100MU) that influenced the regression lines lowered the R-squared values, but slopes did not change appreciably. The 20 MU/100 g equivalent by ELISA was estimated to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams.

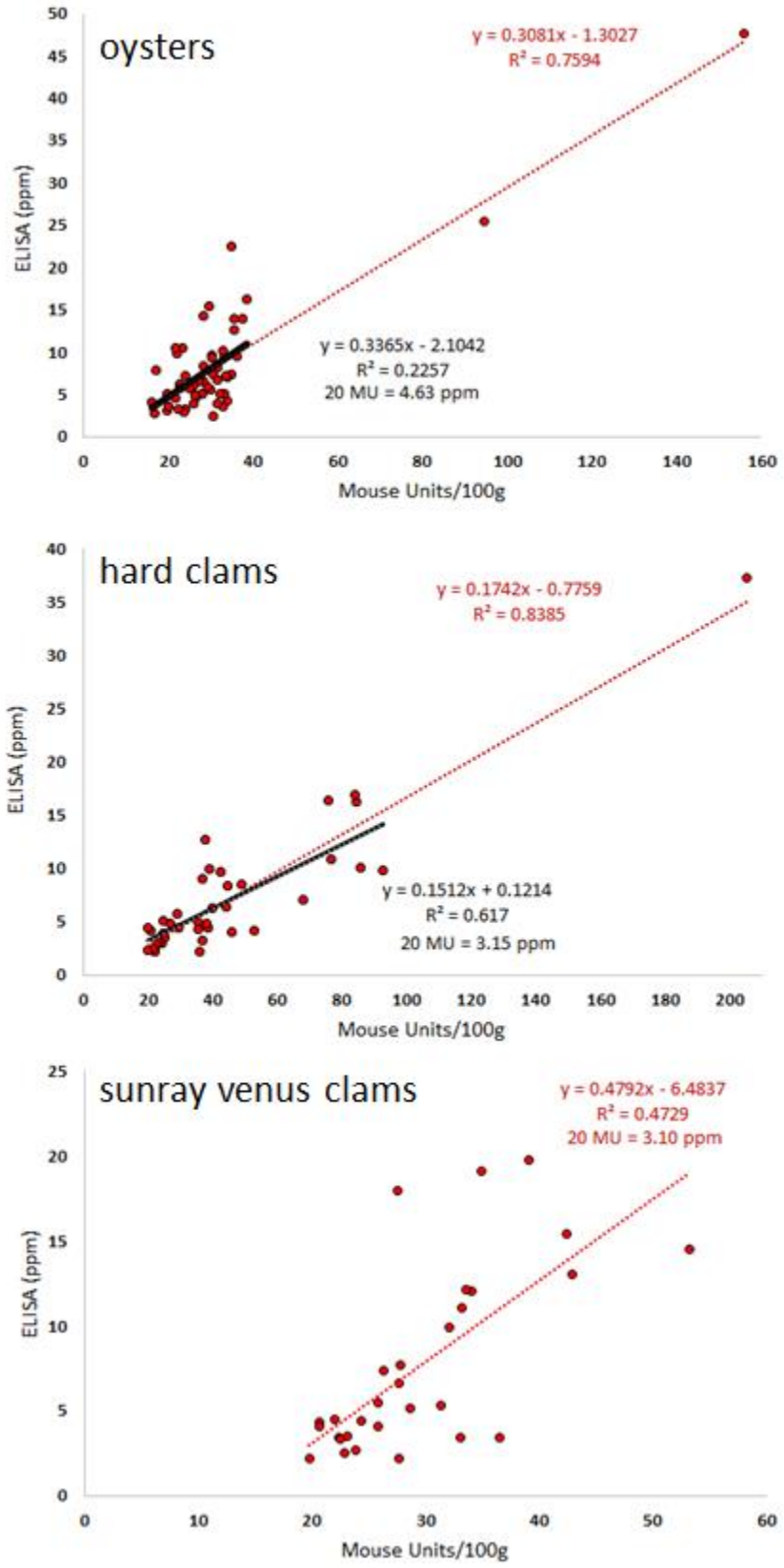

Figure G9. NSP mouse bioassay vs. ELISA results in oyster, hard clams, and sunray venus clams.

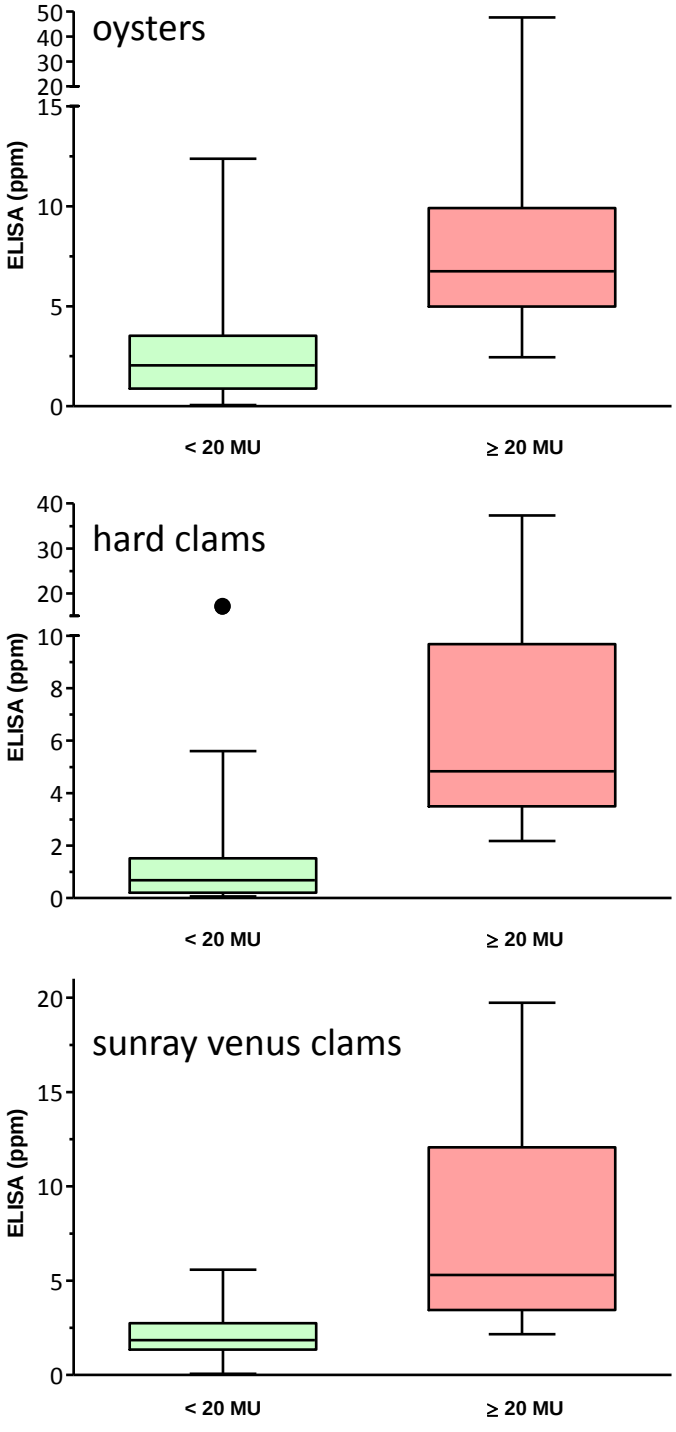

Figure G10. Boxplots (whiskers min to max) of ELISA results for samples testing < 20 MU/100g and ≥ 20 MU/100g in oysters, hard clams, and sunray venus clams. A value of 0.06ppm (half the limit of detection[LOD]) was substituted for ELISA results that were <LOD.

Boxplots were created to visualize the distribution of the data for samples testing < 20 MU/100g and ≥ 20 MU/100g (Fig. G10). There was a very wide range of concentrations measured by ELISA in samples testing < 20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay.

Brevetoxin metabolites are persistent in shellfish, and some level is frequently measured in bivalves from *K. brevis* endemic areas that have tested safe by mouse bioassay. In samples testing <20 MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. The highest concentrations were measured in wild oysters and hard clams, presumably due to repeated exposure to *K. brevis* and retention of the more persisitent metabolites across multiple bloom seasons. Farmed clams are brevetoxin-free when they are placed on lease sites, and their residence time in natural waters is short. These bivalves do not experience multiple successive bloom seasons. The maximum concentration measured in farmed clams that were < 20 MU was 4.6 ppm and in sunray venus clams was 5.6 ppm.

Importantly, across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that “failed” by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.

As the only Approved Method, the NSP mouse bioassay is currently the only yardstick to which new methods can be compared. The mouse bioassay is semi-quantitative, not calibrated, and detects only that subset of compounds in shellfish that are ether-extractable. Analytical and screening NSP methods are unlikely to ever completely agree with mouse bioassay results, and expectations for

comparisons of proposed alternate methods with the mouse bioassay should be guaged accordingly, with a goal of achieving an equal measure of safety rather than perfect alignment of results and management actions on a sample by sample basis.

The results of our Single Lab Validation demonstrate that this assay generates specific, precise, and repeatable results. Additionally, ELISA results of naturally incurred shellfish compare very well with LC-MS analyses targeting the dominant metabolites found in eastern oysters and hard clams from the Gulf of Mexico (S-desoxy-BTX-B2, BTX-B2, and BTX-B1; Fig. G11).

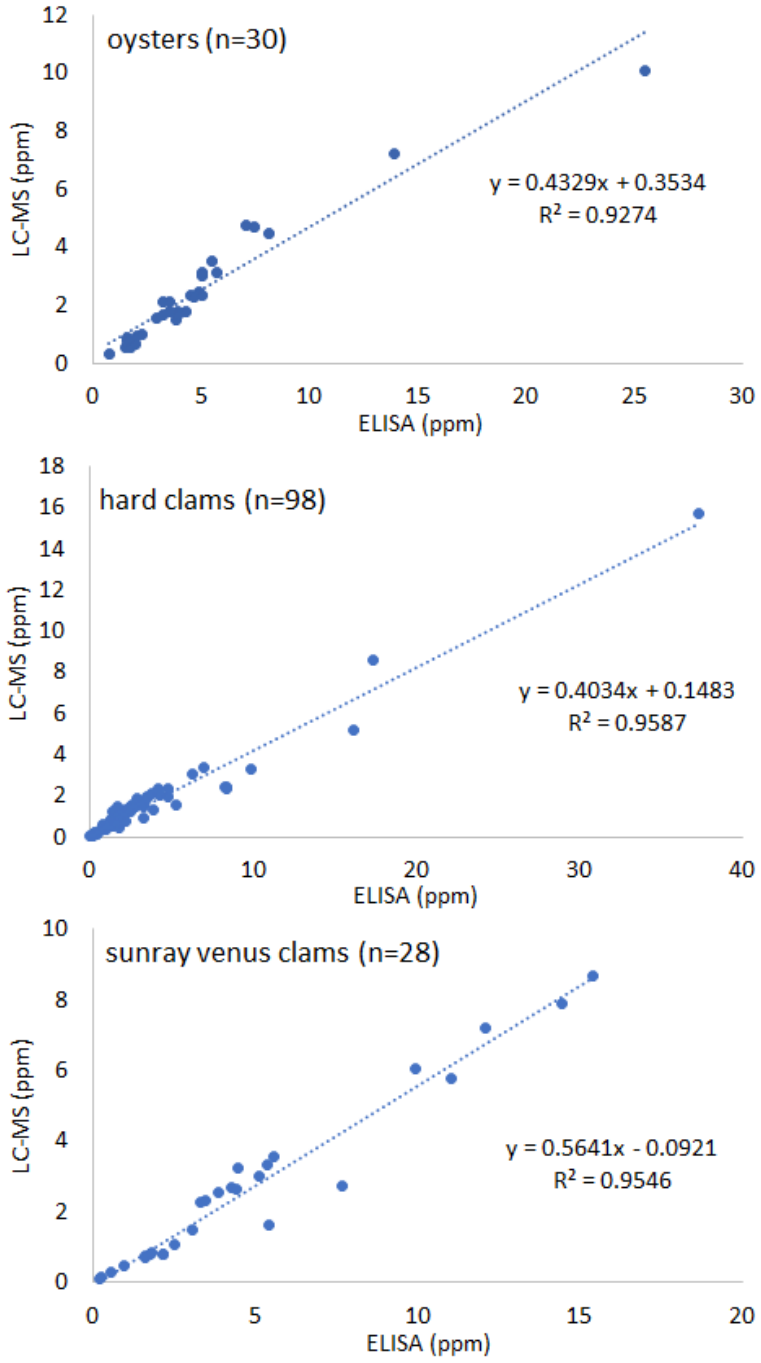

Figure G11. Comparison of NSP ELISA and LC-MS analysis of naturally incurred shellfish. LC-MS data generated and provided by A. Abraham, USFDA Gulf Coast Seafood Laboratory, using samples from this study.

Given the shortcomings and limitations of the mouse bioassay and the hardship this method imposes on both resource managers and industry, the move towards alternate methods must begin.

We propose that ELISA be approved for use as Limited Use Method such that samples would "pass" NSP rapid screening by ELISA when ELISA results are at or below a threshold representing no more than half of the level predicted in samples testing close to 20 MU/100g and below the lowest level measured in samples that have tested greater than or equal to 20 MU/100g (i.e., yielding no false negatives when applied to the existing dataset).

Thresholds of 1.8 ppm in oysters and 1.6 ppm in hard clams and sunray venus clams are proposed. The approach to derive the threshold was to approximate the ELISA equivalent of half of 20 MU/100 g and to ensure that the threshold would yield no false negatives. To protect against false negatives, the proposed thresholds are no more than 75% of the lowest concentration in the dataset that yielded a positive mouse bioassay. In hard clams and sunray venus clams, 1.6 ppm approximates half of the predicted 20 MU/100 g equivalent and is 75% of the lowest level measured in clams that failed mouse bioassay (2.18 ppm). For oysters, 1.80 is less than half of the estimated 20 MU/100 g equivalent and is 73% of the lowest level measured in oysters that failed mouse bioassay (2.45 ppm). These thresholds are not proposed as new guidance or actions limits for NSP, but rather as screening thresholds **specific to the MARBIONC ELISA (using PbTx-3 as a standard)** below which we have confidence that oysters and clams would yield <20 MU/100g and above which testing by mouse bioassay (or other future Approved Method) would be required.

Applying these thresholds to the comparative data set presented here would produce no false negatives (no samples testing greater than or equal to 20 MU/100 exceeded these levels by ELISA). Among the subset of samples testing < 20 MU/100g, ELISA results exceeded the thresholds (and would necessitate additional testing by NSP mouse bioassay) for 56% of oyster samples, 22% of hard clam samples, and 68% of sunray venus clam samples. The high proportion of <20 MU sunray venus clams above the threshold is an artifact of our sample set. Because sunray venus clams are relatively new to Florida aquaculture, our sample size is smaller, and collections during and following *K. brevis* blooms have been targeted in recent years to generate quantitative mouse bioassay data for comparisons.

As a first step away from total reliance on the NSP mouse bioassay, the proposed thresholds are conservative, and they may need to be revised in the future when more data and/or other approved methods are available, but they would have eliminated the need for 246 of the 501 bioassays (49%) conducted and represented in this data set. Having this method available as an approved option for NSP testing would greatly benefit all Gulf States. In 2015, a *K. brevis* affected the entire northern Gulf of Mexico, resulting in simultaneous closures of shellfish harvest areas in Florida, Alabama, Mississippi, and Louisiana. Because Alabama, Mississippi, and Louisiana experience these blooms infrequently, they lack the capacity to conduct NSP mouse bioassays. Therefore, sample testing to reopen harvest areas in these states after the bloom had dissipated was coordinated by our lab in Florida with the assistance of Resource Access International in Maine. While this cooperative effort was successful, it was a heavy burden on Florida, taking five weeks following bloom termination to complete and unnecessarily extending closures in these states. In every case, samples submitted by the other states passed by mouse bioassay (contained < 20 MU/100g), and if screening by ELISA had been an approved option, bioassays would not have been necessary in Mississippi or Louisiana, where NSP levels of oyster samples tested by ELISA ranged from 0.16 to 1.22 ppm.

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VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must be sufficiently rugged to withstand the relatively minor day to day changes likely to occur in routine use. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Ruggedness of the new or modified method is the ability of a particular method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure for testing the ruggedness of new or modified methods: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine method concentration for the target analyte/measurand/organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the new method or modified method's intended application to spike the sample aliquots. However both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days.

Data for demonstrating the ruggedness of the new or modified method:
For this study, results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min).

1) Different lots of ELISA kit reagents:

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	Jun-14 lot	Jun-16 lot
oyster	OY01	0.4	0.38	0.36
oyster	OY02	0.4	0.38	0.36
oyster	OY03	1	0.92	0.90
oyster	OY04	1	0.88	0.88
oyster	OY05	2	1.87	1.88
oyster	OY06	2	1.84	2.04
oyster	OY07	4	3.78	3.66
oyster	OY08	4	3.88	4.54
oyster	OY09	8	7.67	7.86
oyster	OY10	8	8.05	7.73
hard clam	HC01	0.4	0.38	0.39
hard clam	HC02	0.4	0.39	0.38
hard clam	HC03	1	1.08	0.92
hard clam	HC04	1	0.97	0.97
hard clam	HC05	2	1.97	1.92

hard clam	HC06	2	2.16	1.85
hard clam	HC07	4	3.78	4.05
hard clam	HC08	4	3.90	3.83
hard clam	HC09	8	7.86	7.69
hard clam	HC10	8	7.79	8.16
sunray venus clam	SV01	0.4	0.35	0.33
sunray venus clam	SV02	0.4	0.39	0.38
sunray venus clam	SV03	1	1.03	0.89
sunray venus clam	SV04	1	1.05	1.00
sunray venus clam	SV05	2	2.05	1.89
sunray venus clam	SV06	2	1.97	1.95
sunray venus clam	SV07	4	3.62	4.23
sunray venus clam	SV08	4	3.82	4.22
sunray venus clam	SV09	8	7.57	7.38
sunray venus clam	SV10	8	8.34	7.85

2) Incubation of ELISA plates throughout the procedure at ambient laboratory temperature (21-22°C) vs. in a heated plate shaker (25°C):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	21-22°C	25°C
oyster	OY01	0.4	0.36	0.37
oyster	OY02	0.4	0.36	0.40
oyster	OY03	1	0.90	0.88
oyster	OY04	1	0.88	0.84
oyster	OY05	2	1.88	1.96
oyster	OY06	2	2.04	1.94
oyster	OY07	4	3.66	3.72
oyster	OY08	4	4.54	4.56
oyster	OY09	8	7.86	8.08
oyster	OY10	8	7.73	8.31
hard clam	HC01	0.4	0.39	0.39
hard clam	HC02	0.4	0.38	0.37
hard clam	HC03	1	0.92	0.91
hard clam	HC04	1	0.97	0.86
hard clam	HC05	2	1.92	2.07
hard clam	HC06	2	1.85	1.87
hard clam	HC07	4	4.05	4.06
hard clam	HC08	4	3.83	4.17
hard clam	HC09	8	7.69	7.96
hard clam	HC10	8	8.16	8.26
sunray venus clam	SV01	0.4	0.33	0.35
sunray venus clam	SV02	0.4	0.38	0.40
sunray venus clam	SV03	1	0.89	0.92
sunray venus clam	SV04	1	1.00	0.94
sunray venus clam	SV05	2	1.89	2.24
sunray venus clam	SV06	2	1.95	1.86

sunray venus clam	SV07	4	4.23	4.08
sunray venus clam	SV08	4	4.22	4.19
sunray venus clam	SV09	8	7.38	7.03
sunray venus clam	SV10	8	7.85	7.49

3) Duration of sample and primary antibody (reagent C) incubation (60 min vs. 90 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	60 min C	90 min C
oyster	OY01	0.4	0.37	0.38
oyster	OY02	0.4	0.39	0.38
oyster	OY03	1	0.92	0.92
oyster	OY04	1	0.92	0.88
oyster	OY05	2	1.61	1.87
oyster	OY06	2	1.62	1.84
oyster	OY07	4	3.28	3.78
oyster	OY08	4	3.51	3.88
oyster	OY09	8	7.94	7.67
oyster	OY10	8	7.99	8.05
hard clam	HC01	0.4	0.40	0.38
hard clam	HC02	0.4	0.39	0.39
hard clam	HC03	1	1.02	1.08
hard clam	HC04	1	1.07	0.97
hard clam	HC05	2	1.84	1.97
hard clam	HC06	2	1.97	2.16
hard clam	HC07	4	3.65	3.78
hard clam	HC08	4	3.40	3.90
hard clam	HC09	8	7.44	7.86
hard clam	HC10	8	7.89	7.79
sunray venus clam	SV01	0.4	0.37	0.35
sunray venus clam	SV02	0.4	0.40	0.39
sunray venus clam	SV03	1	0.94	1.03
sunray venus clam	SV04	1	0.95	1.05
sunray venus clam	SV05	2	2.11	2.05
sunray venus clam	SV06	2	2.07	1.97
sunray venus clam	SV07	4	3.89	3.62
sunray venus clam	SV08	4	3.73	3.82
sunray venus clam	SV09	8	7.84	7.57
sunray venus clam	SV10	8	7.89	8.34

4) Duration of TMB color development step (7 min vs 13 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	TMB 7 min	TMB 13 min
oyster	OY01	0.4	0.35	0.48
oyster	OY02	0.4	0.36	0.36
oyster	OY03	1	0.91	1.13
oyster	OY04	1	0.91	0.89
oyster	OY05	2	1.69	2.05
oyster	OY06	2	1.86	1.90
oyster	OY07	4	3.84	4.05
oyster	OY08	4	3.88	4.61
oyster	OY09	8	7.89	8.35
oyster	OY10	8	7.90	7.63
hard clam	HC01	0.4	0.34	0.42
hard clam	HC02	0.4	0.37	0.37
hard clam	HC03	1	0.95	1.13
hard clam	HC04	1	0.93	0.93
hard clam	HC05	2	1.78	2.22
hard clam	HC06	2	1.64	1.80
hard clam	HC07	4	3.74	4.45
hard clam	HC08	4	3.62	4.37
hard clam	HC09	8	7.52	7.48
hard clam	HC10	8	7.94	7.55
sunray venus clam	SV01	0.4	0.35	0.39
sunray venus clam	SV02	0.4	0.38	0.44
sunray venus clam	SV03	1	0.94	0.97
sunray venus clam	SV04	1	0.93	1.17
sunray venus clam	SV05	2	1.84	2.13
sunray venus clam	SV06	2	1.76	1.81
sunray venus clam	SV07	4	3.66	3.90
sunray venus clam	SV08	4	3.76	4.04
sunray venus clam	SV09	8	7.88	7.50
sunray venus clam	SV10	8	7.95	8.14

For shellfish samples, repeat for each tissue type of interest.

Data handling to demonstrate the ruggedness of the new or modified method

In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the new or modified method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

To determine whether the new or modified method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be

used depending upon the shape of the distribution produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 >2 indicates a lack of homogeneity of variance.

5. Use either the paired t-test or Welch's t-test for the analysis based on the following considerations.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the analysis.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis.
    - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range of -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for demonstrating the ruggedness of the new or modified method:

See tables on next page

Significant differences were observed with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). 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 of 1.0 ± 30%.

Value for the test of symmetry of the distribution of batch/lot 1 data _____

Value for the test of symmetry of the distribution of batch/lot 2 data _____

Variance of batch/lot 1 data _____

Variance of batch/lot 2 data _____

Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 _____

Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples? Y/N

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	Jun14 lot	Jun16 lot	Jun14 lot	Jun16 lot			
oyster	-0.32402 (0.750)	-0.07704 (0.906)	0.2281	0.2350	1.030	0.302	no
hard clam	-0.10448 (0.916)	-0.26257 (0.856)	0.2366	0.2483	1.049	0.708	no
sunray venus clam	-0.27735 (0.804)	-0.17249 (0.852)	0.2327	0.2471	1.062	0.465	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	21-22°C	25°C	21-22°C	25°C			
oyster	-0.07704 (0.974)	-0.20833 (0.822)	0.2350	0.2465	1.049	0.741	no
hard clam	-0.26257 (0.740)	-0.18657 (0.874)	0.2483	0.2483	1.000	0.287	no
sunray venus clam	-0.17249 (0.820)	-0.37325 (0.764)	0.2471	0.2333	1.059	0.754	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	60 min C	90 min C	60 min C	90 min C			
oyster	-0.13316 (0.866)	-0.32402 (0.780)	0.2160	0.2281	1.056	0.219	no
hard clam	0.25186 (0.772)	-0.10448 (0.912)	0.2301	0.2366	1.028	0.099	no
sunray venus clam	-0.42338 (0.680)	-0.27735 (0.734)	0.2326	0.2327	1.000	0.982	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	TMB 7 min	TMB 13 min	TMB 7 min	TMB 13 min			
oyster	0.07922 (0.892)	-0.13022 (0.922)	0.2388	0.2297	1.040	0.014	yes
hard clam	-0.00274 (0.958)	-0.04028 (0.982)	0.2460	0.2302	1.069	0.030	yes
sunray venus clam	-0.03460 (0.990)	-0.06355 (0.940)	0.2387	0.2187	1.092	0.011	yes

*m-out-of-n bootstrap symmetry test by Miao, Gel, and Gastwirth (2006)

Miao, W., Y. R. Gel, and J. L. Gastwirth. "A New Test of Symmetry about an Unknown Median. Random Walk." *Sequential Analysis and Related Topics-A Festschrift in Honor of Yuan-Shih Chow. Eds.: Agnes Hsiung, Cun-Hui Zhang, and Zhiliang Ying, World Scientific Publisher, Singapore* (2006).

VALIDATION CRITERIA

Linear Range is the range within the working range where the results are proportional to the concentration of the analyte/measurand/organism of interest present in the sample.

Limit of Detection is the minimum concentration at which the analyte/measurand/organism of interest can be identified.

Limit of Quantitation/Sensitivity is the minimum concentration of the analyte/measurand/organism of interest that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take at least six (6) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work and spike five (5) of the six (6) aliquots with five (5) different concentrations (i.e. 10^a, 10^b…10^n) of the target analyte/measurand/organism of interest spanning 50 – 150% of the working range/range of interest for the method under study. Do not spike the sixth or last aliquot of each sample. This is the sample blank. For microbiological methods determine the concentration of the target analyte/measurand/organism of interest used to spike each aliquot of each sample by plating in/on appropriate agar. Do not use aliquots of the same master solution/culture to spike all the samples in this exercise. A separate master solution /culture should be used for each sample. Process each aliquot including the sample blank as usual to determine method concentration for the target analyte/measurand/organism of interest. Do three (3) replicates for each aliquot excluding the sample blank. Do only one blank per sample. For growing waters do ten (10) samples collected from a variety of growing areas. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed (10^a, 10^b…10^n).

Data:
Sample type
Working range/Range of interest: 0.4-8 ppm
Range in spiking levels used: 0.4 ppm, 1 ppm, 4 ppm, 8 ppm, 12 ppm
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

Response is the signal data (absorbance, fluorescence, Ct value), colonies, plaques, etc resulting from the analysis.

For shellfish samples repeat for each tissue type of interest.

DATA HANDLING

Linear Range

To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it.
6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response
7. Multiply the value obtained in step 5 by 0.95 and 1.05.

8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects either of the lines obtained above.
10. The linear range of the method as implemented by the laboratory is comprised of the range in concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organisn of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of "blank + 3σ" and "blank + 10σ" generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

Data Summary: See below for explanation

Linear range of the method as implemented 0.12 ppm to 35.33 ppm
The limit of detection of the method as implemented 0.040 ppm
The limit of quantitation/sensitivity of the method as implemented 0.12 ppm

Data was generated as directed above (ten samples spiked to five levels, analyzed in triplicate plus one blank aliquot) for each matrix type examined, but this data could not be analyzed as described in the data handling portion of this SOP. (Although most of the data was not used to determine linearity and LOQ/LOD, it is provided at the end of this Appendix.)

This ELISA kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the maximum absorbance of the reference wells (Amax). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared and analyzed. Assay response is converted to concentration by comparison to a standard curve, and the final sample concentration is the product of the concentration measured in the assay and the dilution factor. As a result, similar responses (signal data) can be measured for very different sample concentrations.

The overall or dynamic linear range of this method results from a combination of: 1) the linear range of the assay standard curve, 2) the assay limit of quantitation, and 3) the range of sample dilutions on the plate.

1) Linear Range of Assay

To evaluate the linear range of the assay, 7-point PbTx-3 standard curves (ranging from 0.08 to 5.0 ng/mL) from 60 ELISA plates run in this validation were generated using the sigmoidal dose-response (variable slope), or four-parameter logistic, curve fitting equation in Prism 5 (GraphPad Software). The upper and lower plateaus of the curves were then applied to formulae derived by Sebaugh and McCray[1] to define the "bend points" of the standard curves, the beginning and end of the linear concentration--response region, expressed both in terms of % inhibition (1-A/Amax x 100) and concentration (Table E.1). The assays included data generated using two different kit lots: June 2014 (n=34) and June 2016 (n=26). We found that the position of the standard curves and the linear range defined by the bend points differed between the two kit lots (Fig. E1). Such shifts can be achieved with the same kit lot by altering dilutions of key reagents (A and C). Therefore, we believe that the differences we observed in kit lots were due to minor concentration variations in the supplied reagents A and/or C. However, comparative analyses of spiked samples were not significantly different between the two kit lots (see Appendix F: Ruggedness).

Table E1. Average bend points (± standard deviation), expressed as % inhibition and concentration, defining the linear range of standard curves generated using two lots of ELISA kit reagents.

	% inhibition		ng PbTx-3/mL	
	Jun-14 Lot	Jun-16 Lot	Jun-14 Lot	Jun-16 Lot
lower bend point	17.34 ± 2.47	16.76 ± 2.73	0.21 ± 0.04	0.30 ± 0.06
upper bend point	76.91 ± 2.07	74.19 ± 1.68	1.04 ± 0.14	1.38 ± 0.16

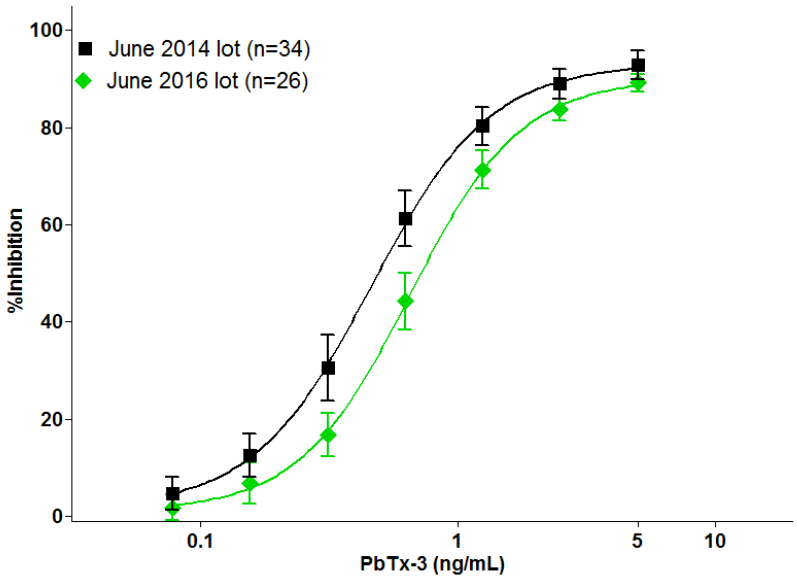


Figure E1. Average of multiple semi-log standard curves generated using two lots of ELISA kit reagents. Error bars represent standard deviation of independent curves prepared and assayed on different plates or days.

Using the June 2014 lot, two additional 14-point standard curves were assayed on different days to generate curves with more points that fell along the linear portion of the curve (Fig. E2). The bend points from these 14-point curves (16%-76%) were similar to those derived from the routine standard curves (Table E1).

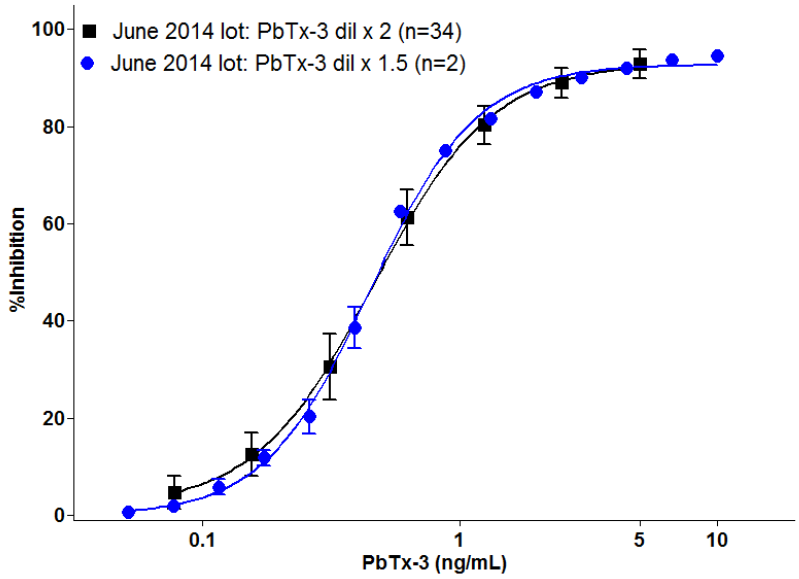


Figure E2. Comparison of 7-point and 14-point PbTx-3 standard curves. Error bars represent standard deviation of independent curves prepared and assays on different plates/days.

To verify linearity within the range defined by the bend points, multiple dilutions of shellfish samples spiked to 0.4 ppm with PbTx-3 were quantified. Ten samples were used for each matrix type, and three replicates per sample were extracted and analyzed. These assays were performed using the June 2014 kit lot. As written in the method protocol, the shellfish extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume). The extract is then diluted another 40 times in ELISA buffer for the initial dilution, and six additional dilutions are prepared by serial dilution by two, yielding a total of seven dilutions (from 1:400 to 1:25,600) for each sample.
At the 0.4 ppm spike level, the expected value of the first three dilutions are 1.0, 0.5, and 0.25 ng/mL, which are all within the linear range of the June 2014 kit lot as defined by the bend points. The expected and mean measured values of the three dilutions are listed in Table E2. Linear regression yielded r^2 values of 0.94-0.97 (Fig. E3).

Table E2. Expected concentrations and mean of concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Shellfish were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed.

	dilution	expected	mean measured	SD	%CV
oyster	400	1.00	0.935	0.079	8.4%
	800	0.50	0.471	0.038	8.1%
	1600	0.25	0.229	0.034	14.8%
hard clam	400	1.00	0.893	0.081	9.1%
	800	0.50	0.456	0.055	12.1%
	1600	0.25	0.233	0.033	14.2%
sunray venus clam	400	1.00	0.911	0.098	10.8%
	800	0.50	0.455	0.059	13.0%
	1600	0.25	0.234	0.030	12.8%

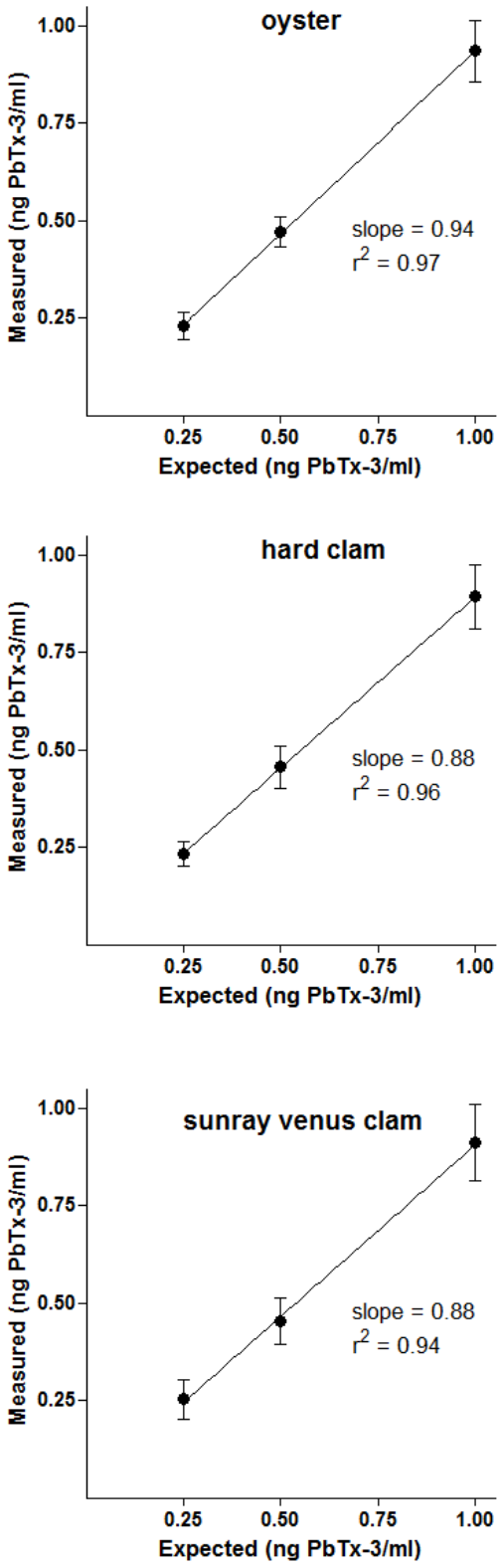


Figure E3. Expected concentrations vs. mean concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Oyster, hard clams, and sunray venus clams were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed. Error bars represent standard deviation.

Limit of Detection and Limit of Quantitation

Blank samples consistently yielded assay responses that were not quantifiable. Therefore, the standard deviation of results from the 1:1600 dilution of shellfish reported in Table E2 above were used to derive the limit of detection (LOD) and limit of quantitation (LOQ) expressed as 3x and 10x the standard deviation, respectively. This dilution consistently yielded a signal (% inhibition) closest to the lower bend point (17%) and was the lowest quantifiable dilution.

For all three matrix types, the standard deviation at the 1:1600 dilution was approximately 0.03. Calculated assay LOD and LOQ are 0.1 and 0.3 ng/mL, respectively. At the lowest sample dilution of 1:400, the LOD and LOQ for brevetoxin in shellfish are 40 and 120 ng/g or 0.04 and 0.12 ppm.

Dynamic linear range

The overall or dynamic linear range of this method is a combination of the linear range of the standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate (from 400 to 25,600). Using the LOQ calculated above, which falls with the linear portion of the standard curve identified for both lots of kits used in this study, and the upper bend points identified for each kit lot, the overall or dynamic linear range of the method is from 120 ng PbTx-3 eq./g or 0.12 ppm up to 26,624 or 26.62ppm for the June 2014 kit lot and up to 35,328 ng PbTx-3 eq. per g or 35.33 ppm for the June 2016 kit lot.

References:

1. Sebaugh JL, McCray PD (2003) Defining the linear portion of a sigmoid-shaped curve: bend points. Pharmaceutical Statistics 2: 167-174.

Results of spiking experiments: ten samples were spiked to five levels and analyzed in triplicate (plus one blank aliquot) for each matrix type examined.

Data Summary:

spike conc (ppm)	Average concentration (ppm) Average recovery (%) Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%
12	10.63	11.03	12.74
	89%	92%	106%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY01	12	10.60	10.97	10.73
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY02	12	10.77	10.26	10.54
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY03	12	10.83	11.13	11.11
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY04	12	10.90	10.08	9.94
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36

oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY05	12	10.17	10.38	9.68
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY06	12	11.62	10.71	11.36
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY07	12	10.68	11.42	10.75
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY08	12	10.68	11.00	10.46
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04
oyster	OY09	12	11.09	10.44	10.78
oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
oyster	OY10	12	9.58	9.75	10.34
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC01	12	9.89	9.80	10.75
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC02	12	11.14	11.33	11.63
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC03	12	10.26	11.20	10.25
hard clam	HC04	0	<LD		

hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC04	12	11.50	11.92	11.74
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC05	12	10.78	9.82	11.27
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC06	12	11.18	11.40	12.08
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC07	12	10.94	10.50	10.70
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC08	12	10.53	10.76	10.98
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40
hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC09	12	12.38	10.77	10.84
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
hard clam	HC10	12	10.99	11.31	12.19
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV01	12	11.61	12.13	11.52
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV02	12	12.57	11.57	12.71

sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV03	12	12.95	12.02	13.17
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV04	12	12.51	11.70	13.43
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV05	12	13.32	12.34	13.47
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV06	12	14.52	12.76	13.90
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV07	12	11.91	13.74	11.53
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70
sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV08	12	11.73	14.16	12.19
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV09	12	13.18	14.53	12.87
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94
sunray venus clam	SV10	12	12.62	12.50	12.98

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable, the new or modified method must be specific for the analyte/measurand/organism of interest. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Specificity of the new or modified method is the ability of this new or modified method to measure only what it is intended to measure. To determine the specificity of new or modified methods, samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/target organism of interest.

Procedure for demonstrating the specificity of the new or modified method: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method/modified method under study) of the target analyte/measurand/organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank , the aliquot spiked with the target analyte/measurand/organism of interest and the aliquot spiked with the target analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method/modified method concentration for the target analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one (1) sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

Data for demonstrating the specificity of the new or modified method:

Potentially interfering substances examined in this study included two types of microalgae – the flagellate *Isochrysis* aff. *galbana* and the cryptophyte *Rhodomonas lens* – that are commonly fed to aquaculture-reared bivalves at the age/size at which they are ready to be relocated from the hatchery to the aquaculture zones. With the capacity to ingest as many as 10^9 cells per day, it is reasonable to predict there may be some bioaccumulation of cell constituents over time, and that they may still be present when the bivalves are harvested. Cells were added to a concentration of 100 million cells per g of shellfish.

Also examined was okadaic acid, a dinoflagellate toxin produced by some species of *Dinophysis* and *Prorocentrum*. These organisms are present in waters where *Karenia brevis* occurs, and potentially both toxins could be present. Both brevetoxin and okadaic acid are polyether toxins, so cross-reactivity with okadaic acid was investigated. Okadaic acid was added to a concentration of 1.5 µg per g of shellfish (or 1.5 ppm), which is roughly ten times above the current US guidance limit of 0.16 ppm.

The final substance to be examined was *Karenia mikimotoi*, a dinoflagellate that is closely related to *Karenia brevis*. *K. mikimotoi* produces bioactive compounds, but brevetoxin production has not been documented in this species. *Karenia* blooms are often mixed species blooms with two or more *Karenia* species present, although *K. brevis* is typically dominant. *K. mikimotoi* cells were added to a concentration of 500,000 cells per g.

Interfering organism/compound/toxin:

A *Isochrysis* aff. *galbana* (100 million cells per g shellfish)
B *Rhodomonas lens* (100 million cells per g shellfish)
C Okadaic acid (1.5 µg per g shellfish)
D *Karenia mikimotoi* (500,000 cells per g shellfish)

PbTx-3 spike concentration: 0.4 ppm

oyster

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.39	0.41	0.41	0.38	0.39	<LD
0.38	0.38	0.41	0.38	0.40	
0.42	0.39	0.39	0.37	0.43	
0.34	0.38	0.42	0.37	0.37	
0.39	0.44	0.40	0.35	0.42	

hard clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.36	0.40	0.40	0.38	0.40	<LD
0.38	0.38	0.39	0.40	0.32	
0.39	0.40	0.37	0.37	0.38	
0.35	0.36	0.38	0.37	0.33	
0.38	0.40	0.39	0.38	0.37	

sunray venus clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.33	0.41	0.42	0.41	0.35	<LD
0.35	0.39	0.38	0.40	0.41	
0.38	0.38	0.36	0.35	0.35	
0.35	0.37	0.34	0.39	0.40	
0.38	0.43	0.40	0.39	0.41	

Data handling for demonstrating specificity of the new or modified method

The specificity index will be used to test the specificity of the new or modified method in the presence of suspected interfering organisms/compounds/toxins. The specificity index (SI) is calculated as indicated below:

$$\text{Specificity index (SI)} = \frac{\text{Sample spiked with only target of interest}}{\text{Sample spiked with target in presence if suspected interferences}}$$

All microbiological count data must be converted to logs before statistical analysis. Samples spiked with both the target analyte/measurand/organism of interest and the target analyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any correction that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index (SI) should equal one (1) in the absence of interferences. To test the significance of a Specificity index (SI) other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test at the .05 significance level is used. For each suspected interfering organism/compound/toxin calculate the average Specificity index (SI_{avg}) for the five (5) replicates analyzed for each sample by obtaining the average concentration for both the aliquot containing the target analyte/measurand/organism of interest only and the aliquot containing the target analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

$$SI_{avg} = \frac{\text{Avg. conc. of sample spiked only with target of interest}}{\text{Avg. conc. of sample spiked with target in the presence of suspected interferences}}$$

Perform the t-test to determine if the average Specificity index (SI) obtained from the five (5) replicates from each analysis differs from one (1). Repeat for all the suspected interfering organisms/compounds/toxins tested.

Data summary for testing the specificity of the new or modified method:

Oyster

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.96	no	0.352
B	*Rhodomonas lens*	0.94	no	0.254
C	Okadaic acid	1.03	no	0.490
D	*Karenia mikimotoi*	0.95	no	0.061

Hard clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.97	no	0.164
B	*Rhodomonas lens*	0.97	no	0.230
C	Okadaic acid	0.98	no	0.374
D	*Karenia mikimotoi*	1.04	no	0.364

Sunray venus clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.91	no	0.055
B	*Rhodomonas lens*	0.95	no	0.311
C	Okadaic acid	0.93	no	0.205
D	*Karenia mikimotoi*	0.94	no	0.230

VALIDATION CRITERIA

Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

Recovery is the fraction or percentage of an analyte/measurand/organism of interest recovered following sample analysis.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take four (4) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work. Spike one of the four aliquots with a low (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Spike the second aliquot of the growing water sample or shellfish homogenate with a medium concentration of the target analyte/measurand/organism of interest. Spike the third aliquot of the growing water sample or shellfish homogenate with a high (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Do not spike the fourth aliquot of the growing water sample or shellfish homogenate. This is the sample blank. Spiking levels must cover the range in concentrations important to the application of the method (working range). For microbiological methods determine the concentration of the target organism of interest used to spike each aliquot by plating in/on appropriate agar. Process each aliquot including the sample blank as usual to determine the method concentration for the target analyte/measurand/organism of interest. Do two (2) replicates for each of the three (3) spiked aliquots. Replicate analysis is unnecessary for the sample blank. Do only one sample blank per sample. For growing waters, do ten (10) samples collected from a variety of growing areas. For shellfish, do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed in this exercise (i.e. 10^1, 10^3 and 10^5).

Data:
Working Range: 0.4 - 4 ppm
Sample Type: Oyster, Hard Clam, Sunray Venus Clam
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

matrix type	sample	spike level	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)
hard clam	HC01	blank	0	<LD	
hard clam	HC01	L	0.4	0.33	0.32
hard clam	HC01	M	1	0.98	0.93
hard clam	HC01	H	4	3.85	3.79
hard clam	HC02	blank	0	<LD	
hard clam	HC02	L	0.4	0.35	0.33
hard clam	HC02	M	1	0.92	0.89
hard clam	HC02	H	4	3.82	3.36
hard clam	HC03	blank	0	<LD	
hard clam	HC03	L	0.4	0.35	0.33
hard clam	HC03	M	1	0.91	0.91
hard clam	HC03	H	4	3.55	3.36
hard clam	HC04	blank	0	<LD	
hard clam	HC04	L	0.4	0.33	0.3
hard clam	HC04	M	1	0.91	0.91
hard clam	HC04	H	4	4.66	3.99
hard clam	HC05	blank	0	<LD	
hard clam	HC05	L	0.4	0.32	0.33
hard clam	HC05	M	1	0.92	0.89

hard clam	HC05	H	4	3.49	4.03
hard clam	HC06	blank	0	<LD	
hard clam	HC06	L	0.4	0.44	0.44
hard clam	HC06	M	1	0.84	0.92
hard clam	HC06	H	4	4.15	4.25
hard clam	HC07	blank	0	<LD	
hard clam	HC07	L	0.4	0.42	0.43
hard clam	HC07	M	1	1	1.01
hard clam	HC07	H	4	4.05	4.12
hard clam	HC08	blank	0	<LD	
hard clam	HC08	L	0.4	0.35	0.37
hard clam	HC08	M	1	0.92	1
hard clam	HC08	H	4	4.23	3.55
hard clam	HC09	blank	0	<LD	
hard clam	HC09	L	0.4	0.4	0.39
hard clam	HC09	M	1	0.93	0.91
hard clam	HC09	H	4	3.98	4.26
hard clam	HC10	blank	0	<LD	
hard clam	HC10	L	0.4	0.36	0.39
hard clam	HC10	M	1	0.97	0.98
hard clam	HC10	H	4	4.54	3.98
oyster	OY01	blank	0	<LD	
oyster	OY01	L	0.4	0.38	0.38
oyster	OY01	M	1	0.99	0.95
oyster	OY01	H	4	4.07	4.12
oyster	OY02	blank	0	<LD	
oyster	OY02	L	0.4	0.39	0.39
oyster	OY02	M	1	0.94	0.95
oyster	OY02	H	4	3.87	3.85
oyster	OY03	blank	0	<LD	
oyster	OY03	L	0.4	0.44	0.42
oyster	OY03	M	1	0.8	0.77
oyster	OY03	H	4	3.57	3.92
oyster	OY04	blank	0	<LD	
oyster	OY04	L	0.4	0.37	0.35
oyster	OY04	M	1	1	0.85
oyster	OY04	H	4	4.17	4.14
oyster	OY05	blank	0	<LD	
oyster	OY05	L	0.4	0.36	0.38
oyster	OY05	M	1	0.77	0.89
oyster	OY05	H	4	4.22	4.06
oyster	OY06	blank	0	<LD	
oyster	OY06	L	0.4	0.31	0.33
oyster	OY06	M	1	0.91	0.92
oyster	OY06	H	4	3.36	3.48
oyster	OY07	blank	0	<LD	

oyster	OY07	L	0.4	0.4	0.4
oyster	OY07	M	1	0.88	1.05
oyster	OY07	H	4	3.9	4.21
oyster	OY08	blank	0	<LD	
oyster	OY08	L	0.4	0.46	0.44
oyster	OY08	M	1	1.05	1.03
oyster	OY08	H	4	3.86	4.03
oyster	OY09	blank	0	<LD	
oyster	OY09	L	0.4	0.43	0.37
oyster	OY09	M	1	1.06	0.92
oyster	OY09	H	4	3.74	3.94
oyster	OY10	blank	0	<LD	
oyster	OY10	L	0.4	0.36	0.38
oyster	OY10	M	1	0.94	0.99
oyster	OY10	H	4	4.24	4.28
sunray venus clam	SV01	blank	0	<LD	
sunray venus clam	SV01	L	0.4	0.36	0.37
sunray venus clam	SV01	M	1	0.94	0.98
sunray venus clam	SV01	H	4	3.89	3.95
sunray venus clam	SV02	blank	0	<LD	
sunray venus clam	SV02	L	0.4	0.32	0.34
sunray venus clam	SV02	M	1	1	0.97
sunray venus clam	SV02	H	4	4.09	3.6
sunray venus clam	SV03	blank	0	<LD	
sunray venus clam	SV03	L	0.4	0.38	0.36
sunray venus clam	SV03	M	1	1	0.98
sunray venus clam	SV03	H	4	4.15	3.71
sunray venus clam	SV04	blank	0	<LD	
sunray venus clam	SV04	L	0.4	0.32	0.32
sunray venus clam	SV04	M	1	1.11	1.01
sunray venus clam	SV04	H	4	4.28	4.45
sunray venus clam	SV05	blank	0	<LD	
sunray venus clam	SV05	L	0.4	0.29	0.3
sunray venus clam	SV05	M	1	1.13	1.08
sunray venus clam	SV05	H	4	4.19	3.98
sunray venus clam	SV06	blank	0	<LD	
sunray venus clam	SV06	L	0.4	0.36	0.33
sunray venus clam	SV06	M	1	0.84	0.87
sunray venus clam	SV06	H	4	4.03	3.67
sunray venus clam	SV07	blank	0	<LD	
sunray venus clam	SV07	L	0.4	0.41	0.41
sunray venus clam	SV07	M	1	0.93	0.91
sunray venus clam	SV07	H	4	4.1	3.62
sunray venus clam	SV08	blank	0	<LD	
sunray venus clam	SV08	L	0.4	0.43	0.42
sunray venus clam	SV08	M	1	0.95	0.92

sunray venus clam	SV08	H	4	4.03	3.82
sunray venus clam	SV09	blank	0	<LD	
sunray venus clam	SV09	L	0.4	0.44	0.35
sunray venus clam	SV09	M	1	0.86	1.03
sunray venus clam	SV09	H	4	4.36	3.87
sunray venus clam	SV10	blank	0	<LD	
sunray venus clam	SV10	L	0.4	0.4	0.38
sunray venus clam	SV10	M	1	1.15	1
sunray venus clam	SV10	H	4	4.22	3.95

DATA HANDLING

Precision

To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Samples	9		
Concentrations in samples	20		
Determinations within concentrations	30		
Total	59		

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations. If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA variance component, **Total**.
3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and,
4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

Recovery

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.
4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.

5. Perform a one way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Concentration	2		
Error	27		
Total	29		

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. Use log transformed data for microbiological methods.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.
4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.
5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.

Data Summary: **Details Below**

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant? **Y**
- If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **See below.**
- Is the one way analysis of variance to determine the consistency of recovery of the method under study significant? **N**
- At what concentrations is the one way analysis of variance significant? **NA**
- What is the overall percent recovery of the MPN based method under study? **Oysters 97.6%, Hard Clams 97.2%, Sunray Venus Clams 99.0 %**

Working Range of the assay

The overall working range of this ELISA assay is a combination of the linear range of the standard curve and the range of sample dilutions on the plate. This kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the Amax (see linearity). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared. The extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume), and the extract is diluted another 40X for the initial ELISA dilution (yielding a starting dilution factor of 400).

With data showing samples that tested at 20 MU were on the order of 3-4 ppm by ELISA, and anticipating a critical threshold value of approximately half of that, the “low” “medium” and “high” levels selected for this portion were 0.4, 1, and 4 ppm. Samples spiked to these levels were quantitated at dilution factors ranging from 1,600-12,800.

Data Handling Results

Precision

Nested ANOVA: Following this data handling procedure and using log transformed data, the variance ratio (F) at the 95% confidence interval for the variance components: samples/concentrations in samples is not significant. The specified variance ratio for the components: concentrations in samples /determinations within concentrations is significant for all matrices. However, even using the best possible *mock* data, the specified variance ratio is significant. Therefore this approach may not be appropriate for evaluating this data set.

Oysters	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.030086	9	0.003343	0.006402	1	0
concentrations in samples	10.443037	20	0.522152	995.3691	5.93E-37	99.80
determinations within concentrations	0.015737	30	0.000525			0.20
total	10.488860	59				100
Hard Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.036326	9	0.004036	0.007381	1	0
concentrations in samples	10.936302	20	0.546815	1038.552	3.14E-37	99.81
determinations within concentrations	0.015796	30	0.000527			0.19
total	10.988423	59				100
Sunray Venus Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.015388	9	0.001710	0.0031	1	0
concentrations in samples	11.028937	20	0.551447	799.5186	1.57E-35	99.75
determinations within concentrations	0.020692	30	0.000690			0.25
total	11.065016	59				100

Similarly, calculating the %CV for the whole data set as specified above may not be appropriate. Theoretically, if the recoveries were all perfectly 100%, the %CV of the full data set per species would be 89%. Within each spike concentration, %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams.

Oysters	n	%CV
all spike levels/reps	**60**	**90.40**
low (0.4ppm)	20	9.74
med (1ppm)	20	9.45
high (4ppm)	20	6.53
Hard Clams	n	%CV
all spike levels/reps	**60**	**91.53**
low (0.4ppm)	20	11.97
med (1ppm)	20	4.69
high (4ppm)	20	9.16
Sunray Venus Clams	n	%CV
all spike levels/reps	**60**	**90.21**
low (0.4ppm)	20	12.06
med (1ppm)	20	8.81
high (4ppm)	20	6.02

Recovery

Performing a one-way ANOVA as specified above yielded F test results for each matrix type that were not significant at the 95% confidence interval, suggesting that the recovery of the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method.

oysters	sum of squares	d.f	mean square	Fs	P
concentration	0.015062	2	0.007531	0.33193	0.72042
error	0.612573	27	0.022688		
total	0.627634	29			
hard clams	sum of squares	d.f	mean square	Fs	P
concentration	0.004995	2	0.002498	0.08340	0.92022
error	0.808525	27	0.029945		
total	0.813520	29			
sunray venus clams	sum of squares	d.f	mean square	Fs	P
concentration	0.005632	2	0.002816	0.24244	0.78640
error	0.313593	27	0.011615		
total	0.319224	29			

The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams.

Matrix	Avg Spike Conc (ppm)	Avg Rep Conc (ppm)	% Recovery
Oysters	1.8	1.76	97.62
Hard Clams	1.8	1.75	97.17
Sunray Venus Clams	1.8	1.78	98.99

VALIDATION CRITERIA

Accuracy/Trueness is the closeness of agreement between test results and the accepted reference value. To determine method accuracy/trueness, the concentration of the targeted analyte/measurand/organism of interest as measured by the analytical method under study is compared to a reference concentration.

Measurement uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissues. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots of either the homogenate or growing water sample appropriately sized for your work and spike one(1) of the two (2) aliquots with a suitable known concentration of the target analyte/measurand/organism of interest. Do not spike the second aliquot. This is the sample blank. For microbiological methods determine the concentration of the target organism of interest used to spike each sample by plating on/in appropriate agar. Process both aliquots of sample as usual to determine the method concentration for the target analyte/measurand/organism of interest. For growing waters do twenty (20) samples collected from a variety of growing areas. For shellfish do twenty (20) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. **Use a variety of concentrations spanning the range of concentrations of importance in the application of the method to spike sample homogenates or growing water samples**. Both the low and high level spike concentrations must yield determinate values when analyzed by the method under study.

Data:
Working Range: 0.4-8ppm
Sample Type: Hard Clam, Oyster, Sunray Venus Clam
Agar used to determine spike concentration _____
Organism used for spiking: PbTx-3

Data used for Accuracy are from 10 samples per matrix type, each spiked to 4 concentrations, extracted and analyzed in triplicate with blanks (for each matrix type, n = 120 plus blanks). The stock solution used for spiking was considered the reference and was used for the ELISA positive control/standard curve.

Data summary:

Average concentration (ppm)
Average recovery (%)

spike conc (ppm)	Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36
oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04

oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC04	0	<LD		
hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40

hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70

sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94

DATA HANDLING

Accuracy/Trueness

The accuracy/trueness of a method consists of two distinct components, the portion due to the method itself regardless of the laboratory performing it and the portion contributed by the laboratory's performance. In a single laboratory method validation, it is impossible to distinguish the contribution of each to the overall accuracy/trueness of the method. Consequently, what is being estimated is the accuracy/trueness of the method as implemented by the laboratory performing the analysis. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work. Poor accuracy/trueness on the other hand indicates the potential unsuitability of the method and/or the laboratory's performance of it for the intended work.
Accuracy /trueness will be determined by calculating the closeness of agreement between the test results and either a known reference value or a reference value obtained by plate count for microbiological methods.

To determine the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is worked-up in the following manner.

1. Convert plate counts to logs.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average reference concentration of the analyte/measurand used to spike the samples; or, for microbiological methods calculate the average plate count of the data in logs. The average plate count represents the average reference concentration for the microbiological method.
4. Calculate the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
5. Divide the average concentration calculated from the spiked samples by the average reference concentration.
6. Multiply the quotient by 100. This provides an estimate in percent of the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations of importance to the intended application of the method.

Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

Data Summary:

Calculated % accuracy/trueness: Oysters: 96.27%
Hard Clams: 98.39%
Sunray Venus Clams: 95.12%

Calculated measurement uncertainty: Oysters: -0.0057 – 0.1137
Hard Clams: 0.0603 – 0.1898
Sunray Venus Clams: 0.0783 – 0.2487

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 horse radish 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:

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 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 sucked 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 polypropylene centrifuge tube.
2. Add 9mL 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 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 to 12 ml PGT; for 2 plates, add 80 µl A 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. 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:

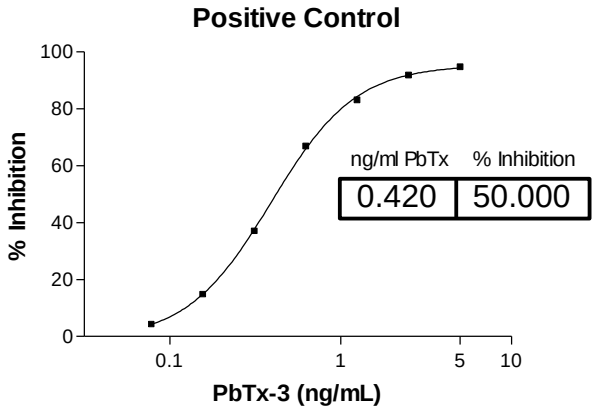
   % inhibition = [1 - (Avg of dups/Amax)] x 100%

   where Amax is the average absorbance of the reference wells (PGT only) oriented below the sample or standard dilutions.

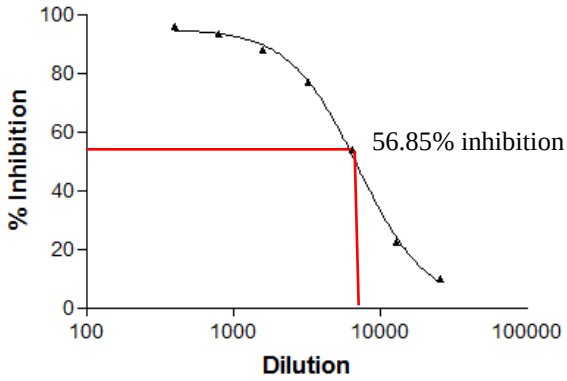
2. Using the 4-parameter logistic (4PL) curve in a curve-fitting program like Prism or SigmaPlot, fit a curve to the positive control with ng toxin/ml on the x-axis (log scale), and % inhibition on the y-axis (linear scale).
3. Determine the concentration for sample dilutions falling within the linear 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 (20-70% inhibition) must be < 20%.

If either criteria 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; 20-70% 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 criteria are not met, re-run the sample.

4. Approved Limited Use Methods for Marine Biotoxin Testing

	Biotoxin Type: Amnesic Shellfish Poisoning (ASP)	Biotoxin Type: Paralytic Shellfish Poisoning (PSP)	Biotoxin Type: Neurotoxic Shellfish Poisoning (NSP)	Application: Growing Area Survey & Classification Sample Type: Shellfish	Application: Dockside Testing Program Sample Type: Shellfish	Application: Controlled Relaying Sample Type: Shellfish	Application: Controlled Harvest end product testing Sample Type: Shellfish
Abraxis Shipboard ELISA[3]		X			X		
JRT[2]		X		X	X	X	
HPLC[1]	X			X		X	
Reveal 2.0 ASP[4]	X			X	X	X	
RBA[5]		X		X	X	X	
MARBIONC Brevetoxin ELISA[6]			X	X		X	X

Footnotes:

[1]M.A. Quilliam, M.Xie and W.R. Hardstaff. 1991. Rapid Extraction and Cleanup Procedure for the Determination of Domoic Acid in Tissue Samples. NRC Institute for Marine Biosciences, Technical Report #64, National Research Council Canada #33001. This method may also be used direct without cleanup.

[2]Jellett Rapid Test for PSP, Jellett Rapid Testing Ltd.

a. Method can be used to determine when to perform a mouse bioassay in a previously closed area.
b. A negative result can be substituted for a mouse bioassay to maintain an area in the open status.
c. A positive result shall be used for a precautionary closure.

[3]Saxitoxin (PSP) ELISA Kit. Method can be used in conjunction with rapid extraction method using 70% isopropanol (rubbing alcohol): 5% acetic acid (white vinegar) 2.5:1. ISSC Summary of Actions, Proposal 05-111 (page 15) and 09-107 (page 140).

[4]Reveal 2.0 ASP. Neogen Corporation. Screening Method for Qualitative Determination of Domoic Acid Shellfish. ISSC 2013 Summary of Actions Proposal 13-112.

[5]Receptor Binding Assay (RBA) for Paralytic Shellfish Poisoning (PSP) Toxicity Determination. Dr. Fran Van Dolah. Method for Clams and Scallops for the Purpose of Screening and Precautionary Closure for PSP. ISSC 2013 Summary of Actions Proposal 13-114

[6]MARBIONC Brevetoxin ELISA, MARBIONC Development Group, LLC. Method can be used in place of an Approved Method for oysters, hard clams, and sunray venus clams within these parameters:

a. A negative result (≤ 1.6 ppm in hard clams and sunray venus clams and ≤ 1.80 ppm in oysters) can substitute for testing by an Approved Method for the purposes of controlled relaying, controlled harvest end-product testing, or to re-open a previously closed area.
b. A positive result (> 1.6 ppm in hard clams and sunray venus clams and > 1.80 ppm in oysters) requires additional testing by an Approved Method or could support the same management actions as samples failing by an Approved Method.

ISSC 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	Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish
Name of the Method Developer	The ELISA Kit was developed by UNCW and is sold through MARBIONC. The method was optimized and submitted for use with molluscan shellfish by Leanne Flewelling, Florida Fish and Wildlife Conservation Commission.
Developer Contact Information	Florida Fish and Wildlife Conservation Commission 100 8th Avenue SE St. Petersburg, FL 33701 (727) 502-4891 leanne.flewelling@myfwc.com

Checklist	Y/N	Submitter Comments
A. Need for the New Method		
1. Clearly define the need for which the method has been developed.		Blooms of the dinoflagellate *Karenia brevis* threaten the productive Gulf of Mexico shellfish industry. Brevetoxins produced by *K. brevis* are toxic to humans and can result in Neurotoxic Shellfish Poisoning (NSP) if contaminated shellfish are eaten. To prevent NSP, shellfish harvesting areas (SHAs) are closed when *K. brevis* concentrations exceed 5,000 cells/L and are re-opened once *K. brevis* levels decrease and testing demonstrates that shellfish are no longer toxic. This biotoxin plan successfully prevents occurrences of NSP from lawfully harvested shellfish, but NSP closures come at a steep economic cost to the shellfish industry. The APHA mouse bioassay - the only NSSP approved method for regulatory NSP testing - has many drawbacks. The delays caused by the time required to analyze samples (two full days) and very low sample throughput delay re-openings and add to economic losses. The assay is nonspecific, imprecise, and not calibrated against known levels of brevetoxins. It is costly in terms of labor and supplies, and the use of live animals is both undesirable and increasingly unacceptable. To mitigate economic harm to the shellfish industry and ensure the continued protection of public health, rapid alternative methods for NSP testing are needed. Among the many chemical and biological methods developed for brevetoxin detection, enzyme-linked immunosorbent assays (ELISAs) have performed well. The method proposed here was the first commercially-available brevetoxin ELISA to be offered. The assay uses goat anti-brevetoxin antibodies developed by Trainer and Baden (1991) and is based on the indirect competitive assay developed in 2002 by Naar et al. (2002). The kit is marketed by MARBIONC Development Group (MDG), which is based at the University of North

		Carolina at Wilmington. This assay is widely and routinely used to monitor brevetoxins in Florida's marine systems and to diagnose human, marine mammal, and other animal exposure to brevetoxins. This method is much faster than the mouse bioassay, more user-friendly, more sensitive, more specific to brevetoxins, less expensive, and does not involve the use of live animals.
2. What is the intended purpose of the method?		The proposed use for the MARBIONC ELISA is as a Limited Use Method for determination of NSP toxin levels in hard clams, sunray venus clams, and oysters. Applications include Growing Area Survey & Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing as permitted within a State Authority's marine biotoxin contingency program. We propose that the ELISA be approved for limited use in NSP testing such that samples with negative results by ELISA (≤ 1.6 ppm in clams and ≤ 1.8 ppm in oysters, at or below the estimated equivalent to one-half the 20 MU/100 g guidance level) would pass, while samples with positive results by ELISA (greater than these levels) would require additional testing by an Approved Method (currently, the NSP mouse bioassay). Samples passing by ELISA would enable the same management actions as samples passing by NSP mouse bioassay including: Growing Area Classification (re-opening closed areas), Controlled Relaying, and Controlled Harvest end product testing. Samples failing by ELISA would either require additional testing by NSP mouse bioassay or could support the same management actions as samples failing by NSP mouse bioassay. ELISA could also be used as a screening method to initiate precautionary closures.
3. Is there an acknowledged need for this method in the NSSP?		Yes, the ISSC Laboratory Committee has specified the need for qualitative or semi-quantitative (screening) and quantitative/confirmatory methods of analysis for all toxins and for each commercially-harvested bivalve species.
4. What type of method? i.e. chemical, molecular, culture, etc.		ELISA is a biological method that uses biological components (antibodies) to detect toxins. Detection relies on structural recognition of a region of the toxin molecule shared by PbTx-2-type brevetoxins (the most abundant forms) and provides an overall estimate of toxin content.
B. Method Documentation		
1. Method documentation includes the following information:		
Method Title		Enzyme-linked Immunosorbent Assay (ELISA) method for the determination of Neurotoxic Shellfish Poisoning (NSP) toxins in molluscan shellfish.
Method Scope		This ELISA is a high-throughput, sensitive, accurate, quantitative assay for NSP toxins in shellfish. The method is being submitted for consideration as an NSSP Approved Limited Use Method for the purposes of screening for NSP toxins in hard clams, sunray venus clams, and oysters.
References		Original method reference: Naar J, Bourdelais A, Tomas C, Kubanek J, Whitney PL, Flewelling LJ, Steidinger KA, Lancaster J, Baden DG. 2002. A competitive ELISA to detect brevetoxins from

		Karenia brevis (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ Health Perspect 110(2):179-185. Antibody development reference: Trainer VL, Baden DG. 1991. An enzyme immunoassay for the detection of Florida red tide brevetoxins. Toxicon 29(11):1387-1394. Epitope identification reference: Melinek R, Rein KS, Schultz DR, Baden DG. 1994. Brevetoxin PbTx-2 immunology: differential epitope recognition by antibodies from two goats. Toxicon 32(8):883-90. Other relevant publications: Dickey RW, Plakas SM, Jester ELE, El Said KR, Johannessen JN, Flewelling LJ, Scott P, Hammond DG, Dolah FMV, Leighfield TA, Dachraoui M-YB, Ramsdell JS, Pierce RH, Henry MS, Poli MA, Walker C, Kurtz J, Naar J, Baden DG, Musser SM, White KD, Truman P, Miller A, Hawryluk TP, Wekell MM, Stirling D, Quilliam MA, Lee JK. 2004. Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger KA, Landsberg JH, Tomas CR, Vargo GA, editors. Harmful Algae 2002. St. Petersburg, FL USA: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. p. 300-302. Plakas SM, Wang Z, El-Said KR, Jester ELE, Granade HR, Flewelling L, Scott P, Dickey RW. 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44:677-685. Plakas SM, Jester EL, El Said KR, Granade HR, Abraham A, Dickey RW, Scott PS, Flewelling LJ, Henry M, Blum P, Pierce R. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed Eastern oyster (Crassostrea virginica). Toxicon 52(1):32-8. Abraham A, El Said KR, Wang Y, Jester EL, Plakas SM, Flewelling LJ, Henry MS, Pierce RH. 2015. Biomarkers of brevetoxin exposure and composite toxin levels in hard clam (Mercenaria sp.) exposed to *Karenia brevis* blooms. Toxicon 96:82-88.
Principle		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. 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 horse radish peroxidase (HRP)-linked secondary antibodies, and addition of an HRP substrate (3,3'5,5'-Tetramethylbenzidine), which yields a blue color that changes to yellow (Amax = 450nm) upon addition of a sulfuric acid stop solution. The intensity of this color is inversely proportional to the amount of brevetoxin present in the well during incubation. Using this method, one ELISA plate can be used to quantitatively assay five shellfish samples. For quick screening, more samples can be run on one plate

		(up to 40).
Any Proprietary Aspects		Methods of production of key kit reagents (brevetoxin-BSA conjugate and anti-brevetoxin antibodies) are proprietary (MDG).
Equipment Required		Equipment required: Balance capable of measuring to 0.1g Number 10 sieve Laboratory blender Vortex mixer Centrifuge capable of 3,000x*g,* with rotor for 15 mL Microplate reader with filter for measurement at 450 nm Multichannel pipettor (50-200 µL) Individual pipettors (10-1000 µL) Orbital microplate shaker Refrigerator/freezer Consumables required: Disposable glass test tubes Disposable plastic dilution tubes (96-well cluster format) 15-ml and 50-ml polypropylene centrifuge tubes Nunc flat-bottom polystyrene 96-well Maxisorp Immunoplates (- substitution NOT recommended) Microplate sealing film Assorted pipet tips Solution basins Aluminum foil
Reagents Required		Included in MARBIONC ELISA Kit: • Reagent A: BSA-linked PbTx-3 • Reagent C: Goat anti-brevetoxin Ab • Reagent D: HRP-linked anti-goat secondary Ab • Brevetoxin standard (PbTx-3) Reagents required but not included: • Methanol • Reagent B: Superblock Blocking Buffer • Phosphate Buffered Saline, pH 7.4 • Phosphate Buffered Saline, 0.05% Tween 20, pH 7.4 • Gelatin • 3,3'5,5'-Tetramethylbenzidine (TMB) • Sulfuric acid stop solution (H_2SO_4, 0.5M) • Nanopure water (or equivalent quality water)
Sample Collection, Preservation and Storage Requirements		At least 12 animals and a total mass of 100-120 grams of meat should be collected per sample. Immediately after collection, shellfish should be placed in dry storage between 0 and 10°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 sucked 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 (see protocol in Appendix A). Samples must be processed within 24 hours of shucking.
Safety Requirements		General chemical safety requirements (e.g., personal protective equipment including gloves, safety glasses,

		and laboratory coat) must be followed.
Clear and Easy to Follow Step-by-Step Procedure		See protocol detailed in Appendix A.
Quality Control Steps Specific for this Method		Acceptance of assay results is dependent on meeting the following criteria: Absorbance of reference wells (Amax) must be ≥ 0.6. %CV of raw absorbance of duplicate wells for standard curve within the linear range of the assay (20-70% inhibition) must be < 20%. 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; 20-70% inhibition) must be <20%. %CV of calculated concentrations of different sample dilutions within the linear range of the assay must be <20%.
C. Validation Criteria		
1. Accuracy / Trueness		Accuracy /trueness was determined by calculating the closeness of agreement between the test results and targeted value. Calculated % accuracy/trueness: Oysters: 96.27% Hard Clams: 98.39% Sunray Venus Clams: 95.12% Data and details in Appendix B
2. Measurement Uncertainty		Two-sided, 95% confidence intervals for the difference in concentrations between the reference and the spiked samples: Oysters: -0.0057 – 0.1137 Hard Clams: 0.0603 – 0.1898 Sunray Venus Clams: 0.0783 – 0.2487 Data and details in Appendix B
3. Precision Characteristics (repeatability and reproducibility)		Repeatability was assessed using duplicate determinations of 10 samples spiked with PbTx-3 to three levels (0.4, 1, and 4 ppm). %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams. Data and details in Appendix C
4. Recovery		The recovery of the method was consistent over the range of concentrations examined to determine Precision. The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams. Data and details in Appendix C
5. Specificity		Potentially interfering substances examined in this study included three types of microalgae (two types commonly used as food for hatchery raised bivalves and a non-brevetoxin producing *Karenia* species) as well as okadaic acid (a potentially co-occurring polyether dinoflagellate toxin). Two-sided t-tests indicated no significant difference in brevetoxin measurements in the presence or absence of these substances. Data and details in Appendix D
6. Working and Linear Ranges		The overall or dynamic linear range of this method results from a combination of the linear range of the assay standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate. The linear range of the ELISA standard curve varied slightly among two lots of kit reagents examined. One lot yielded a range of 0.21-1.04 ng PbTx-3/mL and a second lot yielded a range of 0.30-1.38 ng PbTx-3/mL. The overall or dynamic linear range of the method as

		described for this proposal (in PbTx-3 equivalents) is from 0.12 ppm to 26.62 ppm for the June 2014 kit lot and up to 35.33 ppm for the June 2016 kit lot. Data and details in Appendix E
7. Limit of Detection		The calculated assay LOD is 0.1 ng/mL. At the lowest sample dilution of 1:400, the LOD for brevetoxin in shellfish is 0.04 ppm. Data and details in Appendix E
8. Limit of Quantitation / Sensitivity		The calculated assay LOQ is 0.3 ng/mL. At the lowest sample dilution of 1:400, the LOQ for brevetoxin in shellfish is 0.12 ppm. Data and details in Appendix E
9. Ruggedness		Results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min). Significant differences were observed only with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). 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 of 1.0 ± 30%. Data and details in Appendix F
10. Matrix Effects		Brevetoxin-free samples (10 samples per species) for this study were obtained from shellfish harvest areas along Florida's Gulf coast that infrequently experience *K.. brevis* blooms during periods when *K. brevis* was verified to be absent. Farmed hard clams and sunray venus clams were sourced from Cedar Key, FL and were provided by a Shellfish Aquaculture Extension Agent and as well as local clam farmers. Hard clams were collected from 10 different locations over four days. Sunray venus clams were collected from two locations over six days. Wild oysters were collected by Florida Department of Agriculture and Consumer Services staff from five sites in Apalachicola Bay over nine days. At the lowest dilution (1:400), all samples tested <LOD and no matrix effects were observed.

11. Comparability (if intended as a substitute for an established method accepted by the NSSP)		Comparative data for 501 samples (173 oyster, 277 hard clam, and 51 sunray venus clam) are presented in Appendix G. For several reasons discussed in Appendix G, comparing NSP mouse bioassay and ELISA data is not straightforward, and analytical NSP methods of any type are unlikely to ever completely agree with mouse bioassay results. There was a very wide range of concentrations measured by ELISA in samples testing <20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay. In samples testing < 20MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. Where quantitative results were obtained by both mouse bioassay and ELISA (i.e., in samples testing ≥ 20 MU/100 g), significant positive correlations were observed. Using linear regression, the 20 MU/100 g equivalent by ELISA was predicted to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams (in PbTx-3 equivalents). Across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that "failed" by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.
D. Other Information		
1. Cost of the Method		Kit reagents are sold in bulk. The cost of reagents is currently $2,400 for 15 plates and $1,000 for 5 plates. The cost of additional consumables and reagents not included is approximately $20 per plate. Therefore cost per sample is $36-44 for full quantitation (5 samples per plate) and less than $6 per sample for qualitative screening (40 samples per plate).
2. Special Technical Skills Required to Perform the Method		General laboratory skills are required: reagent preparation, pipetting, basic equipment operation, data analysis using curve-fitting software, basic calculations.
3. Special Equipment Required and Associated Cost		Microplate reader with filter for measurement at 450 nm. Costs range, but basic readers start at approximately $5,000, and a used plate reader can be purchased for less than $1,000.
4. Abbreviations and Acronyms Defined		Ab Antibody BSA Bovine Serum Albumin ELISA Enzyme-linked Immunosorbent Assay HRP Horse radish peroxidase MDG MARBIONC Development Group NSP Neurotoxic Shellfish Poisoning PBS Phosphate Buffered Saline PBS-Tween Phosphate Buffered Saline with Tween 20 (0.05%) PbTx Brevetoxin PGT Phosphate Buffered Saline with gelatin (5%) Tween 20 (0.05%) TMB 3,3'5,5'-Tetramethylbenzidine
5. Details of Turn Around Times (time involved to complete the method)		The ELISA takes approximately 6 hours to complete, and one practiced analyst can comfortably process up to 4 plates per day.
6. Provide Brief Overview of the Quality Systems Used in the Lab		The Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research

		Institute's HAB Biotoxin Laboratory maintains and follows a Quality Assurance Program to ensure the precision, accuracy and reliability of all toxin analyses and for the production of scientifically sound, legally defensible data. Thorough documentation and standardization of laboratory processes, procedures and activities are required. The Laboratory Manager, Laboratory Safety Officer, Laboratory Secondary Staff and field staff are responsible for implementing QA/QC procedures outlined in the manual. Key practices include the use of Standard Operating Procedures, standard methods, training, quality control, and database record keeping and tracking. All QA practices are consistent with Good Laboratory Practices and all applicable safety, environmental and legal regulations and guidelines. **From the manufacturer (MARBIONC):** Each time new kit reagents are made from stocks, QC ELISAs are run and compared to previous assays. A standard ELISA set is retained to compare all new kits back to. New reagent stocks are given lot numbers. When new reagents are made (e.g. purified antibodies or PbTx-BSA conjugate), the ELISAs are designed with the new reagents to maintain continuity with previous kit lots. Kits are manufactured in a controlled environment to maintain cleanliness and avoid any cross contamination. Kits and kit components are validated. Kit and kit components are serialized to maintain traceability. Higher-level Good Manufacturing Processes are in process and as new reagents are produced, they will conform to requirements to allow for overall implementation of quality systems. Supply: MARBIONC Development Group, LLC has a future vision and is currently working to maintain an adequate supply of reagents. Sufficient supplies are on hand to cover current and projected increased demand for the foreseeable future (approximately 10-15 yrs). MARBIONC is committed to providing the kits for research and commercial use and has also committed to provide resources for the resupply of kit components in advance of the time when such components may be required.
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.
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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.

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 horse radish 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:

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 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 sucked 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 polypropylene centrifuge tube.
2. Add 9mL 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 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 to 12 ml PGT; for 2 plates, add 80 µl A 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. 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:

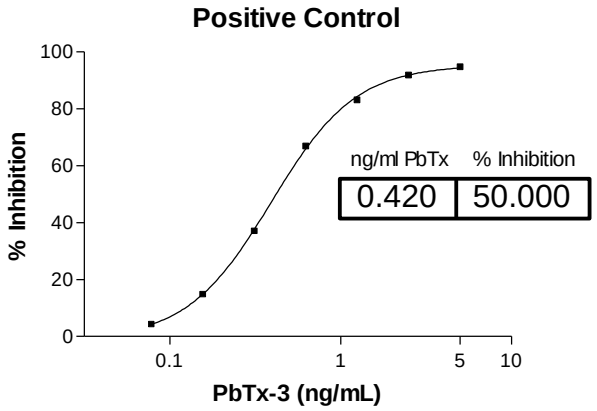
   % inhibition = [1 - (Avg of dups/Amax)] x 100%

   where Amax is the average absorbance of the reference wells (PGT only) oriented below the sample or standard dilutions.

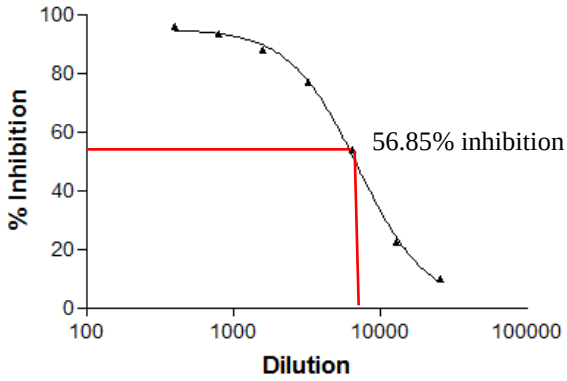
2. Using the 4-parameter logistic (4PL) curve in a curve-fitting program like Prism or SigmaPlot, fit a curve to the positive control with ng toxin/ml on the x-axis (log scale), and % inhibition on the y-axis (linear scale).
3. Determine the concentration for sample dilutions falling within the linear 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 (20-70% inhibition) must be < 20%.

If either criteria 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; 20-70% 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 criteria are not met, re-run the sample.

VALIDATION CRITERIA

Accuracy/Trueness is the closeness of agreement between test results and the accepted reference value. To determine method accuracy/trueness, the concentration of the targeted analyte/measurand/organism of interest as measured by the analytical method under study is compared to a reference concentration.

Measurement uncertainty is a single parameter (usually a standard deviation or confidence interval) expressing the possible range of values around the measured result within which the true value is expected to be with a stated degree of probability. It takes into account all recognized effects operating on the result including: overall precision of the complete method, the method and laboratory bias and matrix effects.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissues. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots of either the homogenate or growing water sample appropriately sized for your work and spike one(1) of the two (2) aliquots with a suitable known concentration of the target analyte/measurand/organism of interest. Do not spike the second aliquot. This is the sample blank. For microbiological methods determine the concentration of the target organism of interest used to spike each sample by plating on/in appropriate agar. Process both aliquots of sample as usual to determine the method concentration for the target analyte/measurand/organism of interest. For growing waters do twenty (20) samples collected from a variety of growing areas. For shellfish do twenty (20) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. **Use a variety of concentrations spanning the range of concentrations of importance in the application of the method to spike sample homogenates or growing water samples**. Both the low and high level spike concentrations must yield determinate values when analyzed by the method under study.

Data:
Working Range: 0.4-8ppm
Sample Type: Hard Clam, Oyster, Sunray Venus Clam
Agar used to determine spike concentration _____
Organism used for spiking: PbTx-3

Data used for Accuracy are from 10 samples per matrix type, each spiked to 4 concentrations, extracted and analyzed in triplicate with blanks (for each matrix type, n = 120 plus blanks). The stock solution used for spiking was considered the reference and was used for the ELISA positive control/standard curve.

Data summary:

Average concentration (ppm)
Average recovery (%)

spike conc (ppm)	Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36
oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04

oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC04	0	<LD		
hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40

hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70

sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94

DATA HANDLING

Accuracy/Trueness

The accuracy/trueness of a method consists of two distinct components, the portion due to the method itself regardless of the laboratory performing it and the portion contributed by the laboratory's performance. In a single laboratory method validation, it is impossible to distinguish the contribution of each to the overall accuracy/trueness of the method. Consequently, what is being estimated is the accuracy/trueness of the method as implemented by the laboratory performing the analysis. Good accuracy/trueness suggests the appropriateness of the method and the laboratory's performance of it for the intended work. Poor accuracy/trueness on the other hand indicates the potential unsuitability of the method and/or the laboratory's performance of it for the intended work.
Accuracy /trueness will be determined by calculating the closeness of agreement between the test results and either a known reference value or a reference value obtained by plate count for microbiological methods.

To determine the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is worked-up in the following manner.

1. Convert plate counts to logs.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average reference concentration of the analyte/measurand used to spike the samples; or, for microbiological methods calculate the average plate count of the data in logs. The average plate count represents the average reference concentration for the microbiological method.
4. Calculate the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
5. Divide the average concentration calculated from the spiked samples by the average reference concentration.
6. Multiply the quotient by 100. This provides an estimate in percent of the accuracy/trueness of the method as implemented by the laboratory over the range in concentrations of importance to the intended application of the method.

Measurement uncertainty

Measurement uncertainty can be determined by subtracting the results for each spiked sample from the reference value for the sample and calculating the 95% confidence interval of these differences. The confidence interval of these differences represents the range in values within which the true measurement uncertainty lies. A narrow range in values indicates that the method as implemented by the laboratory produces reliable results.

Use the log transformed data for both the plate count and the microbial results obtained from the spiked samples. If necessary use the sample blank (converted to logs for microbiological methods) to correct the spiked sample for matrix effects and calculate the two-sided, 95% confidence interval for the difference in concentrations between the reference and the spiked samples. This range in counts represents the measurement uncertainty of the method as implemented by the laboratory.

Data Summary:

Calculated % accuracy/trueness: Oysters: 96.27%
Hard Clams: 98.39%
Sunray Venus Clams: 95.12%

Calculated measurement uncertainty: Oysters: -0.0057 – 0.1137
Hard Clams: 0.0603 – 0.1898
Sunray Venus Clams: 0.0783 – 0.2487

VALIDATION CRITERIA

Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

Recovery is the fraction or percentage of an analyte/measurand/organism of interest recovered following sample analysis.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take four (4) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work. Spike one of the four aliquots with a low (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Spike the second aliquot of the growing water sample or shellfish homogenate with a medium concentration of the target analyte/measurand/organism of interest. Spike the third aliquot of the growing water sample or shellfish homogenate with a high (but determinable by the method under study) concentration of the target analyte/measurand/organism of interest. Do not spike the fourth aliquot of the growing water sample or shellfish homogenate. This is the sample blank. Spiking levels must cover the range in concentrations important to the application of the method (working range). For microbiological methods determine the concentration of the target organism of interest used to spike each aliquot by plating in/on appropriate agar. Process each aliquot including the sample blank as usual to determine the method concentration for the target analyte/measurand/organism of interest. Do two (2) replicates for each of the three (3) spiked aliquots. Replicate analysis is unnecessary for the sample blank. Do only one sample blank per sample. For growing waters, do ten (10) samples collected from a variety of growing areas. For shellfish, do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed in this exercise (i.e. 10^1, 10^3 and 10^5).

Data:
Working Range: 0.4 - 4 ppm
Sample Type: Oyster, Hard Clam, Sunray Venus Clam
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

matrix type	sample	spike level	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)
hard clam	HC01	blank	0	<LD	
hard clam	HC01	L	0.4	0.33	0.32
hard clam	HC01	M	1	0.98	0.93
hard clam	HC01	H	4	3.85	3.79
hard clam	HC02	blank	0	<LD	
hard clam	HC02	L	0.4	0.35	0.33
hard clam	HC02	M	1	0.92	0.89
hard clam	HC02	H	4	3.82	3.36
hard clam	HC03	blank	0	<LD	
hard clam	HC03	L	0.4	0.35	0.33
hard clam	HC03	M	1	0.91	0.91
hard clam	HC03	H	4	3.55	3.36
hard clam	HC04	blank	0	<LD	
hard clam	HC04	L	0.4	0.33	0.3
hard clam	HC04	M	1	0.91	0.91
hard clam	HC04	H	4	4.66	3.99
hard clam	HC05	blank	0	<LD	
hard clam	HC05	L	0.4	0.32	0.33
hard clam	HC05	M	1	0.92	0.89

hard clam	HC05	H	4	3.49	4.03
hard clam	HC06	blank	0	<LD	
hard clam	HC06	L	0.4	0.44	0.44
hard clam	HC06	M	1	0.84	0.92
hard clam	HC06	H	4	4.15	4.25
hard clam	HC07	blank	0	<LD	
hard clam	HC07	L	0.4	0.42	0.43
hard clam	HC07	M	1	1	1.01
hard clam	HC07	H	4	4.05	4.12
hard clam	HC08	blank	0	<LD	
hard clam	HC08	L	0.4	0.35	0.37
hard clam	HC08	M	1	0.92	1
hard clam	HC08	H	4	4.23	3.55
hard clam	HC09	blank	0	<LD	
hard clam	HC09	L	0.4	0.4	0.39
hard clam	HC09	M	1	0.93	0.91
hard clam	HC09	H	4	3.98	4.26
hard clam	HC10	blank	0	<LD	
hard clam	HC10	L	0.4	0.36	0.39
hard clam	HC10	M	1	0.97	0.98
hard clam	HC10	H	4	4.54	3.98
oyster	OY01	blank	0	<LD	
oyster	OY01	L	0.4	0.38	0.38
oyster	OY01	M	1	0.99	0.95
oyster	OY01	H	4	4.07	4.12
oyster	OY02	blank	0	<LD	
oyster	OY02	L	0.4	0.39	0.39
oyster	OY02	M	1	0.94	0.95
oyster	OY02	H	4	3.87	3.85
oyster	OY03	blank	0	<LD	
oyster	OY03	L	0.4	0.44	0.42
oyster	OY03	M	1	0.8	0.77
oyster	OY03	H	4	3.57	3.92
oyster	OY04	blank	0	<LD	
oyster	OY04	L	0.4	0.37	0.35
oyster	OY04	M	1	1	0.85
oyster	OY04	H	4	4.17	4.14
oyster	OY05	blank	0	<LD	
oyster	OY05	L	0.4	0.36	0.38
oyster	OY05	M	1	0.77	0.89
oyster	OY05	H	4	4.22	4.06
oyster	OY06	blank	0	<LD	
oyster	OY06	L	0.4	0.31	0.33
oyster	OY06	M	1	0.91	0.92
oyster	OY06	H	4	3.36	3.48
oyster	OY07	blank	0	<LD	

oyster	OY07	L	0.4	0.4	0.4
oyster	OY07	M	1	0.88	1.05
oyster	OY07	H	4	3.9	4.21
oyster	OY08	blank	0	<LD	
oyster	OY08	L	0.4	0.46	0.44
oyster	OY08	M	1	1.05	1.03
oyster	OY08	H	4	3.86	4.03
oyster	OY09	blank	0	<LD	
oyster	OY09	L	0.4	0.43	0.37
oyster	OY09	M	1	1.06	0.92
oyster	OY09	H	4	3.74	3.94
oyster	OY10	blank	0	<LD	
oyster	OY10	L	0.4	0.36	0.38
oyster	OY10	M	1	0.94	0.99
oyster	OY10	H	4	4.24	4.28
sunray venus clam	SV01	blank	0	<LD	
sunray venus clam	SV01	L	0.4	0.36	0.37
sunray venus clam	SV01	M	1	0.94	0.98
sunray venus clam	SV01	H	4	3.89	3.95
sunray venus clam	SV02	blank	0	<LD	
sunray venus clam	SV02	L	0.4	0.32	0.34
sunray venus clam	SV02	M	1	1	0.97
sunray venus clam	SV02	H	4	4.09	3.6
sunray venus clam	SV03	blank	0	<LD	
sunray venus clam	SV03	L	0.4	0.38	0.36
sunray venus clam	SV03	M	1	1	0.98
sunray venus clam	SV03	H	4	4.15	3.71
sunray venus clam	SV04	blank	0	<LD	
sunray venus clam	SV04	L	0.4	0.32	0.32
sunray venus clam	SV04	M	1	1.11	1.01
sunray venus clam	SV04	H	4	4.28	4.45
sunray venus clam	SV05	blank	0	<LD	
sunray venus clam	SV05	L	0.4	0.29	0.3
sunray venus clam	SV05	M	1	1.13	1.08
sunray venus clam	SV05	H	4	4.19	3.98
sunray venus clam	SV06	blank	0	<LD	
sunray venus clam	SV06	L	0.4	0.36	0.33
sunray venus clam	SV06	M	1	0.84	0.87
sunray venus clam	SV06	H	4	4.03	3.67
sunray venus clam	SV07	blank	0	<LD	
sunray venus clam	SV07	L	0.4	0.41	0.41
sunray venus clam	SV07	M	1	0.93	0.91
sunray venus clam	SV07	H	4	4.1	3.62
sunray venus clam	SV08	blank	0	<LD	
sunray venus clam	SV08	L	0.4	0.43	0.42
sunray venus clam	SV08	M	1	0.95	0.92

sunray venus clam	SV08	H	4	4.03	3.82
sunray venus clam	SV09	blank	0	<LD	
sunray venus clam	SV09	L	0.4	0.44	0.35
sunray venus clam	SV09	M	1	0.86	1.03
sunray venus clam	SV09	H	4	4.36	3.87
sunray venus clam	SV10	blank	0	<LD	
sunray venus clam	SV10	L	0.4	0.4	0.38
sunray venus clam	SV10	M	1	1.15	1
sunray venus clam	SV10	H	4	4.22	3.95

DATA HANDLING

Precision

To determine the precision of the method as implemented by the laboratory over the range in concentrations important to the intended application of the method, the data is manipulated in the following manner:

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for the microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Perform a nested or hierarchical analysis of variance (ANOVA) on the corrected spiked sample data using the following variance components.

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Samples	9		
Concentrations in samples	20		
Determinations within concentrations	30		
Total	59		

4. Calculate the variance ratio (F) at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations. If the variance ratio is significant this indicates that the precision of the method as implemented by the laboratory is not consistent over the range in concentrations important to the intended application.

If the variance ratio is not significant, calculate the coefficient of variation of the spiked sample data by:

1. Calculating the average concentration of the analyte/measurand/organism of interest in the spiked samples. For microbiological methods log transformed data is used for this calculation.
2. Calculate the standard deviation of the spiked sample data by taking the square root of the nested ANOVA variance component, **Total**.
3. Divide the standard deviation of the spiked sample data by the average concentration of the analyte/measurand/organism of interest calculated for the spiked samples. For microbiological methods log transformed data is used for this calculation; and,
4. Multiply the quotient above by 100. This is the coefficient of variation of the method over the range of concentrations of importance in the application of the method as implemented by the laboratory.

Recovery

The recovery of the target analyte/measurand/organisms of interest must be consistently good over the range of concentrations of importance to the application of the method under study to be of benefit in the intended work. To determine whether recovery by the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method, the data is manipulated in the following manner:

1. Convert plate count and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. For each sample determine the average of the replicates at each concentration such that there is only one value, the average of the two replicates at each concentration tested.
4. For each sample subtract the average for the replicates from its associated spike concentration/plate count value.

5. Perform a one way analysis of variance (ANOVA) on the data formatted by sample concentration with the following variance components:

Source of variation	Degrees of freedom	Sum of Squares	Mean Square
Concentration	2		
Error	27		
Total	29		

6. Calculate the variance ratio (F) at the 95% confidence interval for the mean square for concentration divided by the mean square for error. If the variance ratio or F test is significant at the 95% confidence interval, perform Tukey's Honestly Significant Difference (HSD) to compare recovery by concentration. A significant F test suggests that recovery of the method as implemented by the laboratory is not consistent over the range in concentrations important to the application of the method and may not be suitable for the work intended.

   If the variance ratio or F test is not significant at the 95% confidence interval, conclude that the recovery is consistent over the range in concentrations important to the application of the method and calculate the overall percent recovery of the method as implemented by the laboratory.

To determine the percent recovery of the method as implemented by the laboratory, the data is manipulated in the following manner:

1. Use log transformed data for microbiological methods.
2. If necessary use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Calculate the average spike concentration/plate count by summing over concentrations and dividing by 30.
4. Calculate the average concentration of analyte/measurand/organism of interest in the spiked samples from the analysis by summing over concentrations and replicates and dividing by 60.
5. Divide the average concentration of analyte/measurand/organism of interest from the analysis of the spiked samples by the average concentration from the spike/plate counts then multiply by 100. This is the percent recovery of the method as implemented by the laboratory.

Data Summary: **Details Below**

- Is the variance ratio at the 95% confidence interval for the variance components, concentrations in samples/determinations within concentrations significant? **Y**
- If the variability of the method as implemented by the laboratory is consistent over the range in concentrations important to its intended applications, what is the coefficient of variation? **See below.**
- Is the one way analysis of variance to determine the consistency of recovery of the method under study significant? **N**
- At what concentrations is the one way analysis of variance significant? **NA**
- What is the overall percent recovery of the MPN based method under study? **Oysters 97.6%, Hard Clams 97.2%, Sunray Venus Clams 99.0 %**

Working Range of the assay

The overall working range of this ELISA assay is a combination of the linear range of the standard curve and the range of sample dilutions on the plate. This kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the Amax (see linearity). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared. The extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume), and the extract is diluted another 40X for the initial ELISA dilution (yielding a starting dilution factor of 400).

With data showing samples that tested at 20 MU were on the order of 3-4 ppm by ELISA, and anticipating a critical threshold value of approximately half of that, the “low” “medium” and “high” levels selected for this portion were 0.4, 1, and 4 ppm. Samples spiked to these levels were quantitated at dilution factors ranging from 1,600-12,800.

Data Handling Results

Precision

Nested ANOVA: Following this data handling procedure and using log transformed data, the variance ratio (F) at the 95% confidence interval for the variance components: samples/concentrations in samples is not significant. The specified variance ratio for the components: concentrations in samples /determinations within concentrations is significant for all matrices. However, even using the best possible *mock* data, the specified variance ratio is significant. Therefore this approach may not be appropriate for evaluating this data set.

Oysters	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.030086	9	0.003343	0.006402	1	0
concentrations in samples	10.443037	20	0.522152	995.3691	5.93E-37	99.80
determinations within concentrations	0.015737	30	0.000525			0.20
total	10.488860	59				100
Hard Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.036326	9	0.004036	0.007381	1	0
concentrations in samples	10.936302	20	0.546815	1038.552	3.14E-37	99.81
determinations within concentrations	0.015796	30	0.000527			0.19
total	10.988423	59				100
Sunray Venus Clams	sum of squares	d.f.	mean square	Fs	P	variance component (percentage)
among samples	0.015388	9	0.001710	0.0031	1	0
concentrations in samples	11.028937	20	0.551447	799.5186	1.57E-35	99.75
determinations within concentrations	0.020692	30	0.000690			0.25
total	11.065016	59				100

Similarly, calculating the %CV for the whole data set as specified above may not be appropriate. Theoretically, if the recoveries were all perfectly 100%, the %CV of the full data set per species would be 89%. Within each spike concentration, %CV ranged from 6.53% to 9.74% in oysters, 4.69% to 11.97% in hard clams, and 6.02% to 12.06% in sunray venus clams.

Oysters	n	%CV
all spike levels/reps	**60**	**90.40**
low (0.4ppm)	20	9.74
med (1ppm)	20	9.45
high (4ppm)	20	6.53
Hard Clams	n	%CV
all spike levels/reps	**60**	**91.53**
low (0.4ppm)	20	11.97
med (1ppm)	20	4.69
high (4ppm)	20	9.16
Sunray Venus Clams	n	%CV
all spike levels/reps	**60**	**90.21**
low (0.4ppm)	20	12.06
med (1ppm)	20	8.81
high (4ppm)	20	6.02

Recovery

Performing a one-way ANOVA as specified above yielded F test results for each matrix type that were not significant at the 95% confidence interval, suggesting that the recovery of the method as implemented by the laboratory is consistent over the range in concentrations important to the application of the method.

oysters	sum of squares	d.f	mean square	Fs	P
concentration	0.015062	2	0.007531	0.33193	0.72042
error	0.612573	27	0.022688		
total	0.627634	29			
hard clams	sum of squares	d.f	mean square	Fs	P
concentration	0.004995	2	0.002498	0.08340	0.92022
error	0.808525	27	0.029945		
total	0.813520	29			
sunray venus clams	sum of squares	d.f	mean square	Fs	P
concentration	0.005632	2	0.002816	0.24244	0.78640
error	0.313593	27	0.011615		
total	0.319224	29			

The overall percent recovery of the method was 97.62% in oysters, 97.17% in hard clams, and 98.99% in sunray venus clams.

Matrix	Avg Spike Conc (ppm)	Avg Rep Conc (ppm)	% Recovery
Oysters	1.8	1.76	97.62
Hard Clams	1.8	1.75	97.17
Sunray Venus Clams	1.8	1.78	98.99

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable, the new or modified method must be specific for the analyte/measurand/organism of interest. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Specificity of the new or modified method is the ability of this new or modified method to measure only what it is intended to measure. To determine the specificity of new or modified methods, samples containing suspected interferences (interfering organisms/compounds/toxins) are analyzed in the presence of the analyte/measurand/target organism of interest.

Procedure for demonstrating the specificity of the new or modified method: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish tissue type of interest use a minimum of 10-12 animals per sample. For each sample take three (3) aliquots of either the shellfish homogenate or growing water sample appropriately sized for the work and spike two (2) of the three (3) with a low but determinate level (by the method/modified method under study) of the target analyte/measurand/organism of interest. Take one of these two (2) aliquots and also spike it with a moderate to high level of a suspected interfering organism/compound/toxin if not naturally incurred. Do not spike the third aliquot. This is the sample blank. Process each aliquot, the sample blank , the aliquot spiked with the target analyte/measurand/organism of interest and the aliquot spiked with the target analyte/measurand/organism of interest in the presence of the suspected interfering organism/compound/toxin as usual to determine the method/modified method concentration for the target analyte/measurand/organism of interest. Do five (5) replicates for each aliquot excluding the sample blank. Do one (1) sample blank per analysis. Repeat this process for all suspected interfering organisms/compounds/toxins.

Data for demonstrating the specificity of the new or modified method:

Potentially interfering substances examined in this study included two types of microalgae – the flagellate *Isochrysis* aff. *galbana* and the cryptophyte *Rhodomonas lens* – that are commonly fed to aquaculture-reared bivalves at the age/size at which they are ready to be relocated from the hatchery to the aquaculture zones. With the capacity to ingest as many as 10^9 cells per day, it is reasonable to predict there may be some bioaccumulation of cell constituents over time, and that they may still be present when the bivalves are harvested. Cells were added to a concentration of 100 million cells per g of shellfish.

Also examined was okadaic acid, a dinoflagellate toxin produced by some species of *Dinophysis* and *Prorocentrum*. These organisms are present in waters where *Karenia brevis* occurs, and potentially both toxins could be present. Both brevetoxin and okadaic acid are polyether toxins, so cross-reactivity with okadaic acid was investigated. Okadaic acid was added to a concentration of 1.5 µg per g of shellfish (or 1.5 ppm), which is roughly ten times above the current US guidance limit of 0.16 ppm.

The final substance to be examined was *Karenia mikimotoi*, a dinoflagellate that is closely related to *Karenia brevis*. *K. mikimotoi* produces bioactive compounds, but brevetoxin production has not been documented in this species. *Karenia* blooms are often mixed species blooms with two or more *Karenia* species present, although *K. brevis* is typically dominant. *K. mikimotoi* cells were added to a concentration of 500,000 cells per g.

Interfering organism/compound/toxin:

A *Isochrysis* aff. *galbana* (100 million cells per g shellfish)
B *Rhodomonas lens* (100 million cells per g shellfish)
C Okadaic acid (1.5 µg per g shellfish)
D *Karenia mikimotoi* (500,000 cells per g shellfish)

PbTx-3 spike concentration: 0.4 ppm

oyster

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.39	0.41	0.41	0.38	0.39	<LD
0.38	0.38	0.41	0.38	0.40	
0.42	0.39	0.39	0.37	0.43	
0.34	0.38	0.42	0.37	0.37	
0.39	0.44	0.40	0.35	0.42	

hard clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.36	0.40	0.40	0.38	0.40	<LD
0.38	0.38	0.39	0.40	0.32	
0.39	0.40	0.37	0.37	0.38	
0.35	0.36	0.38	0.37	0.33	
0.38	0.40	0.39	0.38	0.37	

sunray venus clam

PbTx-3	PbTx-3 + A	PbTx-3 + B	PbTx-3 + C	PbTx-3 + D	Blank
0.33	0.41	0.42	0.41	0.35	<LD
0.35	0.39	0.38	0.40	0.41	
0.38	0.38	0.36	0.35	0.35	
0.35	0.37	0.34	0.39	0.40	
0.38	0.43	0.40	0.39	0.41	

Data handling for demonstrating specificity of the new or modified method

The specificity index will be used to test the specificity of the new or modified method in the presence of suspected interfering organisms/compounds/toxins. The specificity index (SI) is calculated as indicated below:

$$\text{Specificity index (SI)} = \frac{\text{Sample spiked with only target of interest}}{\text{Sample spiked with target in presence if suspected interferences}}$$

All microbiological count data must be converted to logs before statistical analysis. Samples spiked with both the target analyte/measurand/organism of interest and the target analyte/measurand/organism of interest in the presence of a suspected interfering organism/compound/toxin may have to be corrected for matrix effects before determining the Specificity index (SI). The sample blank accompanying the analysis is used for this purpose. Any correction that may be necessary to microbiological data for matrix effects are done using log transformed data.

The Specificity index (SI) should equal one (1) in the absence of interferences. To test the significance of a Specificity index (SI) other than one (1) for any suspected interfering organism/compound/toxin, a two-sided t-test at the .05 significance level is used. For each suspected interfering organism/compound/toxin calculate the average Specificity index (SI_{avg}) for the five (5) replicates analyzed for each sample by obtaining the average concentration for both the aliquot containing the target analyte/measurand/organism of interest only and the aliquot containing the target analyte/measurand/organism of interest in the presence of suspected interfering organisms/compounds/toxins and using the formula below.

$$SI_{avg} = \frac{\text{Avg. conc. of sample spiked only with target of interest}}{\text{Avg. conc. of sample spiked with target in the presence of suspected interferences}}$$

Perform the t-test to determine if the average Specificity index (SI) obtained from the five (5) replicates from each analysis differs from one (1). Repeat for all the suspected interfering organisms/compounds/toxins tested.

Data summary for testing the specificity of the new or modified method:

Oyster

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.96	no	0.352
B	*Rhodomonas lens*	0.94	no	0.254
C	Okadaic acid	1.03	no	0.490
D	*Karenia mikimotoi*	0.95	no	0.061

Hard clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.97	no	0.164
B	*Rhodomonas lens*	0.97	no	0.230
C	Okadaic acid	0.98	no	0.374
D	*Karenia mikimotoi*	1.04	no	0.364

Sunray venus clam

Interfering organism/compound/toxin		SI_{avg}	significantly different from 1?	*p* value
A	*Isochrysis* aff. *galbana*	0.91	no	0.055
B	*Rhodomonas lens*	0.95	no	0.311
C	Okadaic acid	0.93	no	0.205
D	*Karenia mikimotoi*	0.94	no	0.230

VALIDATION CRITERIA

Linear Range is the range within the working range where the results are proportional to the concentration of the analyte/measurand/organism of interest present in the sample.

Limit of Detection is the minimum concentration at which the analyte/measurand/organism of interest can be identified.

Limit of Quantitation/Sensitivity is the minimum concentration of the analyte/measurand/organism of interest that can be quantified with an acceptable level of precision and accuracy under the conditions of the test.

Procedure: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take at least six (6) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work and spike five (5) of the six (6) aliquots with five (5) different concentrations (i.e. 10^a, 10^b…10^n) of the target analyte/measurand/organism of interest spanning 50 – 150% of the working range/range of interest for the method under study. Do not spike the sixth or last aliquot of each sample. This is the sample blank. For microbiological methods determine the concentration of the target analyte/measurand/organism of interest used to spike each aliquot of each sample by plating in/on appropriate agar. Do not use aliquots of the same master solution/culture to spike all the samples in this exercise. A separate master solution /culture should be used for each sample. Process each aliquot including the sample blank as usual to determine method concentration for the target analyte/measurand/organism of interest. Do three (3) replicates for each aliquot excluding the sample blank. Do only one blank per sample. For growing waters do ten (10) samples collected from a variety of growing areas. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same spiking levels for each of the ten (10) samples analyzed (10^a, 10^b…10^n).

Data:
Sample type
Working range/Range of interest: 0.4-8 ppm
Range in spiking levels used: 0.4 ppm, 1 ppm, 4 ppm, 8 ppm, 12 ppm
Agar used to determine spike concentration: NA
Organism used for spiking: PbTx-3

Response is the signal data (absorbance, fluorescence, Ct value), colonies, plaques, etc resulting from the analysis.

For shellfish samples repeat for each tissue type of interest.

DATA HANDLING

Linear Range

To determine the range within the working range where the results are proportional to the concentration of the target analyte/measurand/organism of interest present, the data is manipulated in the following manner.

1. Convert the plate counts and spiked sample results for the microbiological methods to logs.
2. If necessary, use the sample blank (converted to logs for microbiological methods) to correct the results from the spiked samples for matrix effects.
3. Divide the response obtained for each replicate tested by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it. Use log values for the microbiological data.
4. Plot the data obtained above on the y-axis against the log of the concentration of the spiked analyte/measurand/organism of interest which gave rise to the respective data point on the x-axis. Connect the points. This is the relative response line.
5. Calculate the mean of the values obtained (in step 3) when the response for each replicate tested is divided by the concentration of the spiked analyte/measurand/organism of interest which gave rise to it.
6. Plot this value on the y-axis of the graph obtained in step 4 at each log concentrations of the analyte/measurand/organism of interest spiked into the samples. Connect the points to form a horizontal line. This constitutes the line of constant response
7. Multiply the value obtained in step 5 by 0.95 and 1.05.

8. Plot these values on the y-axis of the graph obtained in steps 4 and 6 at each log concentration of the analyte/measurand /organism of interest spiked into the samples. Connect the points to form two horizontal lines which bracket the line of constant response.
9. The method is linear up to the point where the relative response line (obtained in step 4) intersects either of the lines obtained above.
10. The linear range of the method as implemented by the laboratory is comprised of the range in concentrations obtained by taking the antilogs of the concentrations of the spiked analyte/measurand/organism of interest bracketed within the horizontal lines of the plot obtained in step 8 above.

Limit of Detection and Limit of Quantitation/Sensitivity

To determine the minimum concentration at which the analyte/measurand/organism of interest can be identified and subsequently quantified with an acceptable level of precision and accuracy under the conditions of the test, the data is manipulated in the following manner.

1. Calculate the coefficient of variation or relative standard deviation for each concentration of analyte/measurand/organisn of interest spiked into the samples. Use the log transformed data for manipulating microbiological results.
2. Plot the coefficient of variation/relative standard deviation on the y-axis for each concentration of analyte/measurand/organism of interest spiked into the samples and plotted on the x-axis. Use log transformed concentration values for the microbiological data.
3. Fit the curve and determine from the graph the concentration of analyte/measurand/organism of interest which gave rise to a coefficient of variation/relative standard deviation of 10%. This is the limit of quantitation/sensitivity of the method as implemented by the laboratory.
4. Divide the value for the limit of quantitation/sensitivity obtained from step 3 above by 3.3 or determine the concentration of analyte/measurand/organism of interest that gave rise to a coefficient of variation/relative standard deviation of 33%. This value is the limit of detection of the method as implemented by the laboratory.

For single laboratory validation, the concepts of "blank + 3σ" and "blank + 10σ" generally suffice for determining the limit of detection and the limit of quantitation/sensitivity. Since the blank is in theory zero (0), then the limit of detection and the limit of quantitation /sensitivity become 3σ and 10σ respectively. An absolute standard deviation of 3 and 10 equates to a coefficient of variation/relative standard deviation of 33% and 10% respectively. Accordingly the limit of detection and the limit of quantitation/sensitivity become the concentration of analyte/measurand/organism of interest which give rise to these values.

Data Summary: See below for explanation

Linear range of the method as implemented 0.12 ppm to 35.33 ppm
The limit of detection of the method as implemented 0.040 ppm
The limit of quantitation/sensitivity of the method as implemented 0.12 ppm

Data was generated as directed above (ten samples spiked to five levels, analyzed in triplicate plus one blank aliquot) for each matrix type examined, but this data could not be analyzed as described in the data handling portion of this SOP. (Although most of the data was not used to determine linearity and LOQ/LOD, it is provided at the end of this Appendix.)

This ELISA kit yields a very steep standard curve with a very narrow linear range, typically between 20% and 75% of the maximum absorbance of the reference wells (Amax). Therefore, to ensure quantitative results, serial dilutions (n=7) of each sample are prepared and analyzed. Assay response is converted to concentration by comparison to a standard curve, and the final sample concentration is the product of the concentration measured in the assay and the dilution factor. As a result, similar responses (signal data) can be measured for very different sample concentrations.

The overall or dynamic linear range of this method results from a combination of: 1) the linear range of the assay standard curve, 2) the assay limit of quantitation, and 3) the range of sample dilutions on the plate.

1) Linear Range of Assay

To evaluate the linear range of the assay, 7-point PbTx-3 standard curves (ranging from 0.08 to 5.0 ng/mL) from 60 ELISA plates run in this validation were generated using the sigmoidal dose-response (variable slope), or four-parameter logistic, curve fitting equation in Prism 5 (GraphPad Software). The upper and lower plateaus of the curves were then applied to formulae derived by Sebaugh and McCray[1] to define the "bend points" of the standard curves, the beginning and end of the linear concentration--response region, expressed both in terms of % inhibition (1-A/Amax x 100) and concentration (Table E.1). The assays included data generated using two different kit lots: June 2014 (n=34) and June 2016 (n=26). We found that the position of the standard curves and the linear range defined by the bend points differed between the two kit lots (Fig. E1). Such shifts can be achieved with the same kit lot by altering dilutions of key reagents (A and C). Therefore, we believe that the differences we observed in kit lots were due to minor concentration variations in the supplied reagents A and/or C. However, comparative analyses of spiked samples were not significantly different between the two kit lots (see Appendix F: Ruggedness).

Table E1. Average bend points (± standard deviation), expressed as % inhibition and concentration, defining the linear range of standard curves generated using two lots of ELISA kit reagents.

	% inhibition		ng PbTx-3/mL	
	Jun-14 Lot	Jun-16 Lot	Jun-14 Lot	Jun-16 Lot
lower bend point	17.34 ± 2.47	16.76 ± 2.73	0.21 ± 0.04	0.30 ± 0.06
upper bend point	76.91 ± 2.07	74.19 ± 1.68	1.04 ± 0.14	1.38 ± 0.16

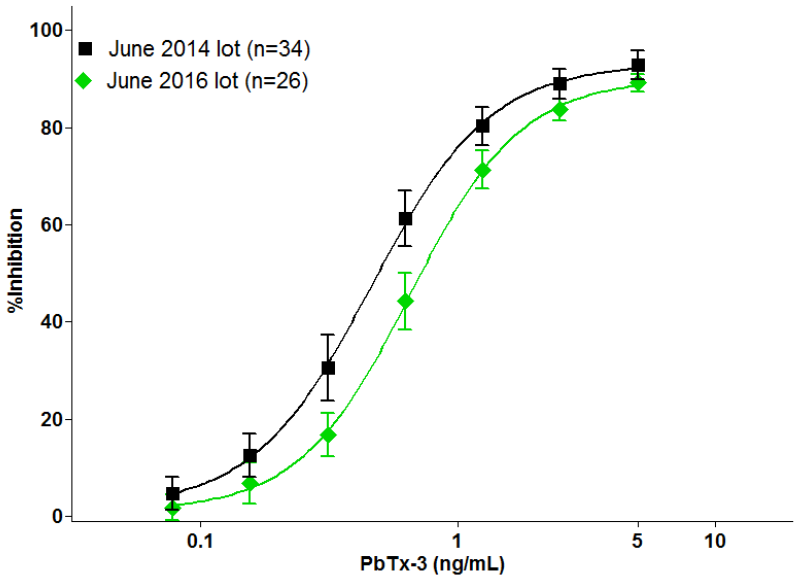


Figure E1. Average of multiple semi-log standard curves generated using two lots of ELISA kit reagents. Error bars represent standard deviation of independent curves prepared and assayed on different plates or days.

Using the June 2014 lot, two additional 14-point standard curves were assayed on different days to generate curves with more points that fell along the linear portion of the curve (Fig. E2). The bend points from these 14-point curves (16%-76%) were similar to those derived from the routine standard curves (Table E1).

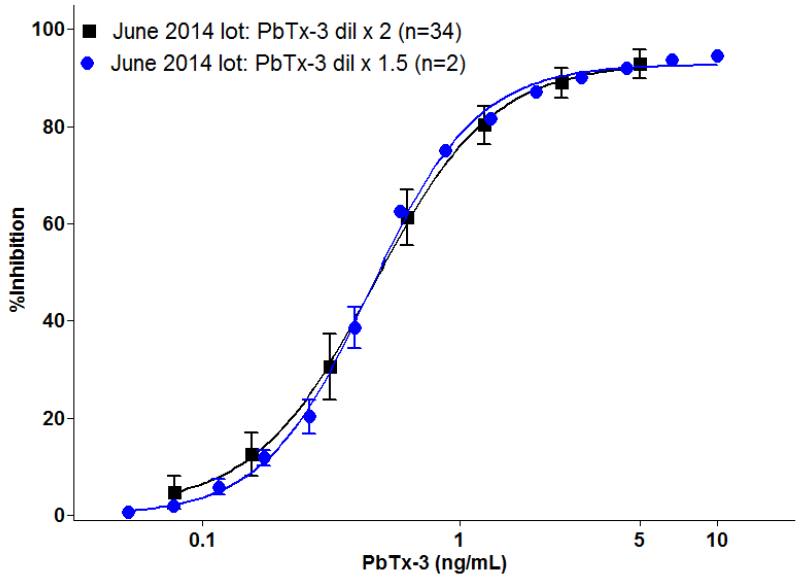


Figure E2. Comparison of 7-point and 14-point PbTx-3 standard curves. Error bars represent standard deviation of independent curves prepared and assays on different plates/days.

To verify linearity within the range defined by the bend points, multiple dilutions of shellfish samples spiked to 0.4 ppm with PbTx-3 were quantified. Ten samples were used for each matrix type, and three replicates per sample were extracted and analyzed. These assays were performed using the June 2014 kit lot. As written in the method protocol, the shellfish extraction protocol yields a 10-fold dilution (1g shellfish in 10 mL final volume). The extract is then diluted another 40 times in ELISA buffer for the initial dilution, and six additional dilutions are prepared by serial dilution by two, yielding a total of seven dilutions (from 1:400 to 1:25,600) for each sample.
At the 0.4 ppm spike level, the expected value of the first three dilutions are 1.0, 0.5, and 0.25 ng/mL, which are all within the linear range of the June 2014 kit lot as defined by the bend points. The expected and mean measured values of the three dilutions are listed in Table E2. Linear regression yielded r^2 values of 0.94-0.97 (Fig. E3).

Table E2. Expected concentrations and mean of concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Shellfish were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed.

	dilution	expected	mean measured	SD	%CV
oyster	400	1.00	0.935	0.079	8.4%
	800	0.50	0.471	0.038	8.1%
	1600	0.25	0.229	0.034	14.8%
hard clam	400	1.00	0.893	0.081	9.1%
	800	0.50	0.456	0.055	12.1%
	1600	0.25	0.233	0.033	14.2%
sunray venus clam	400	1.00	0.911	0.098	10.8%
	800	0.50	0.455	0.059	13.0%
	1600	0.25	0.234	0.030	12.8%

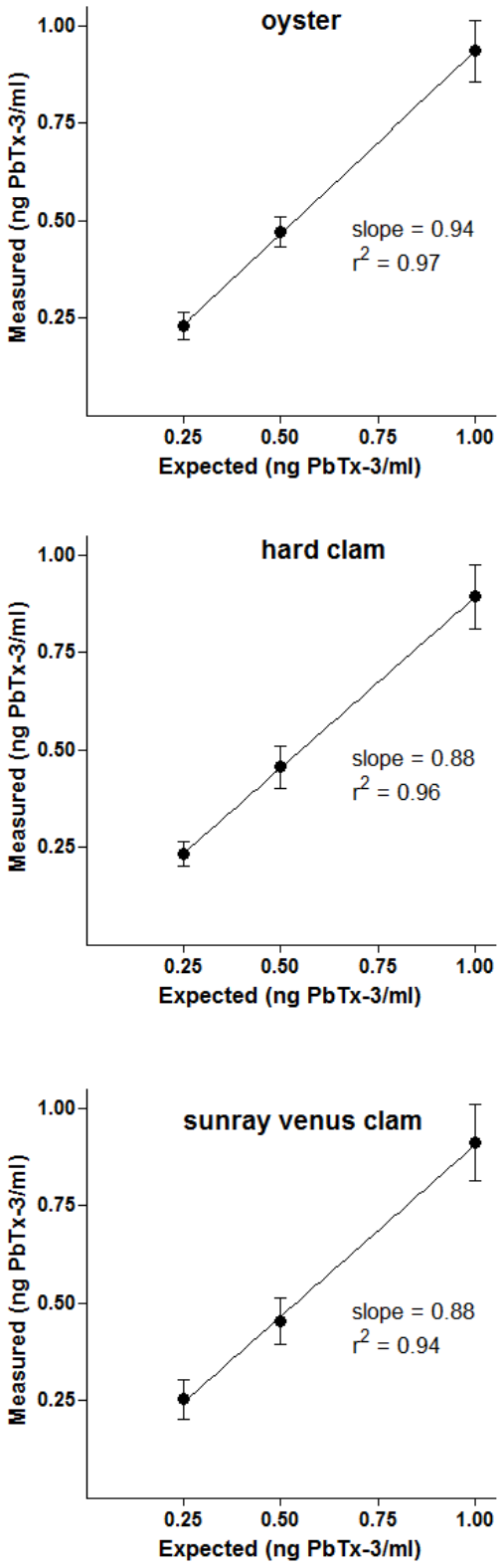


Figure E3. Expected concentrations vs. mean concentrations measured by ELISA (ng PbTx-3/mL) in three dilutions of shellfish extract. Oyster, hard clams, and sunray venus clams were spiked to 0.4ppm with PbTx-3, and three replicates each of ten samples per matrix (n=30) were analyzed. Error bars represent standard deviation.

Limit of Detection and Limit of Quantitation

Blank samples consistently yielded assay responses that were not quantifiable. Therefore, the standard deviation of results from the 1:1600 dilution of shellfish reported in Table E2 above were used to derive the limit of detection (LOD) and limit of quantitation (LOQ) expressed as 3x and 10x the standard deviation, respectively. This dilution consistently yielded a signal (% inhibition) closest to the lower bend point (17%) and was the lowest quantifiable dilution.

For all three matrix types, the standard deviation at the 1:1600 dilution was approximately 0.03. Calculated assay LOD and LOQ are 0.1 and 0.3 ng/mL, respectively. At the lowest sample dilution of 1:400, the LOD and LOQ for brevetoxin in shellfish are 40 and 120 ng/g or 0.04 and 0.12 ppm.

Dynamic linear range

The overall or dynamic linear range of this method is a combination of the linear range of the standard curve, the assay limit of quantitation, and the range of sample dilutions on the plate (from 400 to 25,600). Using the LOQ calculated above, which falls with the linear portion of the standard curve identified for both lots of kits used in this study, and the upper bend points identified for each kit lot, the overall or dynamic linear range of the method is from 120 ng PbTx-3 eq./g or 0.12 ppm up to 26,624 or 26.62ppm for the June 2014 kit lot and up to 35,328 ng PbTx-3 eq. per g or 35.33 ppm for the June 2016 kit lot.

References:

1. Sebaugh JL, McCray PD (2003) Defining the linear portion of a sigmoid-shaped curve: bend points. Pharmaceutical Statistics 2: 167-174.

Results of spiking experiments: ten samples were spiked to five levels and analyzed in triplicate (plus one blank aliquot) for each matrix type examined.

Data Summary:

spike conc (ppm)	Average concentration (ppm) Average recovery (%) Oyster	Hard Clam	Sunray Venus Clam
0.4	0.39	0.36	0.36
	96%	91%	91%
1	0.93	0.93	0.97
	93%	93%	97%
4	3.96	3.98	4.02
	99%	99%	101%
8	7.63	7.91	7.39
	95%	99%	92%
12	10.63	11.03	12.74
	89%	92%	106%

Full Data:

matrix type	sample	spike conc (ppm)	rep1 conc (ppm)	rep2 conc (ppm)	rep3 conc (ppm)
oyster	OY01	0	<LD		
oyster	OY01	0.4	0.38	0.38	0.40
oyster	OY01	1	0.99	0.95	0.93
oyster	OY01	4	4.07	4.12	4.61
oyster	OY01	8	7.84	7.53	7.66
oyster	OY01	12	10.60	10.97	10.73
oyster	OY02	0	<LD		
oyster	OY02	0.4	0.39	0.39	0.38
oyster	OY02	1	0.94	0.95	0.93
oyster	OY02	4	3.87	3.85	3.95
oyster	OY02	8	7.86	8.89	7.97
oyster	OY02	12	10.77	10.26	10.54
oyster	OY03	0	<LD		
oyster	OY03	0.4	0.44	0.42	0.39
oyster	OY03	1	0.80	0.77	0.82
oyster	OY03	4	3.57	3.92	3.58
oyster	OY03	8	7.94	7.86	8.17
oyster	OY03	12	10.83	11.13	11.11
oyster	OY04	0	<LD		
oyster	OY04	0.4	0.37	0.35	0.36
oyster	OY04	1	1.00	0.85	0.87
oyster	OY04	4	4.17	4.14	3.69
oyster	OY04	8	8.89	7.67	7.35
oyster	OY04	12	10.90	10.08	9.94
oyster	OY05	0	<LD		
oyster	OY05	0.4	0.36	0.38	0.36

oyster	OY05	1	0.77	0.89	0.74
oyster	OY05	4	4.22	4.06	4.01
oyster	OY05	8	7.89	7.73	7.04
oyster	OY05	12	10.17	10.38	9.68
oyster	OY06	0	<LD		
oyster	OY06	0.4	0.31	0.33	0.33
oyster	OY06	1	0.91	0.92	0.90
oyster	OY06	4	3.36	3.48	3.98
oyster	OY06	8	7.29	7.11	7.10
oyster	OY06	12	11.62	10.71	11.36
oyster	OY07	0	<LD		
oyster	OY07	0.4	0.40	0.40	0.41
oyster	OY07	1	0.88	1.05	0.99
oyster	OY07	4	3.90	4.21	3.77
oyster	OY07	8	8.51	6.22	7.04
oyster	OY07	12	10.68	11.42	10.75
oyster	OY08	0	<LD		
oyster	OY08	0.4	0.46	0.44	0.45
oyster	OY08	1	1.05	1.03	1.07
oyster	OY08	4	3.86	4.03	4.13
oyster	OY08	8	6.79	8.41	7.12
oyster	OY08	12	10.68	11.00	10.46
oyster	OY09	0	<LD		
oyster	OY09	0.4	0.43	0.37	0.36
oyster	OY09	1	1.06	0.92	0.91
oyster	OY09	4	3.74	3.94	3.89
oyster	OY09	8	7.35	7.13	7.04
oyster	OY09	12	11.09	10.44	10.78
oyster	OY10	0	<LD		
oyster	OY10	0.4	0.36	0.38	0.38
oyster	OY10	1	0.94	0.99	0.97
oyster	OY10	4	4.24	4.28	4.13
oyster	OY10	8	8.07	7.74	7.71
oyster	OY10	12	9.58	9.75	10.34
hard clam	HC01	0	<LD		
hard clam	HC01	0.4	0.33	0.32	0.35
hard clam	HC01	1	0.98	0.93	0.91
hard clam	HC01	4	3.85	3.79	3.48
hard clam	HC01	8	7.29	7.40	7.10
hard clam	HC01	12	9.89	9.80	10.75
hard clam	HC02	0	<LD		
hard clam	HC02	0.4	0.35	0.33	0.34
hard clam	HC02	1	0.92	0.89	0.86
hard clam	HC02	4	3.82	3.36	3.56
hard clam	HC02	8	7.85	8.11	8.52
hard clam	HC02	12	11.14	11.33	11.63
hard clam	HC03	0	<LD		
hard clam	HC03	0.4	0.35	0.33	0.37
hard clam	HC03	1	0.91	0.91	0.87
hard clam	HC03	4	3.55	3.36	3.59
hard clam	HC03	8	7.48	7.74	7.74
hard clam	HC03	12	10.26	11.20	10.25
hard clam	HC04	0	<LD		

hard clam	HC04	0.4	0.33	0.30	0.31
hard clam	HC04	1	0.91	0.91	0.88
hard clam	HC04	4	4.66	3.99	4.34
hard clam	HC04	8	6.99	8.15	8.32
hard clam	HC04	12	11.50	11.92	11.74
hard clam	HC05	0	<LD		
hard clam	HC05	0.4	0.32	0.33	0.29
hard clam	HC05	1	0.92	0.89	0.94
hard clam	HC05	4	3.49	4.03	4.32
hard clam	HC05	8	7.74	8.29	7.75
hard clam	HC05	12	10.78	9.82	11.27
hard clam	HC06	0	<LD		
hard clam	HC06	0.4	0.44	0.44	0.42
hard clam	HC06	1	0.84	0.92	0.99
hard clam	HC06	4	4.15	4.25	4.21
hard clam	HC06	8	7.12	8.19	8.49
hard clam	HC06	12	11.18	11.40	12.08
hard clam	HC07	0	<LD		
hard clam	HC07	0.4	0.42	0.43	0.38
hard clam	HC07	1	1.00	1.01	0.92
hard clam	HC07	4	4.05	4.12	3.82
hard clam	HC07	8	7.54	7.95	8.20
hard clam	HC07	12	10.94	10.50	10.70
hard clam	HC08	0	<LD		
hard clam	HC08	0.4	0.35	0.37	0.39
hard clam	HC08	1	0.92	1.00	0.92
hard clam	HC08	4	4.23	3.55	4.35
hard clam	HC08	8	6.88	7.98	7.63
hard clam	HC08	12	10.53	10.76	10.98
hard clam	HC09	0	<LD		
hard clam	HC09	0.4	0.40	0.39	0.40
hard clam	HC09	1	0.93	0.91	0.94
hard clam	HC09	4	3.98	4.26	4.32
hard clam	HC09	8	9.06	8.51	8.47
hard clam	HC09	12	12.38	10.77	10.84
hard clam	HC10	0	<LD		
hard clam	HC10	0.4	0.36	0.39	0.38
hard clam	HC10	1	0.97	0.98	1.01
hard clam	HC10	4	4.54	3.98	4.39
hard clam	HC10	8	8.02	9.05	7.80
hard clam	HC10	12	10.99	11.31	12.19
sunray venus clam	SV01	0	<LD		
sunray venus clam	SV01	0.4	0.36	0.37	0.36
sunray venus clam	SV01	1	0.94	0.98	0.88
sunray venus clam	SV01	4	3.89	3.95	4.37
sunray venus clam	SV01	8	6.93	7.35	7.27
sunray venus clam	SV01	12	11.61	12.13	11.52
sunray venus clam	SV02	0	<LD		
sunray venus clam	SV02	0.4	0.32	0.34	0.36
sunray venus clam	SV02	1	1.00	0.97	0.98
sunray venus clam	SV02	4	4.09	3.60	4.12
sunray venus clam	SV02	8	6.62	7.97	7.14
sunray venus clam	SV02	12	12.57	11.57	12.71

sunray venus clam	SV03	0	<LD		
sunray venus clam	SV03	0.4	0.38	0.36	0.34
sunray venus clam	SV03	1	1.00	0.98	1.00
sunray venus clam	SV03	4	4.15	3.71	4.12
sunray venus clam	SV03	8	8.96	7.93	7.83
sunray venus clam	SV03	12	12.95	12.02	13.17
sunray venus clam	SV04	0	<LD		
sunray venus clam	SV04	0.4	0.32	0.32	0.29
sunray venus clam	SV04	1	1.11	1.01	0.89
sunray venus clam	SV04	4	4.28	4.45	4.03
sunray venus clam	SV04	8	7.91	8.07	8.40
sunray venus clam	SV04	12	12.51	11.70	13.43
sunray venus clam	SV05	0	<LD		
sunray venus clam	SV05	0.4	0.29	0.30	0.29
sunray venus clam	SV05	1	1.13	1.08	1.08
sunray venus clam	SV05	4	4.19	3.98	4.10
sunray venus clam	SV05	8	8.66	8.06	7.27
sunray venus clam	SV05	12	13.32	12.34	13.47
sunray venus clam	SV06	0	<LD		
sunray venus clam	SV06	0.4	0.36	0.33	0.39
sunray venus clam	SV06	1	0.84	0.87	0.81
sunray venus clam	SV06	4	4.03	3.67	4.19
sunray venus clam	SV06	8	6.49	6.45	6.39
sunray venus clam	SV06	12	14.52	12.76	13.90
sunray venus clam	SV07	0	<LD		
sunray venus clam	SV07	0.4	0.41	0.41	0.42
sunray venus clam	SV07	1	0.93	0.91	0.85
sunray venus clam	SV07	4	4.10	3.62	4.03
sunray venus clam	SV07	8	7.73	6.79	7.91
sunray venus clam	SV07	12	11.91	13.74	11.53
sunray venus clam	SV08	0	<LD		
sunray venus clam	SV08	0.4	0.43	0.42	0.47
sunray venus clam	SV08	1	0.95	0.92	0.91
sunray venus clam	SV08	4	4.03	3.82	3.70
sunray venus clam	SV08	8	7.58	7.67	7.37
sunray venus clam	SV08	12	11.73	14.16	12.19
sunray venus clam	SV09	0	<LD		
sunray venus clam	SV09	0.4	0.44	0.35	0.33
sunray venus clam	SV09	1	0.86	1.03	1.06
sunray venus clam	SV09	4	4.36	3.87	4.30
sunray venus clam	SV09	8	7.29	5.44	6.42
sunray venus clam	SV09	12	13.18	14.53	12.87
sunray venus clam	SV10	0	<LD		
sunray venus clam	SV10	0.4	0.40	0.38	0.34
sunray venus clam	SV10	1	1.15	1.00	1.03
sunray venus clam	SV10	4	4.22	3.95	3.74
sunray venus clam	SV10	8	7.24	7.57	6.94
sunray venus clam	SV10	12	12.62	12.50	12.98

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must be sufficiently rugged to withstand the relatively minor day to day changes likely to occur in routine use. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Ruggedness of the new or modified method is the ability of a particular method to withstand relatively minor changes in analytical technique, reagents or environmental factors likely to arise in different test environments.

Procedure for testing the ruggedness of new or modified methods: This procedure is applicable for use with either growing waters or shellfish tissue. Make every effort to use samples free of the target analyte/measurand/organism of interest. For each shellfish type of interest use a minimum of 10 – 12 animals. For each sample take two (2) aliquots of either the growing water sample or shellfish homogenate appropriately sized for your work. Spike both aliquots with a suitable concentration of the target analyte/measurand/organism of interest. Process both aliquots of the sample as usual to determine method concentration for the target analyte/measurand/organism of interest. For the second aliquot of each sample, however, use a different batch or lot of culture media and/or test reagents as appropriate to process this aliquot. For growing waters, do ten (10) samples collected from a variety of growing waters. For shellfish do ten (10) samples for each shellfish tissue type of interest collected from a variety of growing areas, the same growing area harvested on different days or from different process lots. Use the same two batches or lots of culture media and/or test reagents to process each sample such that "batch or lot 1" is used to process the first aliquot of each sample and "batch or lot 2" is used to process the second aliquot of each sample. Use a range of concentrations which spans the range of the new method or modified method's intended application to spike the sample aliquots. However both aliquots of the same sample must be spiked with the same concentration of the target analyte/measurand/organism of interest. Process samples over a period of several days.

Data for demonstrating the ruggedness of the new or modified method:
For this study, results of sample analyses conducted under varying conditions were compared. Variations examined included: 1) different lots of ELISA kit reagents (June 2014 and June 2016), 2) different temperatures (incubation of ELISA plates throughout the procedure at ambient laboratory temperature [21-22°C] and in a heated plate shaker [25°C]), 3) different durations of sample and primary antibody incubation (60 min vs. 90 min), 4) and duration of final color development step (7 min vs 13 min).

1) Different lots of ELISA kit reagents:

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	Jun-14 lot	Jun-16 lot
oyster	OY01	0.4	0.38	0.36
oyster	OY02	0.4	0.38	0.36
oyster	OY03	1	0.92	0.90
oyster	OY04	1	0.88	0.88
oyster	OY05	2	1.87	1.88
oyster	OY06	2	1.84	2.04
oyster	OY07	4	3.78	3.66
oyster	OY08	4	3.88	4.54
oyster	OY09	8	7.67	7.86
oyster	OY10	8	8.05	7.73
hard clam	HC01	0.4	0.38	0.39
hard clam	HC02	0.4	0.39	0.38
hard clam	HC03	1	1.08	0.92
hard clam	HC04	1	0.97	0.97
hard clam	HC05	2	1.97	1.92

hard clam	HC06	2	2.16	1.85
hard clam	HC07	4	3.78	4.05
hard clam	HC08	4	3.90	3.83
hard clam	HC09	8	7.86	7.69
hard clam	HC10	8	7.79	8.16
sunray venus clam	SV01	0.4	0.35	0.33
sunray venus clam	SV02	0.4	0.39	0.38
sunray venus clam	SV03	1	1.03	0.89
sunray venus clam	SV04	1	1.05	1.00
sunray venus clam	SV05	2	2.05	1.89
sunray venus clam	SV06	2	1.97	1.95
sunray venus clam	SV07	4	3.62	4.23
sunray venus clam	SV08	4	3.82	4.22
sunray venus clam	SV09	8	7.57	7.38
sunray venus clam	SV10	8	8.34	7.85

2) Incubation of ELISA plates throughout the procedure at ambient laboratory temperature (21-22°C) vs. in a heated plate shaker (25°C):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	21-22°C	25°C
oyster	OY01	0.4	0.36	0.37
oyster	OY02	0.4	0.36	0.40
oyster	OY03	1	0.90	0.88
oyster	OY04	1	0.88	0.84
oyster	OY05	2	1.88	1.96
oyster	OY06	2	2.04	1.94
oyster	OY07	4	3.66	3.72
oyster	OY08	4	4.54	4.56
oyster	OY09	8	7.86	8.08
oyster	OY10	8	7.73	8.31
hard clam	HC01	0.4	0.39	0.39
hard clam	HC02	0.4	0.38	0.37
hard clam	HC03	1	0.92	0.91
hard clam	HC04	1	0.97	0.86
hard clam	HC05	2	1.92	2.07
hard clam	HC06	2	1.85	1.87
hard clam	HC07	4	4.05	4.06
hard clam	HC08	4	3.83	4.17
hard clam	HC09	8	7.69	7.96
hard clam	HC10	8	8.16	8.26
sunray venus clam	SV01	0.4	0.33	0.35
sunray venus clam	SV02	0.4	0.38	0.40
sunray venus clam	SV03	1	0.89	0.92
sunray venus clam	SV04	1	1.00	0.94
sunray venus clam	SV05	2	1.89	2.24
sunray venus clam	SV06	2	1.95	1.86

sunray venus clam	SV07	4	4.23	4.08
sunray venus clam	SV08	4	4.22	4.19
sunray venus clam	SV09	8	7.38	7.03
sunray venus clam	SV10	8	7.85	7.49

3) Duration of sample and primary antibody (reagent C) incubation (60 min vs. 90 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	60 min C	90 min C
oyster	OY01	0.4	0.37	0.38
oyster	OY02	0.4	0.39	0.38
oyster	OY03	1	0.92	0.92
oyster	OY04	1	0.92	0.88
oyster	OY05	2	1.61	1.87
oyster	OY06	2	1.62	1.84
oyster	OY07	4	3.28	3.78
oyster	OY08	4	3.51	3.88
oyster	OY09	8	7.94	7.67
oyster	OY10	8	7.99	8.05
hard clam	HC01	0.4	0.40	0.38
hard clam	HC02	0.4	0.39	0.39
hard clam	HC03	1	1.02	1.08
hard clam	HC04	1	1.07	0.97
hard clam	HC05	2	1.84	1.97
hard clam	HC06	2	1.97	2.16
hard clam	HC07	4	3.65	3.78
hard clam	HC08	4	3.40	3.90
hard clam	HC09	8	7.44	7.86
hard clam	HC10	8	7.89	7.79
sunray venus clam	SV01	0.4	0.37	0.35
sunray venus clam	SV02	0.4	0.40	0.39
sunray venus clam	SV03	1	0.94	1.03
sunray venus clam	SV04	1	0.95	1.05
sunray venus clam	SV05	2	2.11	2.05
sunray venus clam	SV06	2	2.07	1.97
sunray venus clam	SV07	4	3.89	3.62
sunray venus clam	SV08	4	3.73	3.82
sunray venus clam	SV09	8	7.84	7.57
sunray venus clam	SV10	8	7.89	8.34

4) Duration of TMB color development step (7 min vs 13 min):

			ELISA (ppm)	
matrix type	sample	spike conc (ppm)	TMB 7 min	TMB 13 min
oyster	OY01	0.4	0.35	0.48
oyster	OY02	0.4	0.36	0.36
oyster	OY03	1	0.91	1.13
oyster	OY04	1	0.91	0.89
oyster	OY05	2	1.69	2.05
oyster	OY06	2	1.86	1.90
oyster	OY07	4	3.84	4.05
oyster	OY08	4	3.88	4.61
oyster	OY09	8	7.89	8.35
oyster	OY10	8	7.90	7.63
hard clam	HC01	0.4	0.34	0.42
hard clam	HC02	0.4	0.37	0.37
hard clam	HC03	1	0.95	1.13
hard clam	HC04	1	0.93	0.93
hard clam	HC05	2	1.78	2.22
hard clam	HC06	2	1.64	1.80
hard clam	HC07	4	3.74	4.45
hard clam	HC08	4	3.62	4.37
hard clam	HC09	8	7.52	7.48
hard clam	HC10	8	7.94	7.55
sunray venus clam	SV01	0.4	0.35	0.39
sunray venus clam	SV02	0.4	0.38	0.44
sunray venus clam	SV03	1	0.94	0.97
sunray venus clam	SV04	1	0.93	1.17
sunray venus clam	SV05	2	1.84	2.13
sunray venus clam	SV06	2	1.76	1.81
sunray venus clam	SV07	4	3.66	3.90
sunray venus clam	SV08	4	3.76	4.04
sunray venus clam	SV09	8	7.88	7.50
sunray venus clam	SV10	8	7.95	8.14

For shellfish samples, repeat for each tissue type of interest.

Data handling to demonstrate the ruggedness of the new or modified method

In the day to day operations of the laboratory there will be changes in the batches/lots of culture media and/or test reagents used to process samples. Environmental factors are also likely to change over time. None of these factors, however, should adversely impact test results if the new or modified method as implemented is sufficiently rugged to be used routinely for regulatory monitoring.

To determine whether the new or modified method as implemented is sufficiently rugged to withstand the types of changes anticipated to occur in routine use, a two-sided t-test at a significance level (α) of .05 will be used on the data to ascertain if results obtained using different culture media and/or test reagent batches/lots under slightly varying environmental conditions are significantly affected by such minor changes. Either a paired t-test or Welch's t-test will be

used depending upon the shape of the distribution produced by the data for each batch/lot and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry of the distribution of results from both batch/lot 1 and batch/lot 2.
2. Calculate the variance of both batch/lot 1 and batch/lot 2 data.
3. Values for the test of symmetry for either batch/lot 1 or batch/lot 2 outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either batch/lot 1 or batch/lot 2 to the smaller of the variances of either batch/lot 1 or batch/lot 2 >2 indicates a lack of homogeneity of variance.

5. Use either the paired t-test or Welch's t-test for the analysis based on the following considerations.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) and there is homogeneity of variance, use a paired t-test for the analysis.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are symmetric (within the range of -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis.
    - If the distribution of the data from batch/lot 1 and batch/lot 2 are skewed (outside the range of -2 to +2) and the skewness for both groups is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis.
    - If the distributions of the data from batch/lot 1 and batch/lot 2 are skewed and the skewness for both groups is either positive for both or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for demonstrating the ruggedness of the new or modified method:

See tables on next page

Significant differences were observed with variant 4, when TMB color development times varied. As the wells grew darker, measured concentrations tended to increase from a maximum absorbance at 450 nm (after stopping the reaction) of approximately 1.0 to a maximum absorbance of 1.5. Variability (%RSD) in replicate reference wells increased moderately with time as well (from 3.9% to 6.3%). 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 of 1.0 ± 30%.

Value for the test of symmetry of the distribution of batch/lot 1 data _____

Value for the test of symmetry of the distribution of batch/lot 2 data _____

Variance of batch/lot 1 data _____

Variance of batch/lot 2 data _____

Ratio of the larger to the smaller of the variances of batch/lot 1 and batch/lot 2 _____

Is there a significant difference between batch/lot 1 samples and batch/lot 2 samples? Y/N

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	Jun14 lot	Jun16 lot	Jun14 lot	Jun16 lot			
oyster	-0.32402 (0.750)	-0.07704 (0.906)	0.2281	0.2350	1.030	0.302	no
hard clam	-0.10448 (0.916)	-0.26257 (0.856)	0.2366	0.2483	1.049	0.708	no
sunray venus clam	-0.27735 (0.804)	-0.17249 (0.852)	0.2327	0.2471	1.062	0.465	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	21-22°C	25°C	21-22°C	25°C			
oyster	-0.07704 (0.974)	-0.20833 (0.822)	0.2350	0.2465	1.049	0.741	no
hard clam	-0.26257 (0.740)	-0.18657 (0.874)	0.2483	0.2483	1.000	0.287	no
sunray venus clam	-0.17249 (0.820)	-0.37325 (0.764)	0.2471	0.2333	1.059	0.754	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	60 min C	90 min C	60 min C	90 min C			
oyster	-0.13316 (0.866)	-0.32402 (0.780)	0.2160	0.2281	1.056	0.219	no
hard clam	0.25186 (0.772)	-0.10448 (0.912)	0.2301	0.2366	1.028	0.099	no
sunray venus clam	-0.42338 (0.680)	-0.27735 (0.734)	0.2326	0.2327	1.000	0.982	no

	Symmetry Test Statistic (*p*-value)*		Variance		Variance Ratio	Paired t-test *p*-value	Sig Dif?
	TMB 7 min	TMB 13 min	TMB 7 min	TMB 13 min			
oyster	0.07922 (0.892)	-0.13022 (0.922)	0.2388	0.2297	1.040	0.014	yes
hard clam	-0.00274 (0.958)	-0.04028 (0.982)	0.2460	0.2302	1.069	0.030	yes
sunray venus clam	-0.03460 (0.990)	-0.06355 (0.940)	0.2387	0.2187	1.092	0.011	yes

*m-out-of-n bootstrap symmetry test by Miao, Gel, and Gastwirth (2006)

Miao, W., Y. R. Gel, and J. L. Gastwirth. "A New Test of Symmetry about an Unknown Median. Random Walk." *Sequential Analysis and Related Topics-A Festschrift in Honor of Yuan-Shih Chow. Eds.: Agnes Hsiung, Cun-Hui Zhang, and Zhiliang Ying, World Scientific Publisher, Singapore* (2006).

VALIDATION CRITERIA

Comparability is the acceptability of a new or modified analytical method as a substitute for an established method in the NSSP. To be acceptable the new or modified method must not produce a significant difference in results when compared to the officially recognized method. Comparability must be demonstrated for each substrate or tissue type of interest by season and geographic area if applicable.

Comparison of Methods:
New or modified methods demonstrating comparability to officially recognized methods must not produce significantly different results when compared

Procedure to compare the new or modified method to the officially recognized method: This procedure is applicable for use with either growing waters or shellfish tissue. For each shellfish type of interest use a minimum of 10-12 animals per sample. For each sample take two (2) aliquots and analyze one by the officially recognized method and the other by the alternative method. Actual samples are preferable; but, in cases where the occurrence of the analyte/measurand/organism of interest is intermittent (such as marine biotoxins), spiked samples can be used. Samples having a variety of concentrations which span the range of the method's intended application should be used in the comparison. Analyze a minimum of thirty (30) paired samples for each season from a variety of growing areas for a total of at least 120 samples over the period of a year for naturally incurred samples. For spiked samples analyze a minimum of ten (10) samples for each season from a variety of growing areas for a total of at least 40 samples over the period of a year.

Data:

A total of 526 samples were tested using both ELISA and the NSP mouse bioassay (Table G1). Results of individual samples are contained in Table G2. Although additional data exists (both published and unpublished) comparing this ELISA with NSP mouse bioassay results, extraction methods have been modified over time. The data presented here includes only samples that were extracted for ELISA using 80% methanol with no additional clean-up. Almost all of the samples (495 of 526, 94%) were extracted and assayed in duplicate, and the mean is reported in the table. The mean %CV of duplicate analyses was 6.2%.

Table G1. Summary of comparative data using both NSP mouse bioassay and ELISA.

Shellfish Matrix	Total Samples	Mouse Bioassay < 20 MU/100g	Mouse Bioassay ≥ 20 MU/100g
Oysters	197	135 (69%)	62 (31%)
Hard Clams	277	238 (86%)	39 (14%)
Sunray Venus Clams	52	22 (42%)	30 (58%)

Table G2. Sample information and results of NSP mouse bioassay and ELISA

Sample ID	Shellfish Matrix	Harvest Area	Sample Date	MU/100g	ELISA (ppm)
HABB070327-017	oyster	Pine Island Sound	3/26/2007	<20	6.60
HABB070403-002	oyster	Pine Island Sound	4/2/2007	<20	5.26
HABB071115-001	oyster	St. Johns	11/14/2007	33.75	7.26
HABB071115-002	oyster	St. Johns	11/14/2007	38.63	16.31
HABB071128-004	oyster	St. Johns	11/27/2007	27.37	6.53
HABB071212-003	oyster	St. Johns	12/11/2007	<20	3.40
HABB080214-001	oyster	Alabama	2/8/2008	<20	0.52
HABB091117-001	oyster	Pine Island Sound	11/16/2009	<20	0.66
HABB091202-001	oyster	Pine Island Sound	12/1/2009	<20	0.42
HABB091202-002	oyster	Pine Island Sound	12/1/2009	<20	0.29
HABB100105-001	oyster	Pine Island Sound	1/4/2010	36.38	9.44
HABB100112-003	oyster	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-004	oyster	Pine Island Sound	1/11/2010	26.04	6.07
HABB100113-001	oyster	Gasparilla Sound	1/12/2010	<20	1.21
HABB100113-002	oyster	Gasparilla Sound	1/12/2010	<20	1.66
HABB100120-001	oyster	Pine Island Sound	1/19/2010	<20	<LOD
HABB100120-002	oyster	Pine Island Sound	1/19/2010	<20	2.34
HABB100224-001	oyster	Pine Island Sound	2/23/2010	<20	1.83
HABB100224-002	oyster	Pine Island Sound	2/23/2010	<20	1.01
HABB111026-003	oyster	Pine Island Sound	10/25/2011	<20	<LOD
HABB111026-004	oyster	Pine Island Sound	10/25/2011	<20	1.99
HABB111103-001	oyster	Gasparilla Sound	11/2/2011	33.31	9.57
HABB111103-002	oyster	Gasparilla Sound	11/2/2011	28.19	6.50
HABB111109-001	oyster	Pine Island Sound	11/8/2011	<20	0.53
HABB111109-002	oyster	Pine Island Sound	11/8/2011	32.93	10.09
HABB111115-001	oyster	Gasparilla Sound	11/14/2011	<20	4.80
HABB111115-002	oyster	Gasparilla Sound	11/14/2011	<20	2.98
HABB111122-002	oyster	Lemon Bay	11/21/2011	<20	7.76
HABB111213-001	oyster	Pine Island Sound	12/12/2011	<20	2.04
HABB111213-002	oyster	Pine Island Sound	12/12/2011	<20	1.71
HABB111220-001	oyster	Pine Island Sound	12/19/2011	<20	10.83
HABB111220-002	oyster	Pine Island Sound	12/19/2011	<20	3.85
HABB120124-003	oyster	Pine Island Sound	1/23/2012	<20	3.94
HABB120124-004	oyster	Pine Island Sound	1/23/2012	<20	1.31
HABB120131-001	oyster	Ten Thousand Islands	1/30/2012	37.70	14.01
HABB120214-001	oyster	Ten Thousand Islands	2/13/2012	22.80	6.19
HABB120214-002	oyster	Pine Island Sound	2/13/2012	<20	8.25
HABB120214-003	oyster	Pine Island Sound	2/13/2012	<20	1.79
HABB120221-001	oyster	Ten Thousand Islands	2/20/2012	27.43	6.72
HABB120228-001	oyster	Ten Thousand Islands	2/27/2012	<20	4.42
HABB121113-002	oyster	Lower Tampa Bay	11/6/2012	34.08	4.32
HABB130212-004	oyster	Lower Tampa Bay	11/14/2012	34.99	22.43
HABB130205-003	oyster	Lower Tampa Bay	2/4/2013	<20	3.28
HABB130409-001	oyster	Gasparilla Sound	4/8/2013	31.56	8.17
HABB130409-002	oyster	Gasparilla Sound	4/8/2013	29.65	15.40
HABB130501-001	oyster	Gasparilla Sound	4/30/2013	32.21	5.07
HABB130501-002	oyster	Gasparilla Sound	4/30/2013	24.07	3.26

HABB130501-003	oyster	Ten Thousand Islands	4/30/2013	<20	0.77
HABB130508-002	oyster	Gasparilla Sound	5/7/2013	<20	4.91
HABB130508-003	oyster	Gasparilla Sound	5/7/2013	<20	3.00
HABB130508-005	oyster	Lemon Bay	5/7/2013	<20	3.92
HABB130515-001	oyster	Pine Island Sound	5/14/2013	<20	3.17
HABB130515-002	oyster	Pine Island Sound	5/14/2013	<20	3.24
HABB130604-002	oyster	Sarasota Bay	6/3/2013	<20	2.43
HABB131210-001	oyster	Gasparilla Sound	12/9/2013	<20	4.52
HABB131210-002	oyster	Gasparilla Sound	12/9/2013	<20	0.79
HABB131210-003	oyster	Pine Island Sound	12/9/2013	<20	1.99
HABB131217-001	oyster	Pine Island Sound	12/16/2013	<20	2.03
HABB131217-002	oyster	Pine Island Sound	12/16/2013	<20	1.51
HABB131217-003	oyster	Matlacha	12/16/2013	<20	0.18
HABB131218-009	oyster	Lemon Bay	12/17/2013	<20	1.63
HABB141021-001	oyster	Suwannee Sound	10/20/2014	<20	4.62
HABB141021-002	oyster	Suwannee Sound	10/20/2014	<20	5.02
HABB141021-003	oyster	Suwannee Sound	10/20/2014	<20	3.34
HABB141022-002	oyster	Horseshoe Beach	10/21/2014	27.89	5.02
HABB141022-003	oyster	Horseshoe Beach	10/21/2014	<20	<LOD
HABB141028-001	oyster	Horseshoe Beach	10/27/2014	<20	4.44
HABB141028-002	oyster	Horseshoe Beach	10/27/2014	<20	5.20
HABB141028-003	oyster	Horseshoe Beach	10/27/2014	22.56	5.73
HABB141104-001	oyster	Horseshoe Beach	11/3/2014	<20	3.53
HABB141118-001	oyster	Gasparilla Sound	11/17/2014	<20	1.07
HABB141118-002	oyster	Gasparilla Sound	11/17/2014	<20	0.45
HABB141124-004	oyster	Pine Island Sound	11/23/2014	<20	2.57
HABB141209-001	oyster	Pine Island Sound	12/8/2014	<20	0.91
HABB141209-002	oyster	Pine Island Sound	12/8/2014	<20	2.49
HABB141216-001	oyster	Ten Thousand Islands	12/15/2014	<20	1.13
HABB151014-002	oyster	Indian Lagoon	10/13/2015	<20	0.84
HABB151119-001	oyster	East Bay	10/29/2015	94.60	25.50
HABB151103-001	oyster	Indian Lagoon	11/2/2015	<20	1.99
HABB151103-002	oyster	Pine Island Sound	11/2/2015	<20	0.98
HABB151103-003	oyster	Pine Island Sound	11/2/2015	<20	<LOD
HABB151110-001	oyster	Gasparilla Sound	11/9/2015	<20	1.34
HABB151110-002	oyster	Gasparilla Sound	11/9/2015	<20	3.87
HABB151117-001	oyster	East Bay	11/16/2015	34.05	7.08
HABB151117-002	oyster	North Bay	11/16/2015	<20	1.59
HABB151124-001	oyster	East Bay	11/23/2015	25.03	5.77
HABB151202-001	oyster	East Bay	12/1/2015	34.84	7.44
HABB151208-001	oyster	West Bay	12/7/2015	33.07	3.57
HABB151208-002	oyster	East Bay	12/7/2015	28.14	5.09
HABB151208-003	oyster	East Bay	12/7/2015	35.47	13.95
HABB151216-001	oyster	East Bay	12/15/2015	33.37	5.04
HABB151216-002	oyster	West Bay	12/15/2015	30.10	5.55
HABB151217-001	oyster	Gasparilla Sound	12/16/2015	<20	2.27
HABB151217-002	oyster	Gasparilla Sound	12/16/2015	26.79	4.73
HABB151217-003	oyster	Pine Island Sound	12/16/2015	31.47	3.96
HABB151217-004	oyster	Pine Island Sound	12/16/2015	20.21	3.56
HABB151222-001	oyster	Gasparilla Sound	12/21/2015	<20	4.31
HABB151222-002	oyster	Gasparilla Sound	12/21/2015	<20	1.77
HABB160105-001	oyster	Pine Island Sound	1/4/2016	<20	2.28

HABB160105-002	oyster	Pine Island Sound	1/4/2016	<20	2.17
HABB160105-003	oyster	Apalachicola Bay	1/4/2016	<20	3.27
HABB160105-004	oyster	Apalachicola Bay	1/4/2016	<20	2.52
HABB160106-001	oyster	East Bay	1/5/2016	30.63	2.45
HABB160106-002	oyster	North Bay	1/5/2016	17.07	7.91
HABB160112-001	oyster	West Bay	1/11/2016	22.35	3.28
HABB160112-002	oyster	North Bay	1/11/2016	23.94	7.28
HABB160112-003	oyster	West Bay	1/11/2016	35.43	12.59
HABB160113-001	oyster	Pensacola Bay	1/12/2016	<20	2.13
HABB160114-001	oyster	Apalachicola Bay	1/12/2016	<20	1.88
HABB160114-002	oyster	Indian Lagoon	1/12/2016	21.84	10.53
HABB160120-001	oyster	East Bay	1/19/2016	<20	2.02
HABB160120-002	oyster	North Bay	1/19/2016	<20	6.41
HABB160120-003	oyster	Mississippi	1/19/2016	<20	0.16
HABB160120-004	oyster	Mississippi	1/19/2016	<20	0.33
HABB160120-005	oyster	Mississippi	1/19/2016	<20	0.23
HABB160120-006	oyster	Mississippi	1/19/2016	<20	0.41
HABB160120-007	oyster	Mississippi	1/19/2016	<20	1.22
HABB160120-008	oyster	Mississippi	1/19/2016	<20	0.88
HABB160121-001	oyster	Indian Lagoon	1/20/2016	22.20	9.84
HABB160126-001	oyster	West Bay	1/25/2016	30.18	9.37
HABB160126-002	oyster	West Bay	1/25/2016	16.69	2.82
HABB160127-001	oyster	Alabama	1/25/2016	<20	3.17
HABB160127-002	oyster	Alabama	1/25/2016	<20	2.23
HABB160127-003	oyster	Alabama	1/25/2016	<20	3.11
HABB160127-004	oyster	Alabama	1/25/2016	<20	0.36
HABB160127-005	oyster	Alabama	1/25/2016	<20	0.42
HABB160128-001	oyster	East Bay	1/27/2016	<20	3.00
HABB160202-001	oyster	West Bay	2/1/2016	29.32	5.96
HABB160203-001	oyster	St. Joseph Bay	2/2/2016	28.40	14.20
HABB160203-002	oyster	Louisiana	2/2/2016	<20	0.29
HABB160203-003	oyster	Louisiana	2/2/2016	<20	0.77
HABB160203-004	oyster	Louisiana	2/2/2016	<20	0.84
HABB160203-005	oyster	Louisiana	2/2/2016	<20	1.08
HABB160203-006	oyster	Louisiana	2/2/2016	<20	0.33
HABB160203-007	oyster	Louisiana	2/2/2016	<20	0.29
HABB160204-001	oyster	Indian Lagoon	2/2/2016	<20	4.22
HABB160211-001	oyster	West Bay	2/10/2016	<20	5.56
HABB160223-001	oyster	Pine Island Sound	2/22/2016	31.66	6.77
HABB160223-005	oyster	St. Joseph Bay	2/22/2016	<20	12.37
HABB160224-001	oyster	Pine Island Sound	2/23/2016	<20	0.94
HABB160301-001	oyster	Alabama	2/29/2016	<20	1.72
HABB160302-001	oyster	Pine Island Sound	3/1/2016	<20	4.02
HABB160303-002	oyster	Gasparilla Sound	3/2/2016	19.81	5.07
HABB160308-001	oyster	Lower Tampa Bay	3/7/2016	23.53	10.51
HABB160309-001	oyster	Choctawhatchee Bay	3/8/2016	<20	0.60
HABB160317-001	oyster	Pine Island Sound	3/16/2016	25.90	3.87
HABB160317-002	oyster	Pine Island Sound	3/16/2016	<20	3.03
HABB160322-001	oyster	Lower Tampa Bay	3/22/2016	<20	4.33
HABB160328-002	oyster	Lower Tampa Bay	3/28/2016	<20	4.87
HABB160330-001	oyster	Pine Island Sound	3/29/2016	26.26	4.88
HABB160330-002	oyster	Pine Island Sound	3/29/2016	<20	2.19

HABB160407-002	oyster	Lower Tampa Bay	4/6/2016	<20	3.99
HABB160407-004	oyster	Pine Island Sound	4/7/2016	<20	3.00
HABB160411-013	oyster	Lower Tampa Bay	4/11/2016	<20	3.83
HABB160418-002	oyster	Lower Tampa Bay	4/18/2016	<20	2.76
HABB160421-002	oyster	Pine Island Sound	4/20/2016	23.66	3.01
HABB160421-003	oyster	Pine Island Sound	4/20/2016	<20	1.71
HABB160427-001	oyster	Pine Island Sound	4/26/2016	<20	3.37
HABB160427-002	oyster	Pine Island Sound	4/26/2016	<20	1.71
HABB160502-001	oyster	Boca Ceiga Bay	5/2/2016	21.65	4.59
HABB160505-001	oyster	Gasparilla Sound	5/4/2016	<20	2.70
HABB160505-002	oyster	Gasparilla Sound	5/4/2016	<20	1.67
HABB160510-001	oyster	Boca Ceiga Bay	5/10/2016	16.23	4.11
HABB161011-002	oyster	Lower Tampa Bay	10/10/2016	<20	0.74
HABB161018-002	oyster	Lower Tampa Bay	10/17/2016	<20	1.57
HABB161114-002	oyster	Lower Tampa Bay	11/14/2016	156.08	47.60
HABB170104-003	oyster	Pine Island Sound	1/3/2017	30.23	9.64
HABB170105-001	oyster	Lower Tampa Bay	1/4/2017	<20	2.31
HABB170110-001	oyster	Lower Tampa Bay	1/9/2017	<20	0.84
HABB170110-004	oyster	Gasparilla Sound	1/9/2017	28.32	8.43
HABB170111-001	oyster	Ten Thousand Islands	1/10/2017	19.63	3.14
HABB170111-002	oyster	Matlacha Pass	1/10/2017	<20	1.58
HABB170111-003	oyster	Pine Island Sound	1/10/2017	30.71	7.37
HABB170118-002	oyster	Gasparilla Sound	1/17/2017	29.46	6.65
HABB170119-003	oyster	Pine Island Sound	1/18/2017	33.87	5.64
HABB170119-004	oyster	Myakka River	1/18/2017	31.00	4.56
HABB170125-001	oyster	Gasparilla Sound	1/24/2017	<20	4.06
HABB170125-003	oyster	Pine Island Sound	1/24/2017	<20	4.31
HABB170131-002	oyster	Gasparilla Sound	1/30/2017	36.73	9.68
HABB170201-002	oyster	Myakka River	1/31/2017	22.45	3.56
HABB170207-002	oyster	Gasparilla Sound	2/6/2017	31.32	8.12
HABB170213-002	oyster	Lower Tampa Bay	2/13/2017	<20	1.47
HABB170214-004	oyster	Pine Island Sound	2/13/2017	<20	2.01
HABB170221-001	oyster	Myakka River	2/20/2017	<20	2.08
HABB170222-001	oyster	Gasparilla Sound	2/21/2017	42.30	10.51
HABB170307-002	oyster	Gasparilla Sound	3/6/2017	29.03	5.11
HABB170314-002	oyster	Gasparilla Sound	3/13/2017	<20	2.55
HABB170315-002	oyster	Lower Tampa Bay	3/14/2017	<20	2.21
HABB170322-002	oyster	Gasparilla Sound	3/21/2017	<20	2.49
HABB170405-001	oyster	Boca Ceiga Bay	4/4/2017	31.35	6.80
HABB170410-005	oyster	Gasparilla Sound	4/10/2017	<20	1.23
HABB170412-001	oyster	Pine Island Sound	4/11/2017	25.73	3.56
HABB170418-001	oyster	Pine Island Sound	4/17/2017	19.01	2.35
HABB170419-001	oyster	Lower Tampa Bay	4/18/2017	<20	5.89
HABB170419-002	oyster	Lower Tampa Bay	4/18/2017	<20	3.72
HABB170425-001	oyster	Gasparilla Sound	4/24/2017	25.81	4.13
HABB170425-002	oyster	Gasparilla Sound	4/24/2017	34.91	8.27
HABB080108-001	hard clam	Volusia County	1/7/2008	<20	0.97
HABB080108-002	hard clam	Volusia County	1/7/2008	<20	0.77
HABB080108-003	hard clam	Mosquito Lagoon	1/7/2008	52.8	4.2
HABB080109-003	hard clam	North Indian River	1/8/2008	<20	2.69
HABB080109-004	hard clam	Indian River Body F	1/8/2008	<20	0.14
HABB080115-001	hard clam	Mosquito Lagoon	1/14/2008	46.26	4

HABB080115-002	hard clam	Indian River Body A	1/14/2008	<20	1.18
HABB080115-003	hard clam	Indian River Body A	1/14/2008	38.66	4.44
HABB080123-022	hard clam	St. Lucie County	1/22/2008	<20	0.93
HABB080123-023	hard clam	Mosquito Lagoon	1/22/2008	<20	3.05
HABB080123-024	hard clam	Indian River Body A	1/22/2008	<20	2.35
HABB080123-025	hard clam	Indian River Body B	1/22/2008	<20	1.16
HABB090519-001	hard clam	Indian River Body F	5/18/2009	<20	<LOD
HABB091109-001	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-002	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB091109-003	hard clam	Pine Island Sound	11/9/2009	<20	<LOD
HABB091109-004	hard clam	Pine Island Sound	11/9/2009	<20	0.06
HABB100105-002	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-003	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-004	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100105-005	hard clam	Pine Island Sound	1/4/2010	<20	<LOD
HABB100112-001	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100112-002	hard clam	Pine Island Sound	1/11/2010	<20	<LOD
HABB100118-001	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-002	hard clam	Pine Island Sound	1/18/2010	<20	0.06
HABB100118-003	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB100118-004	hard clam	Pine Island Sound	1/18/2010	<20	<LOD
HABB111011-001	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-002	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-003	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111011-004	hard clam	Pine Island Sound	10/11/2011	<20	<LOD
HABB111018-001	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-002	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-003	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111018-004	hard clam	Pine Island Sound	10/17/2011	<20	<LOD
HABB111024-001	hard clam	Pine Island Sound	10/23/2011	<20	<LOD
HABB111122-001	hard clam	Gasparilla Sound	11/21/2011	<20	4.13
HABB111206-001	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111206-002	hard clam	Pine Island Sound	12/5/2011	<20	<LOD
HABB111213-003	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB111213-004	hard clam	Pine Island Sound	12/12/2011	<20	<LOD
HABB120104-001	hard clam	Pine Island Sound	1/4/2012	<20	0.63
HABB120104-002	hard clam	Pine Island Sound	1/4/2012	<20	0.66
HABB120109-001	hard clam	Pine Island Sound	1/9/2012	<20	0.63
HABB120109-002	hard clam	Pine Island Sound	1/9/2012	<20	0.48
HABB120117-001	hard clam	Pine Island Sound	1/16/2012	<20	0.24
HABB120117-002	hard clam	Pine Island Sound	1/16/2012	<20	0.23
HABB120124-001	hard clam	Pine Island Sound	1/23/2012	<20	0.14
HABB120124-002	hard clam	Pine Island Sound	1/23/2012	<20	0.13
HABB120131-003	hard clam	Ten Thousand Islands	1/25/2012	<20	1.39
HABB120131-004	hard clam	Ten Thousand Islands	1/25/2012	<20	1.49
HABB121002-001	hard clam	Gasparilla Sound	10/1/2012	37.63	12.68
HABB121002-002	hard clam	Gasparilla Sound	10/1/2012	<20	0.25
HABB121003-001	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121003-002	hard clam	Pine Island Sound	10/3/2012	<20	<LOD
HABB121009-001	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-002	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-003	hard clam	Pine Island Sound	10/8/2012	<20	<LOD

HABB121009-004	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-005	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121009-006	hard clam	Pine Island Sound	10/8/2012	<20	<LOD
HABB121016-001	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-002	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-003	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-004	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-005	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121016-006	hard clam	Pine Island Sound	10/15/2012	<20	<LOD
HABB121017-001	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121017-002	hard clam	Pine Island Sound	10/16/2012	<20	<LOD
HABB121023-005	hard clam	Pine Island Sound	10/22/2012	<20	0.28
HABB121023-006	hard clam	Pine Island Sound	10/22/2012	<20	0.26
HABB121023-007	hard clam	Pine Island Sound	10/22/2012	<20	0.18
HABB121023-008	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-009	hard clam	Pine Island Sound	10/22/2012	<20	0.17
HABB121023-010	hard clam	Pine Island Sound	10/22/2012	<20	0.22
HABB121024-001	hard clam	Lower Tampa Bay	10/23/2012	<20	0.92
HABB121024-002	hard clam	Lower Tampa Bay	10/23/2012	<20	1.05
HABB121024-003	hard clam	Lower Tampa Bay	10/23/2012	<20	0.7
HABB121024-004	hard clam	Lower Tampa Bay	10/23/2012	<20	0.66
Habb121024-005	hard clam	Pine Island Sound	10/23/2012	<20	0.18
HABB121024-006	hard clam	Pine Island Sound	10/23/2012	<20	0.23
HABB121030-001	hard clam	Lower Tampa Bay	10/29/2012	<20	0.5
HABB121030-002	hard clam	Lower Tampa Bay	10/29/2012	<20	0.34
HABB121030-003	hard clam	Pine Island Sound	10/29/2012	<20	1.2
HABB121030-004	hard clam	Pine Island Sound	10/29/2012	<20	0.88
HABB121113-001	hard clam	Lower Tampa Bay	11/6/2012	<20	1.78
HABB130212-003	hard clam	Lower Tampa Bay	11/14/2012	<20	<LOD
HABB121120-001	hard clam	Pine Island Sound	11/19/2012	<20	2.16
HABB121127-001	hard clam	Sarasota Bay	11/26/2012	<20	0.7
HABB121127-002	hard clam	Pine Island Sound	11/26/2012	<20	0.88
HABB121127-003	hard clam	Pine Island Sound	11/26/2012	<20	2.01
HABB121127-004	hard clam	Pine Island Sound	11/26/2012	<20	1.82
HABB121211-001	hard clam	Pine Island Sound	12/10/2012	<20	0.63
HABB121211-002	hard clam	Pine Island Sound	12/10/2012	<20	0.52
HABB121211-003	hard clam	Pine Island Sound	12/10/2012	<20	1.01
HABB121211-004	hard clam	Pine Island Sound	12/10/2012	<20	1.31
HABB121218-001	hard clam	Pine Island Sound	12/17/2012	<20	1.19
HABB121218-002	hard clam	Pine Island Sound	12/17/2012	<20	5.6
HABB121218-003	hard clam	Pine Island Sound	12/17/2012	<20	0.86
HABB121218-004	hard clam	Pine Island Sound	12/17/2012	<20	0.99
HABB121218-005	hard clam	Pine Island Sound	12/17/2012	<20	0.58
HABB121218-006	hard clam	Pine Island Sound	12/17/2012	<20	0.5
HABB121218-007	hard clam	Lower Tampa Bay	12/18/2012	<20	2.01
HABB121218-008	hard clam	Lower Tampa Bay	12/18/2012	<20	2.34
HABB121227-026	hard clam	Lower Tampa Bay	12/26/2012	23.59	3
HABB121227-027	hard clam	Lower Tampa Bay	12/26/2012	22.19	2.34
HABB121227-028	hard clam	Pine Island Sound	12/26/2012	<20	0.45
HABB121227-029	hard clam	Pine Island Sound	12/26/2012	<20	0.44
HABB130103-001	hard clam	Pine Island Sound	1/2/2013	<20	0.74
HABB130103-002	hard clam	Pine Island Sound	1/2/2013	<20	0.82

HABB130103-003	hard clam	Pine Island Sound	1/2/2013	22.09	2.18
HABB130103-004	hard clam	Pine Island Sound	1/2/2013	21.64	2.45
HABB130103-005	hard clam	Pine Island Sound	1/2/2013	<20	0.66
HABB130103-006	hard clam	Pine Island Sound	1/2/2013	<20	0.87
HABB130108-001	hard clam	Pine Island Sound	1/7/2013	<20	0.72
HABB130108-002	hard clam	Pine Island Sound	1/7/2013	<20	0.85
HABB130108-003	hard clam	Pine Island Sound	1/7/2013	<20	1.09
HABB130108-004	hard clam	Pine Island Sound	1/7/2013	<20	0.83
HABB130109-001	hard clam	Lower Tampa Bay	1/8/2013	20.2	4.38
HABB130109-002	hard clam	Lower Tampa Bay	1/8/2013	<20	1.96
HABB130109-003	hard clam	Lower Tampa Bay	1/8/2013	<20	1.51
HABB130115-003	hard clam	Pine Island Sound	1/14/2013	<20	1.07
HABB130115-004	hard clam	Pine Island Sound	1/14/2013	<20	1.74
HABB130122-001	hard clam	Lower Tampa Bay	1/22/2013	<20	1.57
HABB130122-002	hard clam	Lower Tampa Bay	1/22/2013	<20	1.54
HABB130130-001	hard clam	Lower Tampa Bay	1/28/2013	<20	1.8
HABB130130-002	hard clam	Lower Tampa Bay	1/28/2013	<20	1.82
HABB130205-001	hard clam	Lower Tampa Bay	2/4/2013	<20	1.41
HABB130205-002	hard clam	Lower Tampa Bay	2/4/2013	<20	1.44
HABB130212-001	hard clam	Pine Island Sound	2/11/2013	21.01	4.16
HABB130212-005	hard clam	Pine Island Sound	2/11/2013	29.23	5.68
HABB130226-002	hard clam	Pine Island Sound	2/24/2013	49.23	8.44
HABB130226-003	hard clam	Pine Island Sound	2/24/2013	44.71	8.37
HABB130226-004	hard clam	Pine Island Sound	2/24/2013	84.59	16.18
HABB130226-005	hard clam	Pine Island Sound	2/24/2013	39.34	9.89
HABB130226-006	hard clam	Pine Island Sound	2/24/2013	38.23	4.83
HABB130226-007	hard clam	Pine Island Sound	2/24/2013	27.18	4.82
HABB130226-008	hard clam	Pine Island Sound	2/24/2013	68.19	7.04
HABB130226-009	hard clam	Pine Island Sound	2/24/2013	<20	2.55
HABB130226-010	hard clam	Pine Island Sound	2/24/2013	44.16	6.33
HABB151007-002	hard clam	Pine Island Sound	2/25/2013	92.65	9.84
HABB130306-005	hard clam	Pine Island Sound	3/4/2013	<20	4.57
HABB130319-006	hard clam	Pine Island Sound	3/8/2013	<20	2.81
HABB130312-004	hard clam	Pine Island Sound	3/11/2013	205.34	37.33
HABB130312-005	hard clam	Pine Island Sound	3/11/2013	24.95	3.87
HABB130312-006	hard clam	Pine Island Sound	3/11/2013	<20	2.51
HABB130312-007	hard clam	Pine Island Sound	3/11/2013	<20	2.39
HABB130313-007	hard clam	Pine Island Sound	3/11/2013	36.89	3.26
HABB130313-008	hard clam	Pine Island Sound	3/11/2013	<20	1.73
HABB130313-001	hard clam	Pine Island Sound	3/12/2013	<20	1.93
HABB130313-002	hard clam	Pine Island Sound	3/12/2013	<20	2.46
HABB130313-003	hard clam	Pine Island Sound	3/12/2013	<20	2.47
HABB130313-004	hard clam	Pine Island Sound	3/12/2013	<20	2.35
HABB130319-007	hard clam	Pine Island Sound	3/13/2013	<20	2.24
HABB130319-004	hard clam	Pine Island Sound	3/18/2013	<20	2.14
HABB130319-005	hard clam	Pine Island Sound	3/18/2013	<20	4.2
HABB130319-012	hard clam	Pine Island Sound	3/18/2013	22.55	2.79
HABB140725-001	hard clam	Ten Thousand Islands	3/20/2013	<20	3.89
HABB130326-003	hard clam	Pine Island Sound	3/25/2013	<20	1.58
HABB130326-004	hard clam	Pine Island Sound	3/25/2013	<20	1.39
HABB130326-005	hard clam	Pine Island Sound	3/25/2013	<20	1.71
HABB130326-006	hard clam	Pine Island Sound	3/25/2013	<20	1.65

HABB130326-009	hard clam	Pine Island Sound	3/25/2013	<20	1.57
HABB130326-010	hard clam	Pine Island Sound	3/25/2013	<20	1.62
HABB130326-011	hard clam	Pine Island Sound	3/25/2013	<20	1.47
HABB130326-012	hard clam	Pine Island Sound	3/25/2013	<20	1.42
HABB130326-013	hard clam	Gasparilla Sound	3/25/2013	84.16	16.89
HABB130326-014	hard clam	Pine Island Sound	3/25/2013	75.9	16.4
HABB130403-002	hard clam	Pine Island Sound	3/29/2013	<20	2.23
HABB130402-001	hard clam	Pine Island Sound	4/1/2013	<20	2.05
HABB130402-002	hard clam	Pine Island Sound	4/1/2013	<20	1.98
HABB130402-003	hard clam	Pine Island Sound	4/1/2013	25.2	3.5
HABB130402-004	hard clam	Pine Island Sound	4/1/2013	24.3	2.92
HABB130402-005	hard clam	Pine Island Sound	4/1/2013	<20	1.4
HABB130402-006	hard clam	Pine Island Sound	4/1/2013	<20	1.27
HABB130402-007	hard clam	Pine Island Sound	4/1/2013	<20	1.55
HABB130402-008	hard clam	Pine Island Sound	4/1/2013	<20	3.51
HABB130402-009	hard clam	Pine Island Sound	4/1/2013	<20	3.27
HABB130409-003	hard clam	Pine Island Sound	4/8/2013	<20	0.97
HABB130409-004	hard clam	Pine Island Sound	4/8/2013	<20	1.17
HABB130409-008	hard clam	Pine Island Sound	4/8/2013	<20	1.81
HABB130409-009	hard clam	Pine Island Sound	4/8/2013	<20	1.09
HABB130409-010	hard clam	Pine Island Sound	4/8/2013	<20	0.85
HABB130409-011	hard clam	Pine Island Sound	4/8/2013	<20	3.82
HABB130409-012	hard clam	Pine Island Sound	4/8/2013	<20	4.12
HABB130409-013	hard clam	Pine Island Sound	4/8/2013	<20	3.81
HABB130409-014	hard clam	Pine Island Sound	4/8/2013	35.6	4.29
HABB130409-015	hard clam	Pine Island Sound	4/8/2013	<20	1.69
HABB130409-016	hard clam	Pine Island Sound	4/8/2013	<20	1.52
HABB130410-001	hard clam	Pine Island Sound	4/9/2013	<20	1.82
HABB130410-002	hard clam	Pine Island Sound	4/9/2013	<20	1.91
HABB130410-003	hard clam	Pine Island Sound	4/9/2013	<20	1.69
HABB130416-006	hard clam	Pine Island Sound	4/15/2013	<20	0.83
HABB130416-007	hard clam	Pine Island Sound	4/15/2013	<20	0.81
HABB130417-001	hard clam	Pine Island Sound	4/16/2013	<20	1.09
HABB130417-002	hard clam	Pine Island Sound	4/16/2013	<20	1.24
HABB130417-004	hard clam	Pine Island Sound	4/16/2013	<20	1.37
HABB130417-005	hard clam	Pine Island Sound	4/16/2013	<20	1.28
HABB130423-001	hard clam	Pine Island Sound	4/22/2013	<20	1.02
HABB130423-002	hard clam	Pine Island Sound	4/22/2013	<20	1.06
HABB130423-003	hard clam	Pine Island Sound	4/22/2013	<20	0.98
HABB130424-001	hard clam	Pine Island Sound	4/24/2013	<20	0.93
HABB130424-002	hard clam	Pine Island Sound	4/24/2013	<20	1
HABB130424-003	hard clam	Pine Island Sound	4/24/2013	<20	0.86
HABB130508-004	hard clam	Lemon Bay	5/7/2013	<20	17.33
HABB131113-001	hard clam	Pine Island Sound	11/12/2013	<20	0.5
HABB131113-002	hard clam	Pine Island Sound	11/12/2013	<20	0.32
HABB131113-003	hard clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-006	hard clam	Pine Island Sound	11/12/2013	<20	0.41
HABB131113-007	hard clam	Pine Island Sound	11/12/2013	<20	0.38
HABB131113-008	hard clam	Pine Island Sound	11/12/2013	<20	0.44
HABB131119-001	hard clam	Pine Island Sound	11/18/2013	<20	1.96
HABB131119-002	hard clam	Pine Island Sound	11/18/2013	<20	1.71
HABB131119-003	hard clam	Pine Island Sound	11/18/2013	<20	1.78

HABB131126-001	hard clam	Pine Island Sound	11/25/2013	<20	0.3
HABB131126-002	hard clam	Pine Island Sound	11/25/2013	<20	0.29
HABB131126-003	hard clam	Pine Island Sound	11/25/2013	<20	0.28
HABB131126-004	hard clam	Pine Island Sound	11/25/2013	<20	0.59
HABB131126-005	hard clam	Pine Island Sound	11/25/2013	<20	0.69
HABB131126-006	hard clam	Pine Island Sound	11/25/2013	<20	0.68
HABB131203-001	hard clam	Pine Island Sound	12/2/2013	<20	0.23
HABB131203-002	hard clam	Pine Island Sound	12/2/2013	<20	0.24
HABB131203-003	hard clam	Pine Island Sound	12/2/2013	<20	0.21
HABB131203-004	hard clam	Pine Island Sound	12/2/2013	<20	0.31
HABB131203-005	hard clam	Pine Island Sound	12/2/2013	<20	0.33
HABB131203-006	hard clam	Pine Island Sound	12/2/2013	<20	0.38
HABB131210-004	hard clam	Pine Island Sound	12/9/2013	<20	0.35
HABB131210-005	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131210-006	hard clam	Pine Island Sound	12/9/2013	<20	0.33
HABB131211-012	hard clam	Gasparilla Sound	12/10/2013	<20	0.84
HABB131218-010	hard clam	Gasparilla Sound	12/17/2013	36.91	8.96
HABB141014-001	hard clam	Cedar Key	10/13/2014	<20	0.33
HABB141014-002	hard clam	Cedar Key	10/13/2014	<20	0.31
HABB141014-003	hard clam	Cedar Key	10/13/2014	<20	0.42
HABB141113-002	hard clam	Pine Island Sound	11/12/2014	<20	0.34
HABB141113-003	hard clam	Pine Island Sound	11/12/2014	<20	0.44
HABB141113-004	hard clam	Pine Island Sound	11/12/2014	<20	0.69
HABB141113-005	hard clam	Pine Island Sound	11/12/2014	<20	0.7
HABB141113-006	hard clam	Pine Island Sound	11/12/2014	<20	0.66
HABB141113-007	hard clam	Pine Island Sound	11/12/2014	<20	0.62
HABB141119-001	hard clam	Pine Island Sound	11/18/2014	<20	0.15
HABB141119-002	hard clam	Pine Island Sound	11/18/2014	<20	0.13
HABB141119-003	hard clam	Pine Island Sound	11/18/2014	<20	0.2
HABB141119-004	hard clam	Pine Island Sound	11/18/2014	<20	0.18
HABB141119-005	hard clam	Pine Island Sound	11/18/2014	<20	0.23
HABB141119-006	hard clam	Pine Island Sound	11/18/2014	<20	0.25
HABB141124-001	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-002	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB141124-003	hard clam	Pine Island Sound	11/23/2014	<20	0.14
HABB160202-002	hard clam	Pine Island Sound	2/1/2016	<20	0.92
HABB160209-017	hard clam	Gasparilla Sound	2/8/2016	76.77	10.82
HABB160209-018	hard clam	Gasparilla Sound	2/8/2016	42.61	9.68
HABB160209-019	hard clam	Gasparilla Sound	2/8/2016	85.99	10
HABB160223-003	hard clam	Pine Island Sound	2/22/2016	<20	0.44
HABB160301-002	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-003	hard clam	Pine Island Sound	2/29/2016	<20	0.4
HABB160301-004	hard clam	Pine Island Sound	2/29/2016	<20	0.33
HABB160301-005	hard clam	Pine Island Sound	2/29/2016	<20	0.37
HABB160302-002	hard clam	Pine Island Sound	3/1/2016	<20	0.6
HABB160302-003	hard clam	Pine Island Sound	3/1/2016	<20	0.65
HABB160308-002	hard clam	Lower Tampa Bay	3/7/2016	40.05	6.21
HABB160322-002	hard clam	Lower Tampa Bay	3/22/2016	25	5.12
HABB160328-001	hard clam	Lower Tampa Bay	3/28/2016	35.83	4.9
HABB160407-001	hard clam	Lower Tampa Bay	4/6/2016	29.59	4.36
HABB160407-003	hard clam	Pine Island Sound	4/7/2016	<20	0.5
HABB160411-012	hard clam	Lower Tampa Bay	4/11/2016	<20	1.36

HABB160418-001	hard clam	Lower Tampa Bay	4/18/2016	<20	1.76
HABB160601-001	hard clam	Lemon Bay	5/31/2016	<20	0.43
HABB161011-001	hard clam	Lower Tampa Bay	10/10/2016	<20	1.16
HABB161013-001	hard clam	Gasparilla Sound	10/12/2016	<20	0.54
HABB161018-001	hard clam	Lower Tampa Bay	10/17/2016	<20	2.07
HABB170104-001	Hard clam	Pine Island Sound	1/3/2017	<20	1.66
HABB170104-002	Hard clam	Pine Island Sound	1/3/2017	<20	1
HABB170105-002	Hard clam	Lower Tampa Bay	1/4/2017	35.96	2.22
HABB170110-002	hard clam	Lower Tampa Bay	1/9/2017	<20	1.58
HABB170110-003	hard clam	Gasparilla Sound	1/9/2017	20.26	2.35
HABB131125-020	hard clam	Composite		<20	3.9
HABB130115-001	sunray venus clam	Pine Island Sound	1/14/2013	<20	1.85
HABB130212-002	sunray venus clam	Pine Island Sound	2/11/2013	34.13	12.04
HABB130212-005	sunray venus clam	Pine Island Sound	2/11/2013	39.09	19.74
HABB130226-001	sunray venus clam	Pine Island Sound	2/24/2013	42.41	15.41
HABB130226-011	sunray venus clam	Pine Island Sound	2/24/2013	<20	5.58
HABB130228-001	sunray venus clam	Pine Island Sound	2/25/2013	32.17	9.93
HABB130227-001	sunray venus clam	Pine Island Sound	2/26/2013	42.9	13.01
HABB130227-002	sunray venus clam	Pine Island Sound	2/26/2013	34.97	19.09
HABB130228-003	sunray venus clam	Pine Island Sound	2/27/2013	27.54	17.94
HABB130319-009	sunray venus clam	Pine Island Sound	3/8/2013	<20	3.13
HABB130312-001	sunray venus clam	Pine Island Sound	3/11/2013	27.65	6.59
HABB130312-002	sunray venus clam	Pine Island Sound	3/11/2013	26.33	7.39
HABB130312-003	sunray venus clam	Pine Island Sound	3/11/2013	28.7	5.16
HABB130312-009	sunray venus clam	Pine Island Sound	3/11/2013	<20	5.38
HABB150921-001	sunray venus clam	Pine Island Sound	3/11/2013	31.33	5.3
HABB130319-010	sunray venus clam	Pine Island Sound	3/13/2013	<20	3.1
HABB130319-001	sunray venus clam	Pine Island Sound	3/18/2013	22.05	4.48
HABB130319-002	sunray venus clam	Pine Island Sound	3/18/2013	20.67	4.28
HABB130319-003	sunray venus clam	Pine Island Sound	3/18/2013	27.85	7.69
HABB130319-011	sunray venus clam	Pine Island Sound	3/18/2013	25.87	5.43
HABB130326-001	sunray venus clam	Pine Island Sound	3/25/2013	23.16	3.48
HABB130326-002	sunray venus clam	Pine Island Sound	3/25/2013	22.36	3.4
HABB130326-007	sunray venus clam	Pine Island Sound	3/25/2013	24.4	4.44
HABB130326-008	sunray venus clam	Pine Island Sound	3/25/2013	22.5	3.35
HABB130409-006	sunray venus clam	Pine Island Sound	4/8/2013	22.84	2.53
HABB130409-020	sunray venus clam	Pine Island Sound	4/8/2013	<20	2.16
HABB130409-021	sunray venus clam	Pine Island Sound	4/8/2013	23.91	2.69
HABB130410-004	sunray venus clam	Pine Island Sound	4/9/2013	<20	2.18
HABB130410-005	sunray venus clam	Pine Island Sound	4/9/2013	<20	1.84
HABB130416-002	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.47
HABB130416-003	sunray venus clam	Pine Island Sound	4/15/2013	<20	0.99
HABB130416-004	sunray venus clam	Pine Island Sound	4/15/2013	<20	1.48
HABB130417-006	sunray venus clam	Pine Island Sound	4/16/2013	<20	1.62
HABB130604-003	sunray venus clam	Pine Island Sound	6/3/2013	<20	0.56
HABB131113-004	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.26
HABB131113-005	sunray venus clam	Pine Island Sound	11/12/2013	<20	0.24
HABB131125-019	sunray venus clam	Alligator Harbor	11/22/2013	25.88	4.11
HABB151120-001	sunray venus clam	Sarasota Bay	11/18/2015	33.21	11.05
HABB151120-002	sunray venus clam	Sarasota Bay	11/18/2015	33.58	12.11
HABB151207-001	sunray venus clam	Sarasota Bay	12/7/2015	53.21	14.47
HABB160111-002	Sunray venus clam	Lower Tampa Bay	12/15/2015	33.34	6.37

HABB160111-001	sunray venus clam	Sarasota Bay	1/6/2016	<20	2.77
HABB160202-003	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160202-004	sunray venus clam	Pine Island Sound	2/1/2016	<20	2.74
HABB160202-005	sunray venus clam	Pine Island Sound	2/1/2016	19.77	2.14
HABB160202-006	sunray venus clam	Pine Island Sound	2/1/2016	<20	1.62
HABB160223-004	sunray venus clam	Pine Island Sound	2/22/2016	27.66	2.16
HABB160330-004	sunray venus clam	Lower Tampa Bay	3/16/2016	36.48	3.38
HABB160330-005	sunray venus clam	Lower Tampa Bay	3/16/2016	33.04	3.41
HABB161213-019	sunray venus clam	MML lab exposure	9/6/2016	<20	2.63
HABB161213-021	sunray venus clam	MML lab exposure	12/7/2016	20.66	4.04
HABB161213-022	sunray venus clam	MML exp control	12/7/2016	<20	<LOD

Data handling to compare the new or modified method to the officially recognized

Two methods of analysis are considered to be comparable when no significant difference can be demonstrated in their results. To determine whether comparability in methods exists, a two-sided t-test at a significance level (α) of .05 will be used to test the data. Either a paired t-test or Welch's t-test will be used depending upon the shape of the distributions produced by the data for each method and their respective variances. Use log transformed data for the results obtained from microbiological methods. The appropriate t-test to be used for the analysis is determined in the following manner.

1. Test the symmetry for the distribution of results from both the officially recognized analytical method and the proposed alternative analytical method.
2. Calculate the variance of the data for both the officially recognized analytical method and the proposed alternative analytical method.
3. Values for the test of symmetry for either method outside the range of -2 to +2 indicate a significant degree of skewness in the distribution.
4. A ratio of the larger of the variances of either method to the smaller of the variances of either method >2 indicates a lack of homogeneity of variance.
5. Use either the paired t-test or Welch's t-test for the analysis of the data based on the following considerations.
    - If the distribution of the data from the officially recognized analytical method and the proposed alternative analytical method are symmetric (within the range of -2 to +2) and there is homogeneity of variance use a paired t-test for the data analysis.
    - If the distributions of the data for both analytical methods are symmetric (within the range -2 to +2) but there is a lack of homogeneity of variance in the data, use Welch's t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and proposed alternative analytical methods are skewed (outside the range -2 to +2) and the skewness for both methods is either positive for both or negative for both and there is homogeneity of variance in the data, use the paired t-test for the analysis of the data.
    - If the distributions of the data from the officially recognized and the proposed alternative analytical methods are skewed and the skewness for both analytical methods is either positive or negative for both but the data lacks homogeneity of variance, use Welch's t-test to analyze the data.

Data summary for the comparison of the new or modified method to the officially recognized method:

Value for the test of symmetry for the distribution of the data generated by the officially recognized method

Value for the test of symmetry for the distribution of the data generated by the proposed alternative method

Variance of the data generated from the officially recognized analytical method

Variance of the data generated from the proposed alternative analytical method

Ratio of the larger to the smaller of the variances generated by the officially recognized and proposed analytical methods _____

Is there a significant difference between the analytical methods Y/N

Comparative data for NSP mouse bioassays and ELISAs cannot be evaluated as described above. Please see below for additional discussion and comparisons permitted by the data presented above.

Brevetoxins in bivalves

At least nine brevetoxin congeners have been isolated from *K. brevis*[1]. PbTx-1 and PbTx-2 are presumed to be the parent toxins from which all other brevetoxins are derived via substitutions on the terminal ring. Consequently, brevetoxins are grouped into two types according to their backbone structure. Brevetoxin A-type (PbTx-1-type) toxins possess a 10-ring backbone, and brevetoxin B-type (PbTx-2-type) toxins possess an 11-ring backbone (Fig. G1). Although brevetoxin A-type toxins are more potent, the brevetoxin B-type toxins are much more abundant[2]. Polar derivatives identified in both culture and bloom materials have further increased the number of known brevetoxin structures[3,4].

In bivalves, the more reactive forms of brevetoxin are rapidly transformed into brevetoxin metabolites[3,5] that are generally the products of reduction, oxidation, and conjugation to other molecules including taurine, cysteine, cysteine sulfoxide, amino acids and fatty acids[5-7]. Literally dozens of metabolites have been identified in shellfish. Most modifications to brevetoxins occur at the side chain on the terminal ether ring that differentiates the brevetoxin congeners, resulting in an assortment of conjugates with either an A-type or B-type of backbone. Brevetoxin metabolites are known to contribute to NSP toxicity [3,6-8], but their individual potency varies. Toxicity information is available for only a small subset of the dozens of characterized metabolites. Some common shellfish metabolites are less potent than parent brevetoxins, while a few have demonstrated higher toxicities [7,9,10]. Different rates of tissue uptake and elimination of brevetoxin metabolites have also been described and may factor into their variable potencies[11].

The complexity of brevetoxins and their metabolic products is the primary reasons that so little progress has been made on moving away from the NSP mouse bioassay. Of the many chemical and biological methods evaluated for measuring brevetoxins in bivalves, those that recognize molecular structure (i.e., ELISAs and liquid chromatography-mass spectroscopy [LC-MS]) have outperformed activity-based assays (i.e., receptor-binding and cytotoxicity assay), demonstrating less variability and better agreement with mouse bioassays[7,12,13].

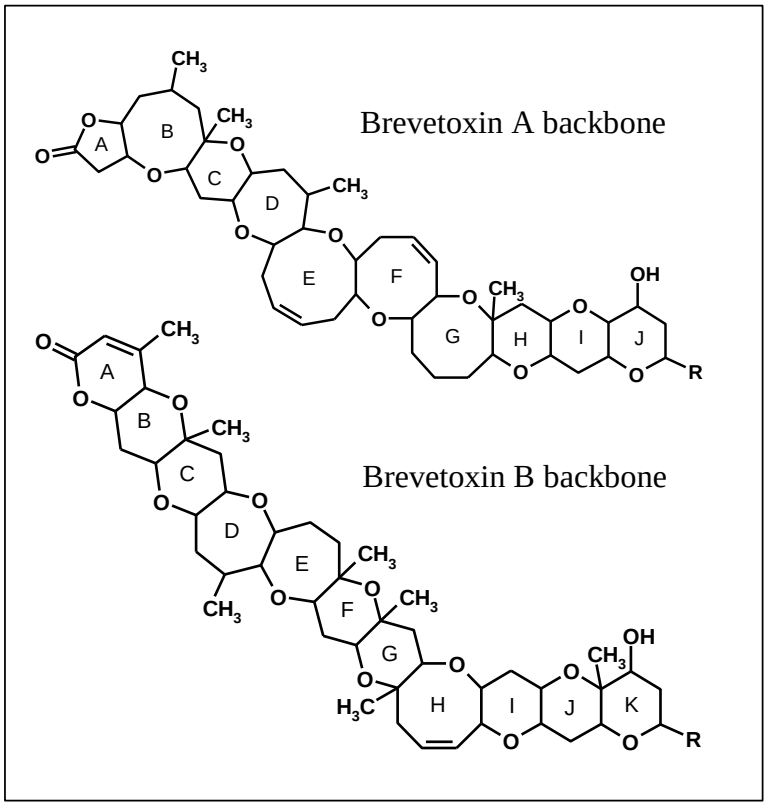


Figure G1. Brevetoxin backbone structures.

An LC-MS method has been developed by the FDA Gulf Coast Seafood Lab and will be submitted to the ISSC for consideration as an alternative to the mouse bioassay. LC-MS can provide confirmation of toxins detected by other assays, and sample throughput is higher compared to the mouse bioassay. However, the large number of brevetoxin metabolites in bivalves will necessitate a targeted approach. For routine analysis as a part of monitoring and management, it is not practical to attempt to identify and quantify them all. Nor is this even possible, given the lack of available standards for almost all metabolites. In the Gulf of Mexico, the most important commercial species are eastern oysters (*Crassostrea virginica*) and hard clams (*Mercenaria mercenaria*). In oysters, the brevetoxin profile is dominated by the cysteine metabolites S-desoxy-BTX-B2 and BTX-B2[3,5,12]. These were also the major metabolites identified in hard clams, along

with BTX-B1, a taurine conjugate[14,15]. Sunray venus clams (*Macrocallista nimbosa*), a relatively new aquaculture product gaining popularity in Florida, have been less well-studied, but analyses thus far indicate that this species metabolizes brevetoxins similarly to hard clams (Fig. G2), with the cysteine and taurine conjugates representing the major metabolites (Fig. G3).

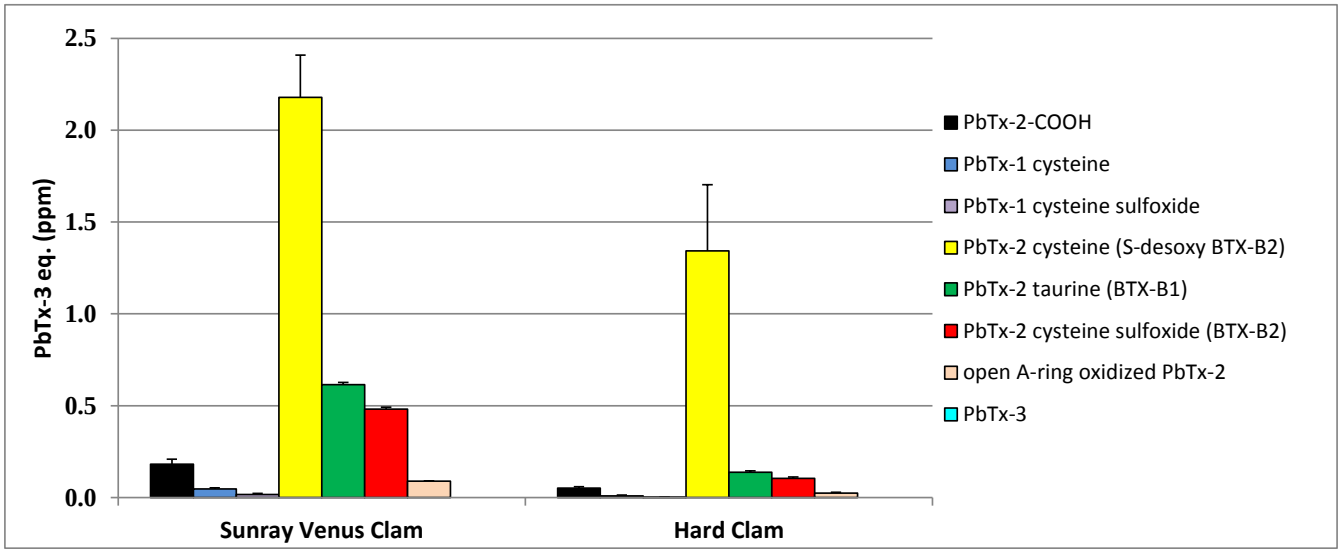


Figure G2. Brevetoxin metabolites identified by LC-MS in laboratory-exposed sunray venus and hard clams. (Error bars=standard deviation, n=3. Unpublished data provided by Dr. R. Pierce, Mote Marine Laboratory.)

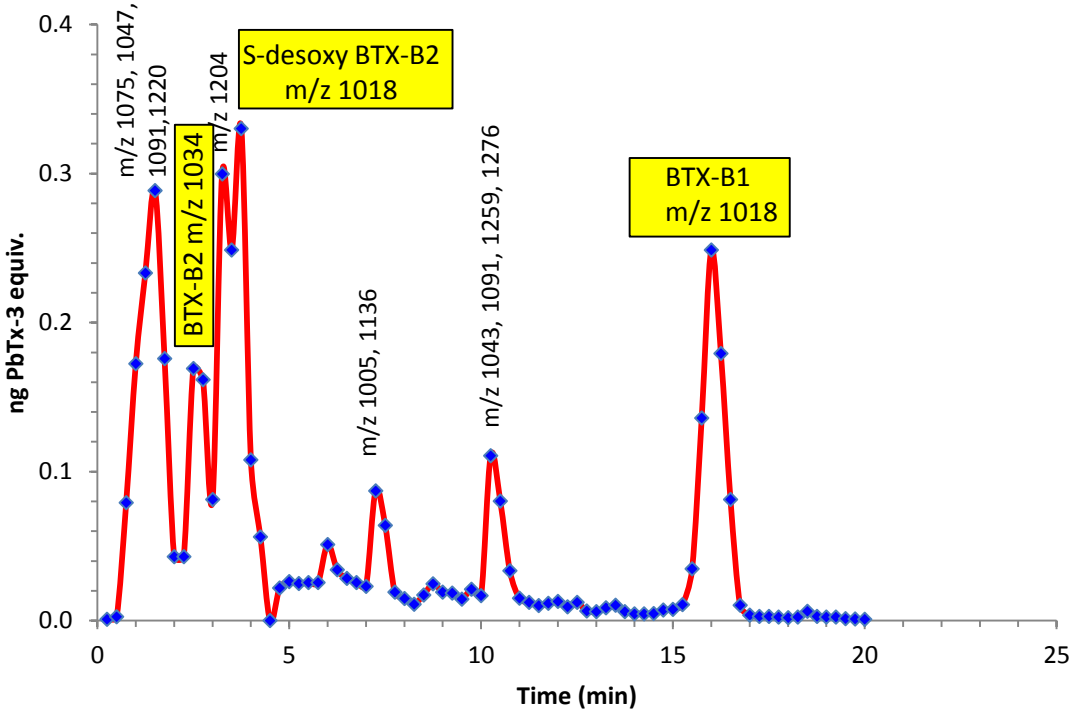


Figure G3. Chromatograms of brevetoxin metabolites in sunray venus clams based on ELISA of LC-fractionated shellfish extracts. (Unpublished data provided by Dr. A. Abraham, USFDA.)

Oral toxicity to mammals has not been assessed for any of the brevetoxin conjugates. Nevertheless, the cysteine and taurine metabolites were found to be excellent biomarkers of composite B-type brevetoxins as determined by ELISA for these species[12,14]. Based on these studies, the FDA's LC-MS protocol targets these three metabolites as biomarkers for NSP toxicity in oysters and clams.

LC-MS analyses require expensive instrumentation and highly technical expertise and are further limited by the time required for each sample to run. Where high throughput is required, the speed and cost-effectiveness of ELISA makes it a more attractive screening method.

MARBIONC Brevetoxin Competitive ELISA

The MARBIONC ELISA kit used in this method validation is the same kit that was used in the method comparisons and bivalve studies cited above. The method is based on the activity of anti-brevetoxin goat polyclonal antibodies, which were produced using a PbTx-3-KLH (keyhole limpet hemocyanin) conjugate[16]. The recognition epitope is believed to include the last four rings (excluding the side chain) of the brevetoxin B type toxins[17,18] (Fig. G4). This specific region is maintained in all brevetoxin B type toxins including in the secondary metabolites identified thus far. However, cross-reactivity of these antibodies have only been assessed for a few metabolites.

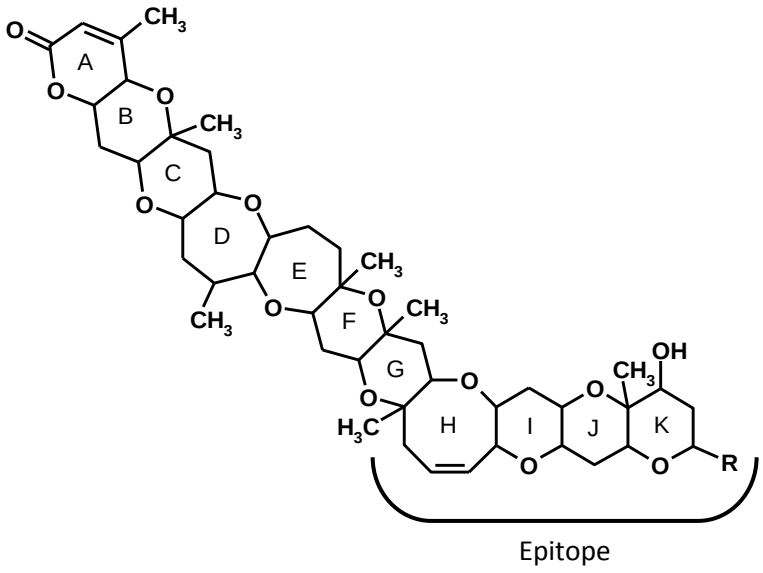


Figure G4. Brevetoxin B backbone with recognition epitope of anti-brevetoxin goat polyclonal antibodies

When this ELISA method was originally published, similar cross-reactivities were reported for PbTx-2, PbTx-3 and PbTx-9, which all share the B-type backbone[18] (Fig G5). MARBIONC reports cross-reactivities of 100% for PbTx-3, 97% for PbTx-2, 105% for oxidized-PbTx-2, and 7% for PbTx-1 at 10 ng/mL (Fig. G6).

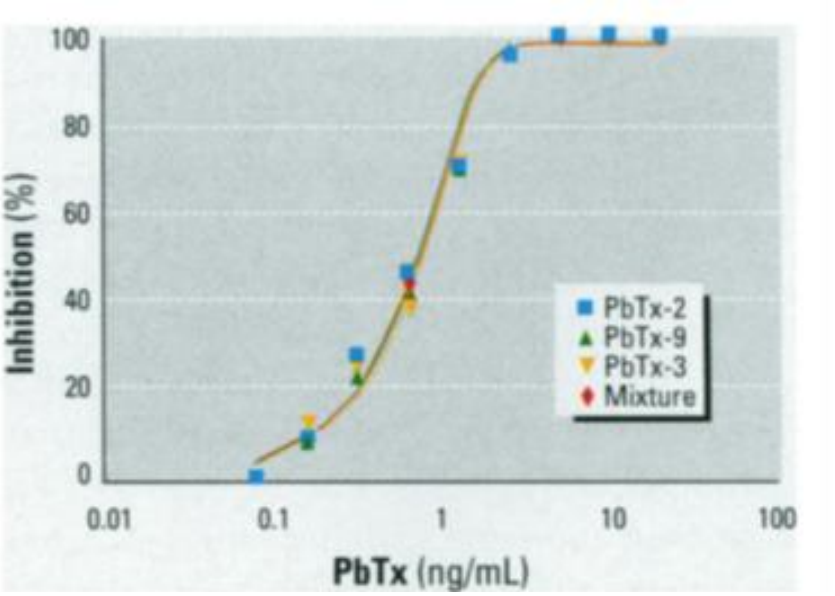


Figure G5. Figure taken from Naar et al.[18]. Anti-brevetoxin antibody cross-reaction with PbTx-2, PbTx-3, PbTx-9, and a mixture of the three toxins.

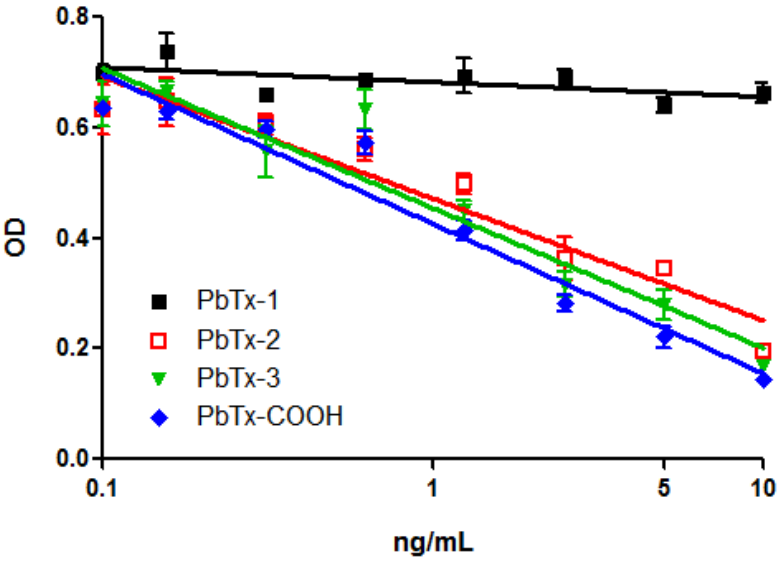


Figure G6. Figure provided by MARBIONC demonstrating degrees of anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2, PbTx-3, and oxidized-PbTx-2 (PbTx-COOH).

Competitive curves generated by L. Flewelling (FWC) are consistent with this, with calculated cross-reactivities (at 50% inhibition) of 97% for PbTx-2 and 2.4% for PbTx-1, relative to PbTx-3 (100%) (Fig. G7).

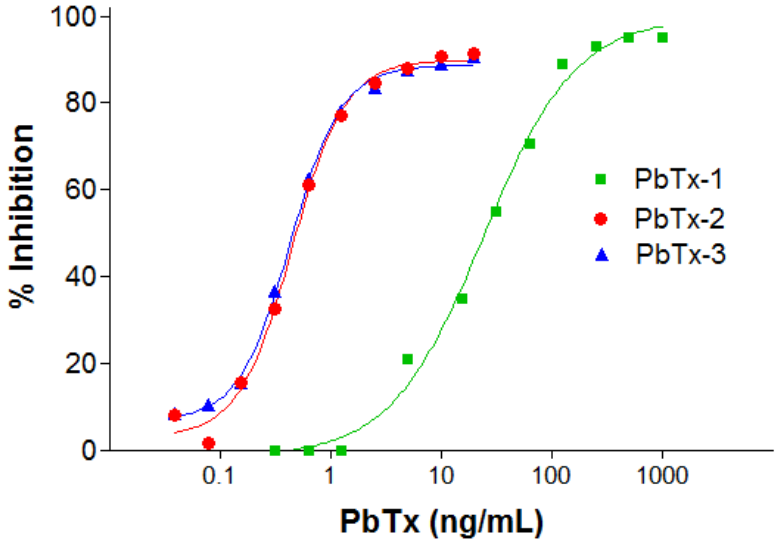


Figure G7. Anti-brevetoxin antibody cross-reaction with PbTx-1 (A-type) and B-type toxins PbTx-2 and PbTx-3 (FWC data).

We also assessed the cross-reactivity of two shellfish metabolites (Fig. G8). The cross-reactivity of the cysteine conjugate S-desoxy BTX-B2 (provided by the FDA Gulf Coast Seafood Laboratory) was found to be 133% relative to PbTx-3. Cross reactivity of the brevetoxin lipid conjugate N-palmitoyl BTX-B2 (or BTX-B4, described in Bottein et al.[19] and provided by NOAA Center for Coastal Environmental Health and Biomolecular Research) was much lower (2.5%).

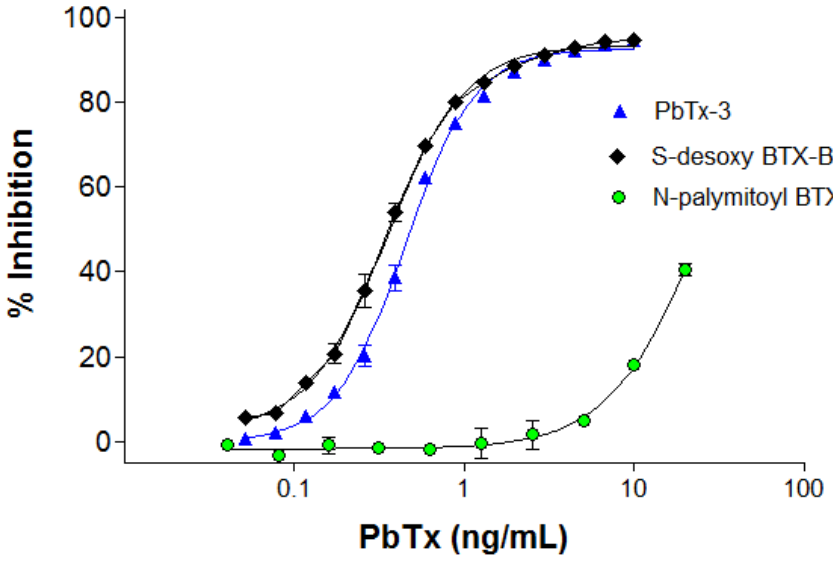


Figure G8. Anti-brevetoxin antibody cross-reaction with B-type brevetoxin metabolites S-desoxy BTX-B2 and N-palymitoyl BTX-B2 compared to PbTx-3 (FWC data).

The relatively low cross-reactivity of the antibodies with PbTx-1 (and presumably PbTx-1-derived conjugates) and with N-palmitoyl BTX-B2 indicates that ELISA results can underestimate of the total amount of brevetoxin and brevetoxin metabolites present in a sample. PbTx-1 is more potent than PbTx-2; however, the parent toxins PbTx-1 and -2 are not found in shellfish, and PbTx-2 type toxins consistently dominate the toxin profile in both *Karenia brevis* cells[20-22] and shellfish[22], typically accounting for 75% or more of the total toxins present. Additionally, although lipid conjugates of brevetoxin are thought to contribute substantially to NSP toxicity, these are derivatives of (and co-occur with) the more abundant amino acid metabolites that dominate the profile of toxic oysters and clams. The ELISA readily detects these forms, which have been identified as excellent biomarkers of NSP toxicity in oysters and clams. Therefore, the limited cross-reactivity of the ELISA with PbTx-1 and with N-palmitoyl BTX-B2 does not diminish the potential for the ELISA to perform successfully within a management program as proposed here.

In recent years, other brevetoxin ELISA kits have been introduced to the market, but prior to implementation into shellfish monitoring each kit would require individual evaluation of antibody cross-reactivity with dominant brevetoxin metabolites and comparisons with currently approved methods.

ELISA vs Mouse Bioassay

Currently, the only approved method for NSP testing is the APHA mouse bioassay[23]. The method is based on the bioassay developed by McFarren et al.[24] more than 50 years ago using toxic shellfish collected during an NSP outbreak in 1963. One mouse unit (MU) is the amount of crude lipid extract that will kill, on average, 50% of 20-g test mice in 15.5 hours. It is important to note that this method has never been validated, and the guidance limit used today (20 MU per 100g) is not based on any toxicological studies, but rather was described as the level of sensitivity of the test for 20g mice observed for 6 hours, which was deemed to be the longest reasonable observation time for the sake of accuracy and expediency. This guidance limit has proven to be effective, as no cases of NSP from legally harvested shellfish have been documented in Florida since the monitoring program began in the 1970's.

Comparing NSP mouse bioassay and ELISA data is not straightforward. The assays measure NSP toxins in very different ways. The mouse bioassay assesses toxicity by measuring the response of mice injected with a crude lipid extract of shellfish. This extract, prepared by repeated partitioning of acidified shellfish homogenate with diethyl ether, contains only a subset of the toxins present[7,25,26]. The method is semi-quantitative, yielding numerical results only at values ≥20 MU per 100g. Conversely, the ELISA is much more sensitive and yields continuous data to much lower concentrations, quantifying (relative to PbTx-3) a more comprehensive collection of brevetoxins and metabolites (regardless of potency) using antibodies that recognize a portion of the brevetoxin B-type backbone structure. Given that the NSP mouse bioassay measures only a subset of the toxins present, is semi-quantitative, has never been appropriately validated, and is not calibrated against known brevetoxin concentrations, a robust agreement of numerical results is unlikely to be achieved by any method.

At present, there is no validated brevetoxin equivalent of 'mouse units' in shellfish. Early work by Baden and Mende [27] established the toxicity of purified PbTx-2 and -3 dissolved in saline to mice intraperitoneally and calculated an LD50 (amount of toxin that kills half of the mice in 24 hours) of 0.2 mg/kg (similar for both toxins). This dose was used to derive a PbTx-2 "equivalent" of 4 µg per 20g-mouse and has since been extended to estimate the brevetoxin concentration in shellfish with a measured toxicity of 20 MU per 100g as 0.8 mg PbTx-2 equivalents per kg shellfish [13,16]. This number appears in several guidance documents; however**,** the extensive metabolism of brevetoxins in shellfish was unknown when the estimated equivalence was first proposed. We now know that shellfish exposed to *K. brevis* blooms contain a mixture of toxins with a multiplicity of potencies. In many cases the metabolites are less toxic, but in some cases they are more toxic. For these reasons, the use of this equivalent for brevetoxins in shellfish is inappropriate and has been acknowledged to be of little use for practical application[7].

Because a biomarker or indicator of toxicity approach is currently necessary for NSP, future NSP guidance limits may vary with the method used and may also vary across shellfish species. An appropriate non-mouse unit guidance limit for brevetoxins in shellfish will provide a level of protection for human health equal to that provided by the existing federal NSP guidance limit of 20 MU per 100 g shellfish. We know from existing data derived from naturally incurred eastern oysters and hard clams that such a limit *as measured using the MARBIONC ELISA with PbTx-3 as a standard* would exceed 0.8 mg per kg shellfish for these species.

Comparison of NSP Mouse Bioassay and ELISA results

Where quantitative results were obtained by both mouse bioassay and ELISA, Spearman rank correlation analysis was used to assess the correlation of brevetoxin concentrations measured by both methods for each shellfish matrix (Table G3). Significant correlations were observed in all cases.

Table G3. Spearman rank correlation coefficients (and p-values) for brevetoxin concentrations measured by NSP bioassay and ELISA

	Spearman rank correlation coefficient	p-value
oysters	0.5590	< 0.0001
hard clams	0.7866	< 0.0001
sunray venus clams	0.6859	< 0.0001

(From this portion on, changes to address early LMC comments are underway, and an updated Appendix G will be submitted.)

Given the differences between the assays and what they measure, strong agreement between numerical results was not expected. Nevertheless, the data were analyzed using linear regression analysis to estimate predicted concentrations by ELISA for samples testing at 20 MU per 100g (Fig. G9). Removal of the outlying (high) mouse bioassay results for oysters (>50MU) and clams (>100MU) that influenced the regression lines lowered the R-squared values, but slopes did not change appreciably. The 20 MU/100 g equivalent by ELISA was estimated to be 4.6 ppm in oysters, 3.2 ppm in hard clams, and 3.1 ppm in sunray venus clams.

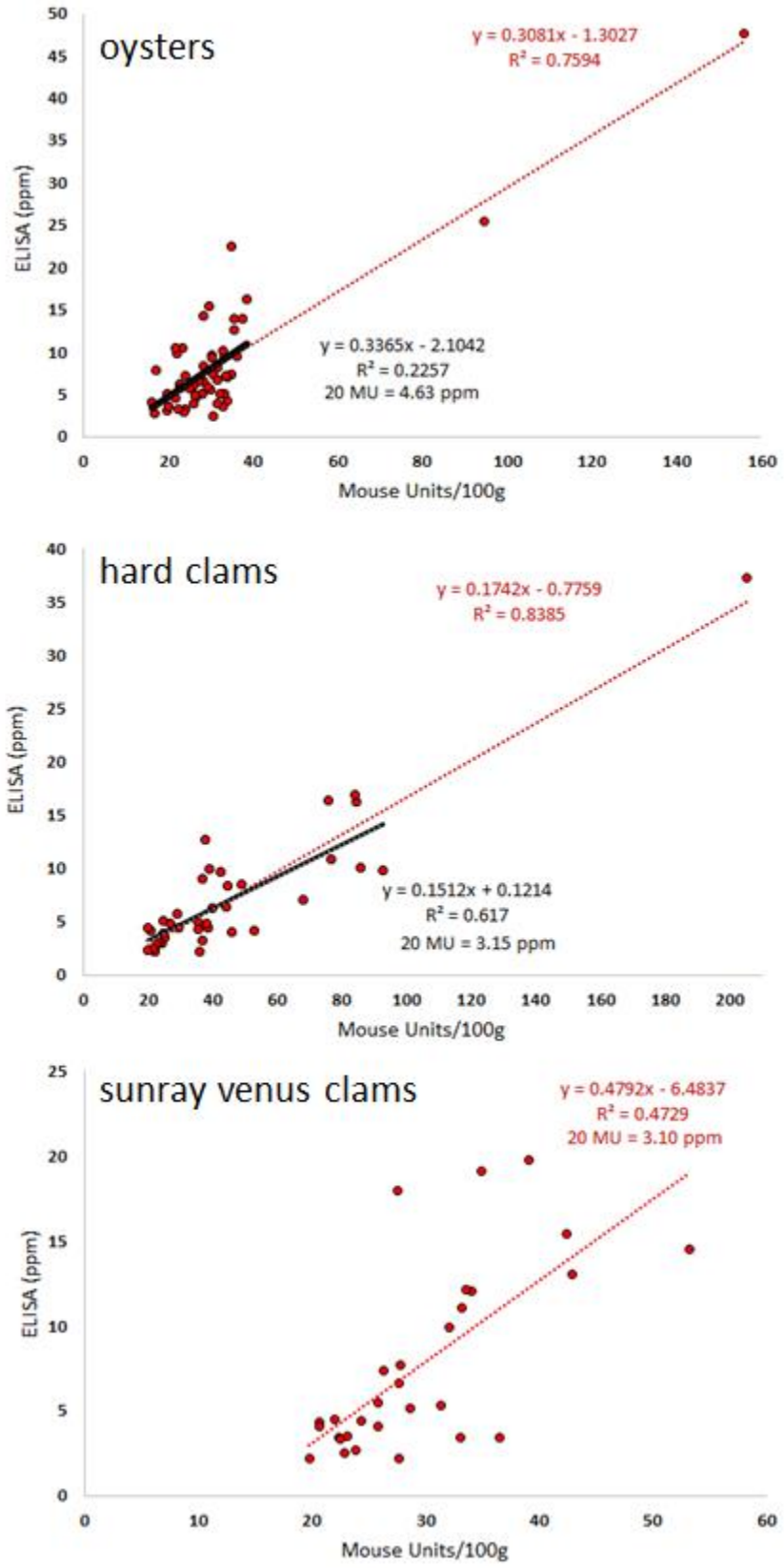


Figure G9. NSP mouse bioassay vs. ELISA results in oyster, hard clams, and sunray venus clams.

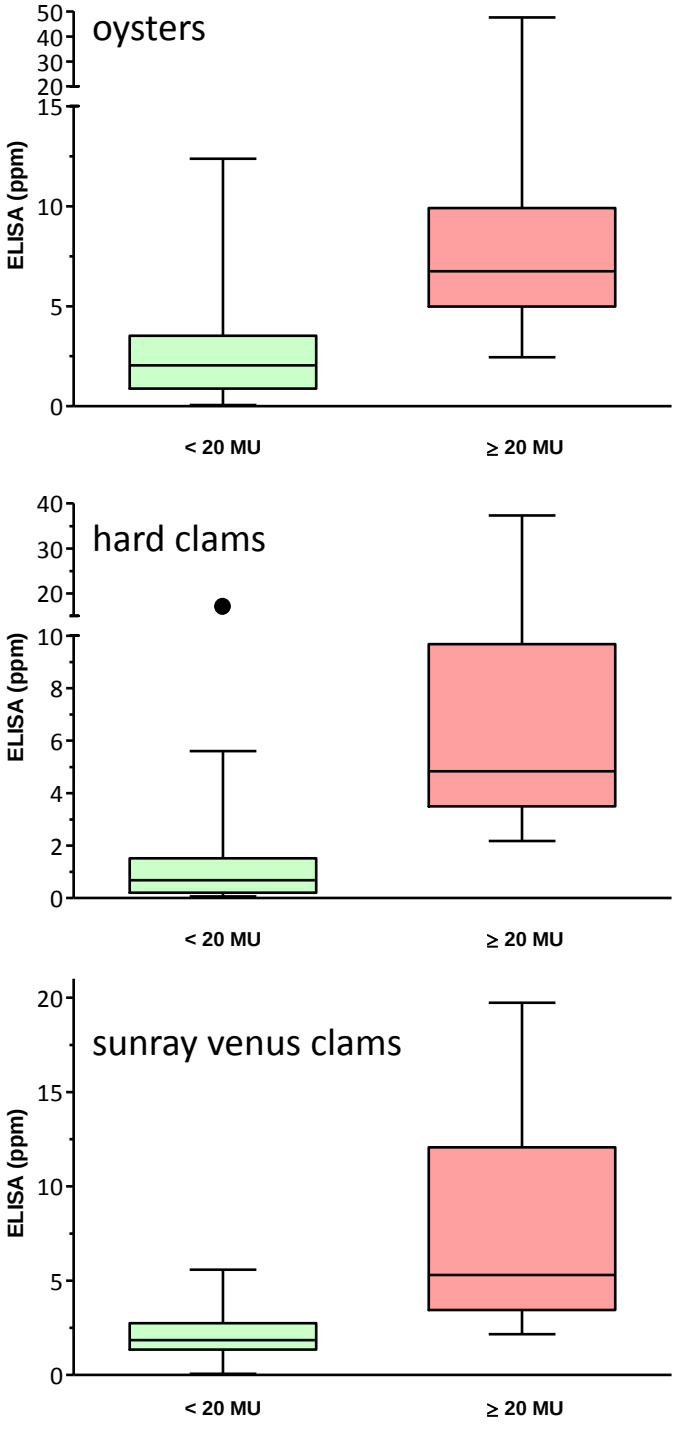


Figure G10. Boxplots (whiskers min to max) of ELISA results for samples testing < 20 MU/100g and ≥ 20 MU/100g in oysters, hard clams, and sunray venus clams. A value of 0.06ppm (half the limit of detection[LOD]) was substituted for ELISA results that were <LOD.

Boxplots were created to visualize the distribution of the data for samples testing < 20 MU/100g and ≥ 20 MU/100g (Fig. G10). There was a very wide range of concentrations measured by ELISA in samples testing < 20 MU. This was expected since those samples represent a range of lower NSP concentrations that are not quantifiable by mouse bioassay.

Brevetoxin metabolites are persistent in shellfish, and some level is frequently measured in bivalves from *K. brevis* endemic areas that have tested safe by mouse bioassay. In samples testing <20 MU the median value was 2.04 ppm in oysters, 0.66 in hard clams, and 1.85 in sunray venus clams. The highest concentrations were measured in wild oysters and hard clams, presumably due to repeated exposure to *K. brevis* and retention of the more persisitent metabolites across multiple bloom seasons. Farmed clams are brevetoxin-free when they are placed on lease sites, and their residence time in natural waters is short. These bivalves do not experience multiple successive bloom seasons. The maximum concentration measured in farmed clams that were < 20 MU was 4.6 ppm and in sunray venus clams was 5.6 ppm.

Importantly, across species, there were similar minima in samples testing ≥ 20 MU/100g. ELISA concentrations in samples that “failed” by mouse bioassay were never below 2.4 ppm in oysters and 2.1 ppm in hard clams or sunray venus clams.

As the only Approved Method, the NSP mouse bioassay is currently the only yardstick to which new methods can be compared. The mouse bioassay is semi-quantitative, not calibrated, and detects only that subset of compounds in shellfish that are ether-extractable. Analytical and screening NSP methods are unlikely to ever completely agree with mouse bioassay results, and expectations for

comparisons of proposed alternate methods with the mouse bioassay should be guaged accordingly, with a goal of achieving an equal measure of safety rather than perfect alignment of results and management actions on a sample by sample basis.

The results of our Single Lab Validation demonstrate that this assay generates specific, precise, and repeatable results. Additionally, ELISA results of naturally incurred shellfish compare very well with LC-MS analyses targeting the dominant metabolites found in eastern oysters and hard clams from the Gulf of Mexico (S-desoxy-BTX-B2, BTX-B2, and BTX-B1; Fig. G11).

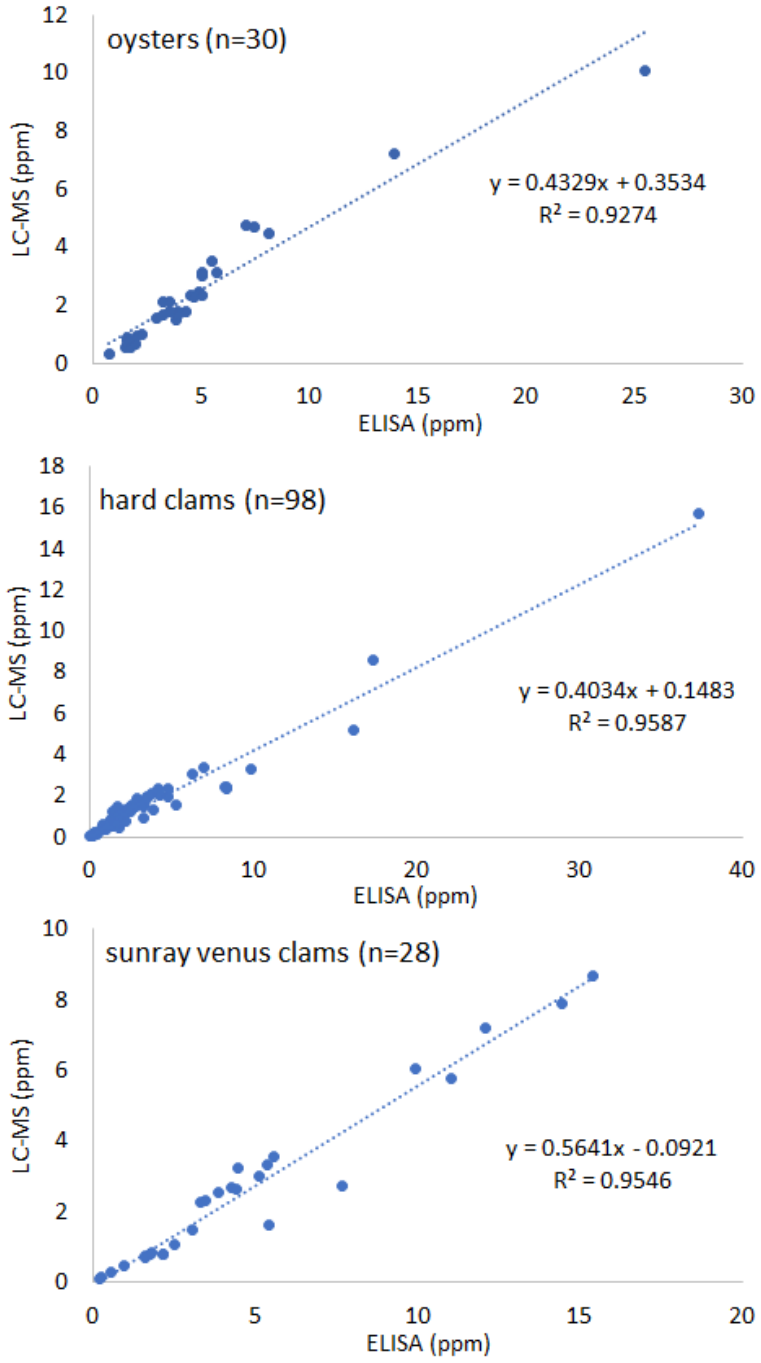


Figure G11. Comparison of NSP ELISA and LC-MS analysis of naturally incurred shellfish. LC-MS data generated and provided by A. Abraham, USFDA Gulf Coast Seafood Laboratory, using samples from this study.

Given the shortcomings and limitations of the mouse bioassay and the hardship this method imposes on both resource managers and industry, the move towards alternate methods must begin.

We propose that ELISA be approved for use as Limited Use Method such that samples would "pass" NSP rapid screening by ELISA when ELISA results are at or below a threshold representing no more than half of the level predicted in samples testing close to 20 MU/100g and below the lowest level measured in samples that have tested greater than or equal to 20 MU/100g (i.e., yielding no false negatives when applied to the existing dataset).

Thresholds of 1.8 ppm in oysters and 1.6 ppm in hard clams and sunray venus clams are proposed. The approach to derive the threshold was to approximate the ELISA equivalent of half of 20 MU/100 g and to ensure that the threshold would yield no false negatives. To protect against false negatives, the proposed thresholds are no more than 75% of the lowest concentration in the dataset that yielded a positive mouse bioassay. In hard clams and sunray venus clams, 1.6 ppm approximates half of the predicted 20 MU/100 g equivalent and is 75% of the lowest level measured in clams that failed mouse bioassay (2.18 ppm). For oysters, 1.80 is less than half of the estimated 20 MU/100 g equivalent and is 73% of the lowest level measured in oysters that failed mouse bioassay (2.45 ppm). These thresholds are not proposed as new guidance or actions limits for NSP, but rather as screening thresholds **specific to the MARBIONC ELISA (using PbTx-3 as a standard)** below which we have confidence that oysters and clams would yield <20 MU/100g and above which testing by mouse bioassay (or other future Approved Method) would be required.

Applying these thresholds to the comparative data set presented here would produce no false negatives (no samples testing greater than or equal to 20 MU/100 exceeded these levels by ELISA). Among the subset of samples testing < 20 MU/100g, ELISA results exceeded the thresholds (and would necessitate additional testing by NSP mouse bioassay) for 56% of oyster samples, 22% of hard clam samples, and 68% of sunray venus clam samples. The high proportion of <20 MU sunray venus clams above the threshold is an artifact of our sample set. Because sunray venus clams are relatively new to Florida aquaculture, our sample size is smaller, and collections during and following *K. brevis* blooms have been targeted in recent years to generate quantitative mouse bioassay data for comparisons.

As a first step away from total reliance on the NSP mouse bioassay, the proposed thresholds are conservative, and they may need to be revised in the future when more data and/or other approved methods are available, but they would have eliminated the need for 246 of the 501 bioassays (49%) conducted and represented in this data set. Having this method available as an approved option for NSP testing would greatly benefit all Gulf States. In 2015, a *K. brevis* affected the entire northern Gulf of Mexico, resulting in simultaneous closures of shellfish harvest areas in Florida, Alabama, Mississippi, and Louisiana. Because Alabama, Mississippi, and Louisiana experience these blooms infrequently, they lack the capacity to conduct NSP mouse bioassays. Therefore, sample testing to reopen harvest areas in these states after the bloom had dissipated was coordinated by our lab in Florida with the assistance of Resource Access International in Maine. While this cooperative effort was successful, it was a heavy burden on Florida, taking five weeks following bloom termination to complete and unnecessarily extending closures in these states. In every case, samples submitted by the other states passed by mouse bioassay (contained < 20 MU/100g), and if screening by ELISA had been an approved option, bioassays would not have been necessary in Mississippi or Louisiana, where NSP levels of oyster samples tested by ELISA ranged from 0.16 to 1.22 ppm.

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