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VALIDATION AND ASSESSMENT OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR USE IN MONITORING AND MANAGING NEUROTOXIC SHELLFISH POISONING

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ABSTRACT Neurotoxic shellfish poisoning caused by the ingestion of shellfish contaminated with brevetoxins threatens human health and economies reliant on the Gulf of Mexico. In the United States, the only federally approved method to test shellfish for brevetoxins is a mouse bioassay. Although effective in protecting public health, this assay has considerable shortcomings, including low throughput, lack of sensitivity and specificity, and reliance on live animal testing. Here, a commercially available enzyme-linked immunosorbent assay (ELISA) (MARBIONC) was validated, and results of field samples tested using ELISA and the mouse bioassay were compared. In this validation of the ELISA method for eastern oysters *Crassostrea virginica* and clams (*Mercenaria* spp. and sunray venus *Macrocallista nimbosa*), ELISA demonstrated strong recoveries, specificity, and ruggedness. Quantitative results were obtained for more than 90% of the 526 field samples tested by ELISA compared with only 26% of the samples tested by mouse bioassay. When quantitative data were available for both assays, significant correlations across methods were found. Based on this work, in 2017, the Interstate Shellfish Sanitation Conference approved the limited use of the MARBIONC ELISA in neurotoxic shellfish poisoning management.

KEY WORDS: brevetoxins, eastern oyster, ELISA, *Karenia brevis*, *Mercenaria* spp., NSP, sunray venus clam

INTRODUCTION

The Gulf of Mexico produces an estimated 55% of the United States shellfish aquaculture product (NMFS 2018), supporting the economies of numerous Gulf coast communities. Bivalve molluscs make up a major portion of this supply. Indeed, eastern oysters *Crassostrea virginica* harvested in the northern Gulf of Mexico account for 80%–90% of the total oyster production in the United States (Tunnell 2017), and commercially grown clams (*Mercenaria* spp. or ''hard clams'') are the most important aquaculture product in Florida (Adams et al. 2014). Both the Gulf of Mexico shellfish industry and human health are threatened by nearly annual blooms (red tides) of the marine dinoflagellate *Karenia brevis*, which produces a family of neurotoxins called brevetoxins (Tester & Steidinger 1997, Steidinger et al. 1998, Magaña et al. 2003, Thyng et al. 2013). Filter-feeding bivalves, including oysters, clams, and mussels, accumulate brevetoxins during *K. brevis* blooms, and ingestion of contaminated shellfish by humans can cause neurotoxic shellfish poisoning (NSP) (Watkins et al. 2008).

To prevent NSP, the National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish prohibits the harvest of shellfish when concentrations of *Karenia brevis* in the water increase or when the tissue concentration of brevetoxins equals or exceeds 20 mouse units (MU) per 100 g of raw shellfish, determined via the American Public Health Association (APHA) mouse bioassay (NSSP 2017). Shellfish harvest areas (SHA) managed by the Gulf states reopen once *K. brevis* abundance decreases and testing demonstrates that NSP toxicity in shellfish is again less than 20 MU/100 g shellfish. When enacted, the NSSP biotoxin plan effectively prevents occurrences of NSP from lawfully harvested bivalves; however, NSP closures come at a steep cost to Gulf economies and communities. Along the Gulf coast of Florida, for example, shellfish closures occur annually and often during the peak harvest season, resulting in considerable economic losses (NOAA 2004, Collins et al. 2019).

Losses to the industry can be minimized by expediently reopening SHA after closures. Yet, the APHA mouse bioassay—the only NSP toxicity method approved for use in the NSSP—is a time-consuming and low-throughput method. Because of limitations in the speed and availability of approved laboratories conducting testing, SHAs may be closed for weeks longer than necessary. The APHA method also has other drawbacks. The assay is nonspecific, imprecise, and not calibrated against known levels of brevetoxins (Plakas & Dickey 2010). It is costly in terms of labor and supplies, and the use of live animals is both undesirable and increasingly unacceptable (Plakas & Dickey 2010, Turner et al. 2015). The use of an alternative rapid NSP test could reduce delays in reopening harvest areas after a bloom, decreasing economic losses, and remove these other analytical limitations.

Despite the development of multiple methods for detecting brevetoxin in bivalves, no method has been approved to replace the mouse bioassay for regulatory NSP testing. The chemical and toxicokinetic complexity of brevetoxins and their metabolic products in bivalves is one of the primary reasons that little progress has been made to replace the mouse bioassay. Brevetoxins (PbTxs or BTXs) are a suite of cyclic polyether compounds that have either an A-type or a B-type backbone structure, containing 10 or 11 rings, respectively (Fig. 1). At least nine brevetoxin congeners with varying potencies have been isolated from *Karenia brevis* (Baden et al. 2005). A-type brevetoxins (PbTx-1, PbTx-7, and PbTx-10) are the more

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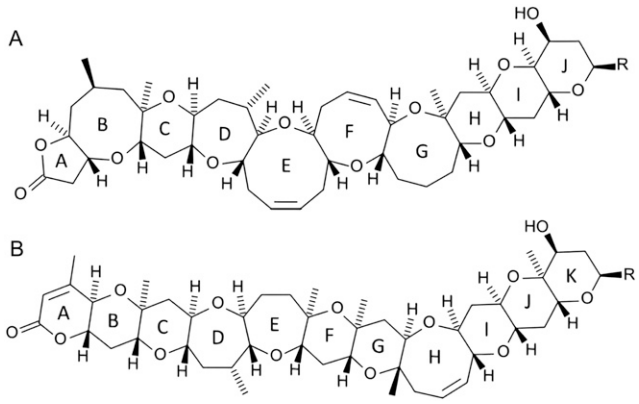

Figure 1. Structures of brevetoxin A (A) and B (B) backbones.

potent, but B-type brevetoxins (PbTx-2, PbTx-3, PbTx-5, PbTx-6, PbTx-8, and -9) are much more abundant in the environment (Baden 1989). In bivalves, the more reactive forms of brevetoxins are rapidly transformed into brevetoxin metabolites that are generally the products of reduction, oxidation, or conjugation to other molecules, including taurine, cysteine, cysteine sulfoxide, amino acids, and fatty acids (Wang et al. 2004, Ishida et al. 2006, Plakas & Dickey 2010). Dozens of brevetoxin metabolites have been identified in shellfish, but information on their potency is available for only a small subset (Munday 2014). In oysters, the brevetoxin profile is dominated by the cysteine metabolites S-desoxy-BTX-B2 and BTX-B2 (Plakas et al. 2004, Wang et al. 2004, Plakas et al. 2008). These are also the major metabolites identified in *Mercenaria* spp., along with BTX-B1, a taurine conjugate (Abraham et al. 2012, Abraham et al. 2015). Sunray venus clams *Macrocallista nimbosa*, a relatively new aquaculture product gaining popularity in Florida, metabolizes brevetoxins similarly to *Mercenaria* spp. (Flewelling 2017). These metabolites were found to be excellent biomarkers of composite B-type brevetoxins (Plakas et al. 2008, Abraham et al. 2015).

Another significant obstacle to advancing alternate methods for NSP toxicity testing has been the lack of clear guidance on requirements for submission of methods for use in the NSSP. The Interstate Shellfish Sanitation Conference (ISSC) comprises state and federal agencies as well as shellfish industry members. The ISSC is responsible for establishing and updating regulatory guidelines and procedures for uniform state application of the NSSP and, together with the U.S. Food and Drug Administration, determines which methods are approved for use in the NSSP.

Of the many chemical and biological methods evaluated for measuring brevetoxins in bivalves, those that recognize molecular structure [i.e., enzyme-linked immunosorbent assays (ELISA) and liquid chromatography–mass spectroscopy] have demonstrated better reproducibility and agreement with the mouse bioassay than activity-based assays (i.e., receptor binding and cytotoxicity assays) (Dickey et al. 2004, Plakas et al. 2008, Plakas & Dickey 2010). In addition, the speed and cost-effectiveness of ELISA make it an attractive, high-throughput screening method. The first commercially available brevetoxin ELISA, marketed by MARBIONC Development Group LLC (Wilmington, NC), is based on the assay developed by Naar et al. (2002) and is an indirect competitive ELISA that uses goat polyclonal anti-brevetoxin antibodies to detect toxins in a sample. This assay has been a useful tool in advancing understanding of the dynamics of brevetoxin accumulation and depuration in bivalves (Naar et al. 2004, Plakas et al. 2004, Plakas et al. 2008, Griffith et al. 2013) and is widely and routinely used to monitor brevetoxins in Florida's marine systems (Naar et al. 2002, Flewelling et al. 2005, Fire et al. 2008) and to diagnose human (Abraham et al. 2008), marine mammal (Flewelling et al. 2005, Fire et al. 2007), and other animal (Naar et al. 2007, Flewelling et al. 2010, Fauquier et al. 2013a, 2013b) exposure to brevetoxins. The present study details a single-laboratory validation (SLV) of a method that couples rapid extraction and detection of brevetoxins in bivalves using the MARBIONC ELISA. The study focused on three species of bivalves, demonstrating the applicability of ELISA across multiple shellfish species and its potential for improving the effectiveness and efficiency of NSP monitoring and management.

MATERIALS AND METHODS

Reagents and Standards

Chemicals used for ELISA and mouse bioassays were of ACS grade or better. Methanol, sulfuric acid, SuperBlock blocking buffer (reagent B), diethyl ether, and vegetable (soybean or safflower) oil were purchased from Thermo Fisher Scientific (Waltham, MA). Phosphate-buffered saline (PBS), gelatin, and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO). Brevetoxin-3 (PbTx-3) and brevetoxin ELISA kits containing PbTx-3, protein-linked brevetoxin (reagent A), polyclonal goat anti-brevetoxin antibodies (reagent C), and horseradish peroxidase (HRP)-linked secondary antibodies (reagent D) were purchased from MARBIONC. Water was prepared using a NANOpure Diamond Ultrapure water-purification system with a 0.2-µm filter (Thermo Fisher Scientific).

Sample Collection and Preparation

This study focused on eastern oysters *Crassostrea virginica* and clams (*Mercenaria* spp. and sunray venus *Macrocallista nimbosa*). For the spike-and-recovery experiments, nontoxic shellfish were collected from multiple sites within two SHAs that infrequently experience *Karenia brevis* blooms along Florida's Gulf coast. Wild eastern oysters were collected from Apalachicola Bay, in northwest Florida, in January 2014 and February 2014, and clams were sourced from clam farms on Cedar Key, FL, in May 2014 to October 2014. To compare the results of ELISA with those of the APHA mouse bioassay, naturally incurred toxic shellfish samples collected between 2007 and 2017 by the Florida Department of Agriculture and Consumer Services for regulatory NSP testing and by aquaculture industry members during or following *K. brevis* blooms were used. Shellfish were either shucked and drained as described in the following paragraphs and stored in a plastic jar on ice before shipping or shipped overnight to the laboratory live on ice. Samples were processed immediately on receipt or were stored at –20°C until processed.

Each sample consisted of at least 12 animals and 120–150 g of meat. Shellfish were rinsed with freshwater, shucked, and then drained for 5 min on a #10 sieve before homogenizing for

2 min in a stainless-steel laboratory blender. For the ELISA validation, multiple 1-g aliquots of each sample were weighed into 15-mL graduated polyethylene centrifuge tubes (BD Falcon) before spiking (see the Validation Parameters section). To ensure that samples were free of brevetoxin, a 1-g aliquot of each sample was screened by ELISA before spiking. Naturally incurred toxic shellfish samples for comparison of ELISA and the mouse bioassay were prepared as aforementioned, and aliquots were weighed for ELISA (1.0 g, in duplicate) and mouse bioassay (100 g).

Brevetoxin Measurement by ELISA

Aliquots of spiked or field samples for ELISA were extracted using 80% aqueous methanol: spiked aliquots received 8.5 mL and blank aliquots and naturally incurred toxic samples received 9 mL of methanol. Tubes were vortexed at maximum speed for 2 min and then centrifuged at 3,000 × g (20°C) for 10 min. Supernatants were transferred to clean graduated 15-mL centrifuge tubes, and final extract volume was adjusted to 10 mL with 80% methanol. Extracts were transferred to glass vials and stored at –20°C until assayed.

Brevetoxin ELISA assays were conducted according to the manufacturer's instructions with modifications as described here. All incubation steps were conducted at room temperature on an orbital microplate shaker. Flat-bottomed 96-well ELISA plates (Nunc MaxiSorp) were coated with protein-linked brevetoxin (reagent A diluted by 300 in PBS, pH 7.4, 100 µL per well) and incubated for 1 h. After coating, the plates were washed three times with PBS (300 µL per well), and any remaining binding sites in the wells were blocked by incubating the plates for 30 min with blocking buffer (reagent B, 250 µL per well). After washing the plates three times with PBS containing 0.05% Tween 20 (PBS-Tween, 300 µL per well), polyclonal goat anti-brevetoxin antibodies [reagent C diluted by 300 in PBS-Tween with 0.5% gelatin (PGT), 100 µL per well] were incubated with samples or standards (100 µL per well) for 90 min. The plates were again washed three times with PBS-Tween and then incubated with HRP-linked secondary antibodies (reagent D diluted by 800 in PGT, 100 µL per well). After 1 h, the plates were washed three times with PBS-Tween and once with PBS to remove any remaining detergent. The HRP substrate TMB was added to the wells in rapid succession. The plates were protected from light with aluminum foil, and the reaction was stopped after 5–7 min by addition of 0.5 M sulfuric acid (100 µL per well). The absorbance of the solution in the wells was read at 450 nm using a µQuant microplate spectrophotometer (Biotek, Winooski, VT). The intensity of the color is inversely proportional to the amount of brevetoxin that was present in the well during incubation. Results were expressed as µg PbTx-3/g for spiked samples and µg PbTx-3 equivalents/g (hereafter abbreviated as µg/g) for naturally incurred toxic samples.

For full quantitation, five samples and a PbTx-3 standard curve (0.08–5 ng/mL) were analyzed on each plate. Shellfish extracts (1-g eq in 10 mL) were diluted 40-fold with PGT and then serially diluted by 2 with PGT six times for a final dilution factor range of 400–25,600. All sample and standard dilutions were assayed in duplicate wells. Wells containing only PGT were assayed to obtain the maximum absorbance (A_{max}). For each dilution, percent inhibition was calculated as [1 – (average of duplicate wells/A_{max})] × 100. Standard curves were plotted using the sigmoidal dose–response (variable slope), or four-parameter logistic, curve in Prism (v7, GraphPad Software, La Jolla, CA) and were used to determine the concentration for sample dilutions falling within the linear portion of the standard curve.

Quality-control criteria for acceptance of individual sample results were (1) coefficient of variation (CV) of raw absorbance of duplicate wells for sample dilutions used for quantitation within the linear range of the assay (20%–70% inhibition) less than 20%; (2) CV of calculated concentrations of different sample dilutions within the linear range of the assay less than 20%; and (3) $A_{max} \geq 0.6$. If any of the criteria were not met, the assay was rerun.

Validation Parameters

The performance characteristics evaluated were those specified in the ISSC SLV Protocol (ISSC 2018), included accuracy, precision, linearity, limit of detection (LOD), limit of quantitation (LOQ), specificity, ruggedness, and comparability. Brevetoxin-3 was used for all spike-and-recovery experiments because of its stability in shellfish and its commercial availability.

To evaluate accuracy and precision, 10 brevetoxin-free samples per species were spiked with a range of PbTx-3 amounts, and subsequent recovery was quantified. Spiking solutions were prepared by diluting PbTx-3 with 100% methanol to 0.8, 2.0, 8.0, and 16.0 µg PbTx-3/mL, and these were used to fortify shellfish aliquots (0.5 mL per 1-g aliquot) to final concentrations of 0.4, 1.0, 4.0, and 8.0 µg PbTx-3/g. Spiked samples were vortexed for 30 sec, and extraction commenced within 30 min of spiking. One blank (nonspiked aliquot) of each sample was also prepared. Accuracy (closeness of agreement between test results and the accepted reference value) was assessed via percent recovery, and precision (closeness of agreement between independent measurements obtained under stipulated conditions) was assessed as the CV of assay results obtained from independently prepared spiked samples. The target spike concentration of PbTx-3 was considered the reference value, and the stock solution used for spiking was used to generate ELISA standard curves for quantitation.

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 were generated. The upper and lower plateaus of the curves were then applied to formulae derived by Sebaugh and McCray (2003) to define the bend points of the standard curves (i.e., the beginning and end of the linear concentration-response region), which were expressed both in terms of percent inhibition ($1 - A/A_{max} \times 100$) and concentration. The LOD and LOQ were calculated by multiplying the SD of results from the lowest quantifiable samples (samples spiked to 0.4 µg/g, $n = 30$ for each matrix) by 3 and by 10, respectively. The overall or dynamic linear range of the method was determined from a combination of the linear range of the assay standard curve, the assay LOQ, and the range of sample dilutions on the plate.

To test the specificity of ELISA, samples containing potentially interfering compounds or organisms were analyzed in the presence of PbTx-3. Cross-reactivity with okadaic acid (OA), a structurally similar dinoflagellate polyether toxin produced by some species of *Dinophysis* and *Prorocentrum* (Van Dolah 2000), was investigated, along with the potential interference

of related (*Karenia mikimotoi*) and unrelated (*Isochrysis* aff. *galbana* and *Rhodomonas lens*) algal species to which bivalves are likely to be exposed. Okadaic acid–producing dinoflagellate genera and *K. mikimotoi* (a close relative of *Karenia brevis* that does not produce brevetoxins) are observed in Gulf of Mexico waters where *K. brevis* occurs (Steidinger & Meave del Castillo 2018), whereas the flagellate *I.* aff. *galbana* and the cryptophyte *R. lens* are commonly fed to aquacultured bivalves.

For each potentially interfering substance, 1-g aliquots of five toxin-free samples of each shellfish species were spiked with a high concentration of the substance and a low concentration of PbTx-3 (0.4 µg/g). An aliquot of each sample received the PbTx-3 spike only. Okadaic acid (National Research Council, Canada) was added to a concentration of 1.5 µg/g, roughly 10 times the current U.S. guidance limit of 0.16 ppm, *Karenia mikimotoi* cells from established clonal cultures were added to a concentration of 0.5 million cells/g, and *Isochrysis galbana* and *Rhodomonas lens* provided by Bay Shellfish Company (Terra Ceia Island, FL) were each added to a concentration of 100 million cells/g of shellfish.

To assess ruggedness, duplicate aliquots of 10 samples per shellfish matrix were spiked to five concentrations (0.4, 1, 2, 4, and 8 µg PbTx-3/g), and the results of ELISA analyses conducted under varying conditions were compared. Variations examined included (1) lot of ELISA kit reagents, (2) plate incubation temperature (21°C–22°C and 25°C), (3) sample and antibody incubation time (60 min and 90 min), and (4) final color development time after TMB addition (7 min and 13 min).

To assess comparability of ELISA and the mouse bioassay, naturally incurred toxic shellfish samples were prepared as aforementioned, and aliquots were weighed for ELISA (1.0 g in duplicate) and mouse bioassay (100 g). Shellfish homogenates for the mouse bioassay were extracted four times with 100 mL of diethyl ether. The extracts were combined in 500-mL beakers, allowed to evaporate under a fume hood overnight, and the lipid residue was dissolved in vegetable oil to a final volume of 10 mL. Bioassay procedures were conducted in accordance with policies set forth by the National Institutes of Health Office of Laboratory Animal Welfare (2002, 2015). Animal care and use policies were adapted from guidelines set forth by the National Research Council (2011) and the American Veterinary Medical Association (Leary et al. 2013). As required by the NSSP, the Fish and Wildlife Research Institute laboratory performance conducting APHA mouse bioassays is evaluated regularly by the U.S. Food and Drug Administration.

Male Swiss–Webster outbred mice (CFW or ND4), 17–23 g, were purchased from Charles River Laboratories Inc. (Wilmington, MA) or Envigo (Indianapolis, IN). Animals were allowed at least 24 h after arrival to acclimate and rehydrate. Five mice per sample received intraperitoneal injections of 1 mL of crude extract via a 25-gauge needle. Time to death and a weight correction, if needed, were used to determine toxicity per unit dose. If all mice died, the mean MU of the five mice was calculated; if any mice survived, the median was calculated. Bioassays were terminated after a 6-h observation period. Using 20-g mice, a 6-h observation time yields a lower LOD of approximately 20 MU/100 g shellfish meat, the guidance level at or above which shellfish are considered unsafe for human consumption. If three or more mice survived the 6-h observation period, results were recorded as less than 20 MU/100 g. Results for the subset of samples that measured less than 20 MU/100 g, yet that indicated some toxicity based on the death of one or two mice, were transformed to increase the number of quantitative mouse bioassay results for a more robust comparison. For these samples, a value of 10 MU (half the LOD for the assay) was assigned to individual mice surviving the assay, and the mean MU of the five mice was calculated, resulting in a small subset of samples that spanned the 10–20 MU/100 g range.

Data Analysis

For all metrics, measurement uncertainty, expressed as a single parameter (i.e., SD), encompassed all effects operating on the results, including overall precision of the method, laboratory bias, and matrix influences. To test the effects of shellfish species and spike concentration on recoveries, a two-way ANOVA was used. The specificity index was calculated as the ratio of the toxin concentration in samples spiked with only the target of interest to the toxin concentration in samples spiked with the target in the presence of potential interferences. A one-sample *t*-test was performed to determine whether the average specificity index obtained from the five replicates from each analysis differed from 1. For tests of ruggedness under each of the conditions (i.e., kit lot, temperature, incubation time, and color development time), skewness of the data were tested using the symmetry test of Miao et al. (2006), homogeneity of variance was assessed by calculating variance ratios, and paired Student's *t*-tests were conducted for each set of test conditions. Spearman rank correlation and weighted Deming regression analyses were used to compare results of ELISA with those of the mouse bioassay. All analyses were performed in R (v3.3.2) and Prism.

RESULTS

Accuracy and Precision

Accuracy ranged from 90.9% to 100.5% across all spike levels and matrices (Table 1). Average recoveries of all replicates across the range of spike concentrations in oysters, *Mercenaria* spp., and sunray venus clams were 96.3%, 98.4%, and 95.1%, respectively, and the effect of matrix on percent recovery was not significant [two-way ANOVA, $F_{(2,119)} = 0.06$, $P = 0.946$]. The CV of spiked samples extracted and assayed in triplicate ranged from 0.8% to 16.0% (Table 1). Across spike concentrations, the average CV of replicate extracts ranged from 3.8%–6.1% in oysters, 3.6%–6.1% in *Mercenaria* spp., and 5.0%–6.2% in sunray venus clams (Table 1).

Linearity, LOD, and Dynamic Linear Range

To determine the linearity of the assay, multiple 7-point standard curves were generated using data obtained from different ELISA plates analyzed over multiple days. The assays included standard curves generated using two different kit lots, one acquired in June 2014 ($n = 34$) and another in June 2016 ($n = 26$). The position of the standard curves and the linear range defined by the bend points differed slightly between the two kit lots (Fig. 2). The linear range of the standard curve determined from the mean upper and lower bend points was 0.2–1.0 ng PbTx-3/mL (17%–77% inhibition) for the 2014 reagents and 0.3–1.4 ng PbTx-3/mL (17%–74%) for the 2016 reagents. Such shifts can occur with the same kit lot by altering

TABLE 1.

Recovery of PbTx-3 from each of the three bivalve matrices tested.

Matrix	Spike concentration (μg PbTx-3/g)	Average measured concentration (μg PbTx-3/g)	Average percent recovery	Average CV (and range) percent
Oyster	0.4	0.39	96.3	3.8 (1.1–10.3)
	1.0	0.93	92.5	5.0 (0.9–10.2)
	4.0	3.96	99.0	4.6 (1.4–9.1)
	8.0	7.63	95.4	6.1 (1.5–16.0)
	All concentrations		96.3	4.9 (0.9–16.0)
Mercenaria spp.	0.4	0.36	91.0	4.3 (2.5–7)
	1.0	0.93	92.9	3.6 (1.8–7.8)
	4.0	3.98	99.5	6.1 (1.1–10.7)
	8.0	7.91	98.9	5.4 (2.0–9.2)
	All concentrations		98.4	4.8 (1.1–10.7)
Sunray venus clam	0.4	0.36	90.9	5.7 (1.1–15.9)
	1.0	0.97	97.2	5.0 (1.0–10.9)
	4.0	4.02	100.5	5.8 (2.6–7.4)
	8.0	7.39	92.4	6.2 (0.8–14.5)
	All concentrations		95.1	5.7 (0.8–15.9)

Columns list spike concentration and the mean measured brevetoxin concentration in samples, percent recovery, and the CV across replicate extracts. For each matrix and spike level, $n = 10$.

dilutions of key reagents (A and C), and the differences observed between kit lots were likely due to minor concentration variations in the supplied reagents. Comparative analyses of spiked samples, however, were not significantly different between the two kit lots (see the **Ruggedness** section). In addition, two 14-point standard curves were assayed on different days to increase the number of points falling along the linear portion of the curve (Fig. 2). The bend points from these 14-point curves (16%–76%) were similar to those derived from the routine standard curves.

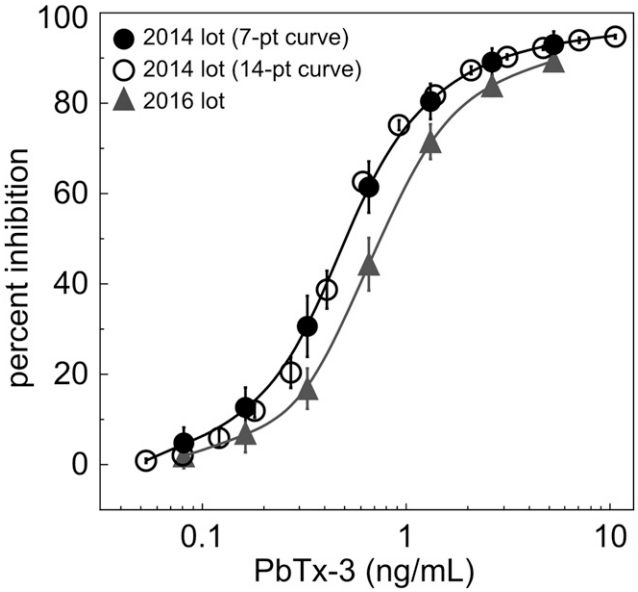

Figure 2. Average of multiple standard curves generated using two lots of ELISA kit reagents and comparison of 7-point and 14-point PbTx-3 standard curves. Error bars represent the SD of independent curves prepared and assayed on different plates/days (2014 lot 7-pt curve, $n = 34$; 2014 lot 14-pt curve, $n = 2$; 2016 lot, $n = 26$).

Blank samples consistently yielded assay responses that were not quantifiable (i.e., not on the linear portion of the standard curve). Therefore, the SD of results from the greatest quantifiable dilution of samples spiked to 0.4 μg/g was used to derive the LOD and LOQ, expressed as 3× and 10× the SD, respectively. This dilution (1:1,600) consistently yielded a signal (percent inhibition) closest to the lower bend point (17%). For all three matrix types, the SD at the 1:1,600 dilution was approximately 0.03. Calculated assay LOD and LOQ were 0.1 and 0.3 ng/mL, respectively. At the starting sample dilution of 1:400, the LOD and LOQ for brevetoxin in shellfish were 0.04 and 0.12 μg/g, respectively.

The overall or dynamic linear range of this method is a combination of the linear range of the standard curve, the assay LOQ, and the range of sample dilutions on the plate (from 400 to 25,600). Using the LOQ calculated earlier, which falls within the linear portion of the standard curve identified for both kit lots used, and the upper bend points identified for each kit lot, the overall or dynamic linear range of the method is from 0.1 to 26.6 μg/g for the June 2014 kit lot and to 35.3 μg/g for the June 2016 kit lot.

Specificity

The specificity index of samples spiked with PbTx-3 at 0.4 μg/g and supplemented with OA (1.5 μg/g shellfish), *Karenia mikimotoi* (0.5 million cells/g shellfish), *Isochrysis galbana* (100 million cells/g shellfish), and *Rhodomonas lens* (100 million cells/g shellfish) ranged from 0.91 to 1.04 with an average (±SD) of 0.96 ± 0.04 (Table 2). The average specificity index from each analysis was not significantly different from 1 (one-sample *t*-test, Table 2).

Ruggedness

Under all conditions, the symmetry test failed to reject the null hypothesis for symmetry, and the variance ratios indicated homogeneity of variance (Table 3). There were no clear statistical differences in assay results for any of the varying test conditions (paired *t*-test, Table 3).

TABLE 2.

Average (±SD) PbTx-3 concentration, specificity index (SI), and result of one-sample *t*-test in each of three matrices with and without (i.e., control) added contaminants ($n = 5$ for each set of conditions).

	Oyster		*Mercenaria* spp.		Sunray venus clam	
	Measured concentration (μg PbTx-3/g)	SI *t*-value (*P* value)	Measured concentration (μg PbTx-3/g)	SI *t*-value (*P* value)	Measured concentration (μg PbTx-3/g)	SI *t*-value (*P* value)
Control	0.38 ± 0.03	–	0.37 ± 0.02	–	0.36 ± 0.02	–
OA	0.37 ± 0.01	1.03 ± 0.09	0.38 ± 0.01	0.98 ± 0.05	0.39 ± 0.02	0.93 ± 0.10
	–	0.8 (0.49)	–	1.0 (0.37)	–	1.5 (0.20)
Karenia mikimotoi	0.40 ± 0.02	0.95 ± 0.04	0.36 ± 0.03	1.04 ± 0.10	0.38 ± 0.03	0.94 ± 0.09
	–	2.6 (0.06)	–	1.0 (0.36)	–	1.4 (0.23)
Isochrysis galbana	0.40 ± 0.03	0.96 ± 0.09	0.39 ± 0.02	0.97 ± 0.04	0.40 ± 0.02	0.91 ± 0.07
	–	1.1 (0.35)	–	1.7 (0.16)	–	2.7 (0.06)
Rhodomonas lens	0.41 ± 0.01	0.94 ± 0.10	0.39 ± 0.01	0.97 ± 0.06	0.38 ± 0.03	0.95 ± 0.11
	–	1.3 (0.25)	–	1.4 (0.23)	–	1.16 (0.31)

Comparability

A total of 526 samples were tested using both ELISA and the mouse bioassay (Table 4). Most samples (495 of 526, 94%) were extracted and assayed by ELISA in duplicate. The mean CV of duplicate analyses was 6.2%. Quantitative mouse bioassay results were obtained for 138 samples (26%). In seven cases, because of weight correction factors applied to smaller mice (<20 g), calculated bioassay results were less than 20 MU/100 g (range 16.2–19.8 MU). By contrast, toxin concentrations in 479 samples (91%) could be quantified by ELISA. All 47 samples testing below the LOD by ELISA were negative (<20 MU/100 g) by mouse bioassay.

A wide range of brevetoxin concentrations was measured by ELISA in the 388 samples that tested less than 20 MU/100 g (Fig. 3). The concentrations in most of these samples were much lower than those measured in samples that tested 20 MU/100 g or greater, with median values of 2.2 μg/g in oysters, 0.7 μg/g in *Mercenaria* spp., and 1.9 μg/g in sunray venus clams (Fig. 3). In samples testing 20 MU/100 g or greater, the range of brevetoxin concentrations measured by ELISA was 2.4–47.6 μg/g in oysters, 2.2–37.3 μg/g in *Mercenaria* spp., and 2.0–19.7 μg/g in sunray venus clams (with no negative ELISA results). Across species, there were similar minima in samples testing 20 MU/100 g or greater and limited overlap in samples passing and failing by the mouse bioassay (Fig. 3).

Spearman rank correlation analysis of data for which quantitative results were obtained using both ELISA and the mouse bioassay yielded significant correlations ($P < 0.0001$) in all cases, with correlation coefficients of 0.559 in clams, 0.787 in *Mercenaria* spp., 0.686 in sunray venus clams, and 0.604 for all samples combined. Weighted Deming regression analysis, performed to assess the linearity of the relationship and to estimate predicted concentrations by ELISA for samples testing at 20 MU/100 g, used an error ratio of 2.3:1 based on results of nine samples that were analyzed in duplicate by both methods.

TABLE 3.

Ruggedness of the MARBIONC ELISA assessed by a symmetry test and paired *t*-test of results obtained for each set of test conditions.

Factor	Symmetry test statistic (*P* value)		Variance ratio	*t*-value (*P* value)
Kit lot	June 2014	June 2016	–	–
Oyster	–0.324 (0.75)	–0.077 (0.90)	1.03	0.7 (0.51)
Mercenaria spp.	–0.104 (0.91)	–0.262 (0.85)	1.05	0.2 (0.86)
Sunray venus clam	–0.277 (0.80)	–0.172 (0.85)	1.06	0.1 (0.94)
Temperature	21°C–22°C	25°C	–	–
Oyster	–0.077 (0.97)	–0.208 (0.82)	1.05	1.4 (0.20)
Mercenaria spp.	–0.262 (0.74)	–0.186 (0.87)	1.00	1.7 (0.12)
Sunray venus clam	–0.172 (0.82)	–0.373 (0.76)	1.06	1.0 (0.36)
Incubation time	60 min	90 min	–	–
Oyster	–0.133 (0.86)	–0.324 (0.78)	1.06	1.4 (0.20)
Mercenaria spp.	0.251 (0.77)	–0.104 (0.91)	1.03	1.9 (0.09)
Sunray venus clam	–0.423 (0.68)	–0.277 (0.73)	1.00	0.01 (0.99)
Color development time	7 min	13 min	–	–
Oyster	0.079 (0.89)	–0.130 (0.92)	1.04	2.1 (0.07)
Mercenaria spp.	–0.002 (0.95)	–0.040 (0.98)	1.07	1.7 (0.12)
Sunray venus clam	–0.034 (0.99)	–0.063 (0.94)	1.09	1.6 (0.13)

The variance ratio for each set of test conditions is also listed.

TABLE 4.

Summary of the naturally incurred toxic shellfish samples analyzed by both NSP mouse bioassay and ELISA.

Shellfish matrix	Total samples	Samples measuring <20 MU/100 g by mouse bioassay	Samples measuring ≥20 MU/100 g by mouse bioassay
Oyster	197	135 (69%)	62 (31%)
Mercenaria spp.	277	238 (86%)	39 (14%)
Sunray venus clam	52	22 (42%)	30 (58%)

Because sunray venus clams and *Mercenaria* spp. share a similar toxin profile (Flewelling 2017) and because the number of sunray venus clams was limited, both species of clams were combined for these analyses. There was a high percentage of nonquantitative mouse bioassay results (<20 MU/100 g) in the data set (74%); therefore, in addition to analyzing only those samples that yielded quantitative results by both methods, the regression analyses were repeated including the transformed results of the subset of samples that measured less than 20 MU/100 g but indicated some toxicity based on the death of one or two mice. Regression models were used to interpolate the ELISA concentration at 20 MU/100 g and the 95% confidence intervals for these values (Fig. 4). The 20 MU/100 g equivalent calculated by ELISA in oysters was 3.7 μg/g, using only determinate mouse bioassay data, or 4.9 μg/g, including the transformed data. In clams, the 20 MU/100 g equivalent calculated by ELISA was 3.1 μg/g, using only determinate mouse bioassay data, or 3.5 μg/g, including the transformed data.

DISCUSSION

Despite the global health risk of marine biotoxins, many regulatory entities still rely on the mouse bioassay as a standard method for toxin detection (Daguer et al. 2018). In the United States, the APHA mouse bioassay is the only approved method for NSP testing in support of the NSSP. One MU is the amount of crude lipid extract that will kill approximately 50% of 20-g test mice in 15.5 h. This method has not been validated, and the current guidance limit of 20 MU/100 g is not rooted in toxicology. Instead, the limit was described as the level of sensitivity of the test for 20-g mice observed for 6 h, deemed to be the longest reasonable observation time for the sake of accuracy and expediency. This guidance limit has proven to be effective; e.g., in Florida, no NSP cases from legally harvested bivalve shellfish have been documented since the NSP monitoring program began in the 1970s (Heil 2009). However, the mouse bioassay has considerable drawbacks, including limitations on the number of samples that can be analyzed, lack of specificity and precision, cost, and ethical ramifications.

This SLV was conducted to advance the use of a method that does not use live animals. Accuracy and precision, recovery, linearity, specificity, and ruggedness of ELISA were determined, demonstrating the suitability of the method for monitoring shellfish for brevetoxins to protect human health across a range of shellfish matrices.

With technological advancements, improved (i.e., more specific, faster) methods for measuring biological or chemical compounds are constantly being developed. Method validations for use in a regulatory testing framework rely heavily on comparability of data across accepted methods. Although ELISA offers improvements over the mouse bioassay in its speed, throughput, specificity, and sensitivity, assessing the comparability of this method with the mouse bioassay has presented challenges because the assays measure the suite of brevetoxins in different ways. The mouse bioassay assesses toxicity by measuring the response of mice injected with a crude lipid extract of toxic shellfish. This extract, prepared by repeated extractions of acidified shellfish homogenate with diethyl ether, contains only a portion of the brevetoxins present (Dickey et al. 1999, Naar et al. 2004, Plakas & Dickey 2010).

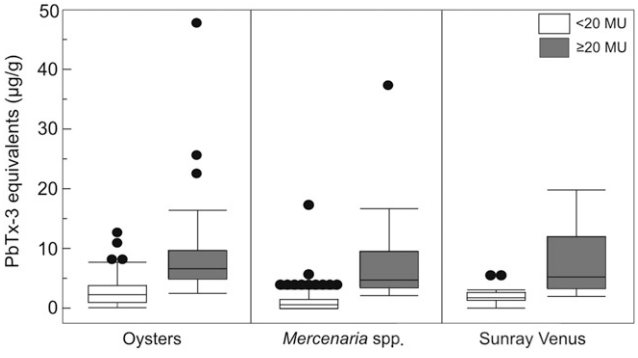

Figure 3. Box and whisker plot illustrating the range of brevetoxin concentrations (μg PbTx-3 eq/g) measured in naturally incurred toxic shellfish samples testing <20 MU/100 g and ≥20 MU/100 g.

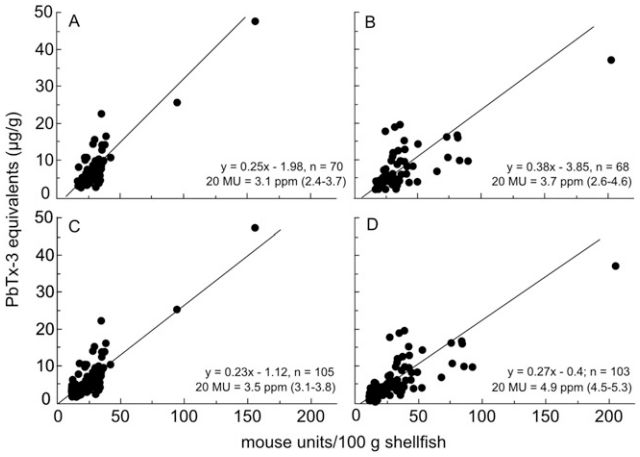

Figure 4. Weighted Deming regressions of brevetoxin concentrations (μg PbTx-3 eq/g) measured by ELISA versus results of the NSP mouse bioassay in naturally incurred toxic clams (left, A & C) and oysters (right, B & D). Plots show only quantitative mouse bioassay data (top, A & B) and data with subset of transformed results included (bottom, C & D).

The method is semiquantitative, yielding numerical results only at values greater than or equal to 20 MU/100 g. Conversely, ELISA is much more sensitive and produces quantitative results at much lower concentrations, capturing a more comprehensive collection of brevetoxins (relative to PbTx-3) and metabolites (regardless of potency) by using antibodies that recognize a portion of the brevetoxin B-type backbone structure (Naar et al. 2002). Given the differences between the mouse bioassay and ELISA (e.g., functional versus structural recognition, units) and differences in the subset of brevetoxins the assays measure (nonpolar, ether-extractable toxins versus predominantly PbTx-2-type toxins with a wider range of polarities), strong agreement between numerical results was not expected.

Variable cross-reactivities of the antibodies with brevetoxin congeners and metabolites likely contributed to the lack of strong agreement with the mouse bioassay. The recognition epitope of the antibodies in this ELISA is believed to include the last four rings, excluding the side chain, shared by all brevetoxin B-type toxins, including the secondary metabolites identified thus far (Melinek et al. 1994, Naar et al. 2002). The antibodies cross-react similarly with B-type brevetoxins PbTx-2, PbTx-3, and PbTx-9 (Naar et al. 2002). Cross-reactivity with PbTx-1 (an A-type brevetoxin) is much lower (2.4%) (Flewelling 2017). Cross-reactivity has only been assessed for a few shellfish metabolites, including the cysteine conjugate S-desoxy BTX-B2, found to be 133% relative to PbTx-3, and the brevetoxin lipid conjugate N-palmitoyl BTX-B2, found to be 2.5% (Flewelling 2017).

The relatively low cross-reactivity of the anti-brevetoxin antibodies with PbTx-1 (and presumably PbTx-1-derived conjugates) and N-palmitoyl BTX-B2 indicates that ELISA results can underestimate the total amount of brevetoxin and brevetoxin metabolites present in a sample. Yet, this limited cross-reactivity does not diminish the potential for ELISA to perform successfully within a management program for a number of reasons. First, although PbTx-1 is more potent than PbTx-2, the parent toxins PbTx-1 and PbTx-2 are not found in shellfish, and PbTx-2 type toxins consistently dominate the toxin profile in both *Karenia brevis* cells (Baden & Tomas 1988, Pierce & Henry 2008, Corcoran et al. 2014) and shellfish (Pierce & Henry 2008), typically accounting for 75% or more of the total toxins present. In addition, although lipid conjugates of brevetoxin are thought to contribute substantially to NSP toxicity, these conjugates are derivatives of, and co-occur with, the more abundant amino acid metabolites that dominate the profile of toxic oysters and clams. Enzyme-linked immunosorbent assay readily detects the latter forms, which have been identified as excellent biomarkers of NSP toxicity in oysters and clams (Plakas et al. 2008, Abraham et al. 2015).

A key challenge in comparing the methods was the lack of a validated brevetoxin equivalent of MU in shellfish. Early work by Baden and Mende (1982) established the intraperitoneal toxicity to mice of purified PbTx-2 and PbTx-3 and calculated an LD50 (amount of toxin that kills half of the mice in 24 h) of 0.2 mg/kg (similar for both toxins). This dose was used to derive a PbTx-2 equivalent of 4 μg/20 g-mouse and has since been extended to estimate the brevetoxin concentration in shellfish with a measured toxicity of 20 MU/100 g shellfish as 0.8 mg PbTx-2 equivalents/kg shellfish or 0.8 μg/g (Dickey et al. 2004). This number appears in several guidance documents (NSSP 2017, USFDA 2019), but the complexity of the metabolism of brevetoxins in shellfish was unknown when the estimated equivalence was first proposed. Shellfish exposed to *Karenia brevis* blooms contain a mixture of brevetoxins with a multiplicity of potencies, and applying this estimated equivalent requires information on both the concentrations of the various forms present and their toxicity equivalence factors, which have yet to be established for many brevetoxin metabolites. ELISA provides a composite concentration of the most abundant brevetoxins in shellfish with no information on the identity or toxicity of the forms present. The estimated equivalents for 20 MU/100 g shellfish using ELISA with PbTx-3 as a standard exceeded 3 μg/g, and the comparative data presented here illustrate that brevetoxin concentrations in shellfish that measure below 20 MU/100 g often exceed 0.8 μg/g by ELISA (Fig. 3).

Transitioning toward a nonmouse-unit guidance limit for brevetoxins in shellfish requires ensuring a level of protection for human health equal to that provided by the existing federal NSP guidance limit of 20 MU/100 g shellfish. As a conservative, initial step away from complete reliance on the mouse bioassay, values for oysters and clams measured by ELISA were derived that were (1) not more than half the concentrations interpolated at 20 MU/100 g and (2) below the lowest level measured in samples that have tested greater than or equal to 20 MU/100 g (i.e., yielding no false negatives when applied to the existing data set). These values (1.6 μg/g for clams and 1.8 μg/g for oysters) were incorporated into a proposal to the ISSC to approve the MARBIONC brevetoxin ELISA as a limited-use method for NSP testing such that samples with negative results by ELISA ($\leq$1.6 μg/g in clams and $\leq$1.80 μg/g in oysters) would pass, whereas samples with positive results by ELISA (greater than these levels) would require additional testing by the APHA mouse bioassay or a future approved method. A passing ELISA result would enable the same management actions as a passing mouse bioassay result. The ISSC approved this proposal in 2017, and the NSSP Guide for the Control of Molluscan Shellfish now includes the use of the MARBIONC ELISA in NSP management as described earlier (NSSP 2017). Applying the aforementioned thresholds to the comparative data set presented here would produce no false negatives (i.e., no samples testing 20 MU/100 g or greater exceeded these thresholds by ELISA). Among that subset of samples testing less than 20 MU/100 g, ELISA results were at or below the thresholds and would have eliminated the need for testing by mouse bioassay for 41% of oyster samples, 88% of *Mercenaria* spp. samples, and 32% of sunray venus clam samples.

Given the shortcomings and limitations of the APHA mouse bioassay and the hardship it imposes on resource managers and industry, the move toward cost-effective, efficient, and reliable alternative methods must begin. As a first step away from total reliance on the mouse bioassay, ELISA is useful, but the thresholds are conservative. Analytical and screening NSP methods that will be developed and evaluated in the future are also unlikely to show strong agreement with mouse bioassay results, and expectations for comparisons of proposed alternative methods with the mouse bioassay should be gauged accordingly, with a goal of achieving an equal measure of safety rather than perfect, sample-by-sample alignment of results.

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LITERATURE CITED

Abraham, A., K. R. El Said, Y. Wang, E. L. Jester, S. M. Plakas, L. J. Flewelling, M. S. Henry & R. H. Pierce. 2015. Biomarkers of brevetoxin exposure and composite toxin levels in hard clam (*Mercenaria* sp.) exposed to *Karenia brevis* blooms. *Toxicon* 96:82–88.

Abraham, A., S. M. Plakas, L. J. Flewelling, K. R. El Said, E. L. E. Jester, H. R. Granade, K. D. White & R. W. Dickey. 2008. Biomarkers of neurotoxic shellfish poisoning. *Toxicon* 52:237–245.

Abraham, A., Y. Wang, K. R. El Said & S. M. Plakas. 2012. Characterization of brevetoxin metabolism in *Karenia brevis* bloom-exposed clams (*Mercenaria* sp.) by LC-MS/MS. *Toxicon* 60:1030–1040.

Adams, C., L. Sturmer & A. Hodges. 2014. Tracking the economic benefits generated by the hard clam aquaculture industry in Florida. University of Florida IFAS Electronic Data Information Source, EDIS FE96. Accessed September 1, 2016. Available at: http://shellfish.ifas.ufl.edu/wp-content/uploads/2012-Clam-Economic-Impact.pdf.

Baden, D. G. 1989. Brevetoxins: unique polyether dinoflagellate toxins. *FASEB J*. 3:1807–1817.

Baden, D. G., A. J. Bourdelais, H. Jacocks, S. Michelliza & J. Naar. 2005. Natural and derivative brevetoxins: historical background, multiplicity, and effects. *Environ. Health Perspect*. 113:621–625.

Baden, D. G. & T. J. Mende. 1982. Toxicity of two toxins from the Florida red tide marine dinoflagellate, *Ptychodiscus brevis*. *Toxicon* 20:457–461.

Baden, D. G. & C. R. Tomas. 1988. Variations in major toxin composition for six clones of *Ptychodiscus brevis*. *Toxicon* 26:961–963.

Collins, A. B., C. M. Adams, A. Hodges & B. Hurt. 2019. Red tide stinks: economic impacts of shellfish closures associated with harmful algal blooms in southwest Florida. Presented at Aquaculture 2019, New Orleans, LA, March 7–11, 2019. World Aquaculture Society. Accessed March 10, 2020. Available at: https://www.was.org/meetings/ShowAbstract.aspx?Id=135764.

Corcoran, A. A., B. Richardson & L. J. Flewelling. 2014. Effects of nutrient-limiting supply ratios on toxin content of *Karenia brevis* grown in continuous culture. *Harmful Algae* 39:334–341.

Daguer, H., R. B. Hoff, L. Molognoni, C. R. Kleemann & L. V. Felizardo. 2018. Outbreaks, toxicology, and analytical methods of marine toxins in seafood. *Curr. Opin. Food Sci*. 24:43–55.

Dickey, R., E. Jester, R. Granade, D. Mowdy, C. Moncreiff, D. Rebarchik, M. Robl, S. Musser & M. Poli. 1999. Monitoring brevetoxins during a *Gymnodinium breve* red tide: comparison of sodium channel specific cytoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. *Nat. Toxins* 7:157–165.

Dickey, R. W., S. M. Plakas, E. L. E. Jester, K. R. El Said, J. N. Johannessen, L. J. Flewelling, P. Scott, D. G. Hammond, F. M. V. Dolah, T. A. Leighfield, M.-Y. B. Dachraoui, J. S. Ramsdell, R. H. Pierce, M. S. Henry, M. A. Poli, C. Walker, J. Kurtz, J. Naar, D. G. Baden, S. M. Musser, K. D. White, P. Truman, A. Miller, T. P. Hawryluk, M. M. Wekell, D. Stirling, M. A. Quilliam & J. K. Lee. 2004. Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger, K. A., J. H. Landsberg, C. R. Tomas & G. A. Vargo, editors. Harmful algae 2002. St. Petersburg, FL: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 300–302.

Fauquier, D. A., L. J. Flewelling, J. Maucher, C. A. Manire, V. Socha, M. J. Kinsel, B. A. Stacy, M. Henry, J. Gannon, J. S. Ramsdell & J. H. Landsberg. 2013a. Brevetoxin in blood, biological fluids, and tissues of sea turtles naturally exposed to *Karenia brevis* blooms in central west Florida. *J. Zoo Wildl. Med*. 44:364–375.

Fire, S. E., D. Fauquier, L. J. Flewelling, M. Henry, J. Naar, R. Pierce & R. S. Wells. 2007. Brevetoxin exposure in bottlenose dolphins (*Tursiops truncatus*) associated with *Karenia brevis* blooms in Sarasota Bay, Florida. *Mar. Biol*. 152:827–834.

Fire, S. E., L. J. Flewelling, J. Naar, M. J. Twiner, M. S. Henry, R. H. Pierce, D. P. Gannon, Z. Wang, L. Davidson & R. S. Wells. 2008. Prevalence of brevetoxins in prey fish of bottlenose dolphins in Sarasota Bay, Florida. *Mar. Ecol. Prog. Ser*. 368:283–294.

Flewelling, L. J. 2017. MARBIONC ELISA for the determination of neurotoxic shellfish poisoning (NSP) toxins in molluscan shellfish (ISSC proposal 17–107, supporting documents). Interstate Shellfish Sanitation Conference. Accessed February 3, 2020. Available at: http://www.issc.org/Data/Sites/1/media/00-2017biennialmeeting/–taskforcei2017/17-107-supporting-documentation.pdf.

Flewelling, L. J., D. H. Adams, J. P. Naar, K. A. Atwood, A. A. Granholm, S. N. O'Dea & J. H. Landsberg. 2010. Brevetoxins in sharks and rays (Chondrichthyes, Elasmobranchii) from Florida coastal waters. *Mar. Biol*. 157:1937–1953.

Flewelling, L. J., J. P. Naar, J. P. Abbott, D. G. Baden, N. B. Barros, G. D. Bossart, M.-Y. D. Bottein, D. G. Hammond, E. M. Haubold, C. A. Heil, M. S. Henry, H. M. Jacocks, T. A. Leighfield, R. H. Pierce, T. D. Pitchford, S. A. Rommel, P. S. Scott, K. A. Steidinger, E. W. Truby, F. M. Van Dolah & J. H. Landsberg. 2005. Red tides and marine mammal mortalities. *Nature* 435:755–756.

Griffith, A. W., S. E. Shumway & A. K. Volety. 2013. Bioaccumulation and depuration of brevetoxins in the eastern oyster (*Crassostrea virginica*) and the northern quahog (= hard clam, *Mercenaria mercenaria*). *Toxicon* 66:75–81.

Heil, D. C. 2009. *Karenia brevis* monitoring, management, and mitigation for Florida molluscan shellfish harvesting areas. *Harmful Algae* 8:608–610.

Interstate Shellfish Sanitation Conference. Single lab validation for laboratory methods. Accessed September 28, 2018. Available at: http://www.issc.org/single-lab-validation-for-laboratory-methods.

Ishida, H., A. Nozawa, H. Nukaya, L. Rhodes, P. McNabb, P. T. Holland & K. Tsuji. 2006. Brevetoxin metabolism in shellfish associated with neurotoxic shellfish poisoning. In: Njapeu, H., S. Trujillo, H. P. van Egmond & D. L. Park, editors. Mycotoxins and phycotoxins: advances in determination, toxicology, and exposure management. Amsterdam, The Netherlands: Wageningen Academic Publishers. pp. 297–307.

Leary, S., W. Underwood, R. Anthony, S. Cartner, D. Corey, T. Grandin, C. Greenacre, S. Gwaltney-Brant, M. A. McCrackin, V. R. Meyer, D. Miller, J. Shearer & R. Yanong. 2013. AVMA guidelines for the euthanasia of animals, 2013 edition. Schaumburg, IL: American Veterinary Medical Association.

Magaña, H. A., C. Contreras & T. A. Villareal. 2003. A historical assessment of *Karenia brevis* in the western Gulf of Mexico. *Harmful Algae* 2:163–171.

Melinek, R., K. S. Rein, D. R. Schultz & D. G. Baden. 1994. Brevetoxin PbTx-2 immunology: differential epitope recognition by antibodies from two goats. *Toxicon* 32:883–890.

Miao, W., Y. R. Gel & J. L. Gastwirth. 2006. A new test of symmetry about an unknown median. In: Hsiung, A., C.-H. Zhang & Z. Ying, editors. Random walk, sequential analysis and related topics—a festschrift in honor of yuan-shih chow. Singapore, Singapore: World Scientific Publisher. pp. 199–215.

Munday, R. 2014. Toxicology of seafood toxins: a critical review. In: Botana, L. M., editor. Seafood and freshwater toxins: pharmacology, physiology, and detection, 3rd edition. Boca Raton, FL: CRC Press. pp. 197–290.

Naar, J., A. Bourdelais, C. Tomas, J. Kubanek, P. L. Whitney, L. J. Flewelling, K. A. Steidinger, J. Lancaster & D. G. Baden. 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. *Environ. Health Perspect*. 110:179–185.

Naar, J. P., L. J. Flewelling, A. Lenzi, J. P. Abbott, A. Granholm, H. M. Jacocks, D. Gannon, M. Henry, R. Pierce, D. G. Baden, J. L. Wolny & J. L. Landsberg. 2007. Brevetoxins, like ciguatoxins, are potent ichthyotoxic neurotoxins that accumulate in fish. *Toxicon* 50:707–723.

Naar, J., J. Kubanek, A. L. Weidner, L. J. Flewelling, A. Bourdelais, K. Steidinger & D. G. Baden. 2004. Brevetoxin depuration in shellfish via production of non-toxic metabolites: consequences for seafood safety and the environmental fate of brevetoxins. In: Steidinger, K. A., J. H. Landsberg, C. R. Tomas & G. A. Vargo, editors. Harmful algae 2002. St. Petersburg, FL: Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO. pp. 488–490.

National Institutes of Health. 2002. Public Health Service policy on humane care and use of laboratory animals. Bethesda, MD: National Institutes of Health.

National Institutes of Health. 2015. Public Health Service policy on humane care and use of laboratory animals, revised. Bethesda, MD: National Institutes of Health. Accessed March 10, 2020. Available at: https://grants.nih.gov/grants/olaw/references/PHSPolicyLabAnimals.pdf.

National Marine Fisheries Service. 2018. Fisheries of the United States, 2017. U.S. Department of Commerce, NOAA Current Fishery Statistics No. 2017. Accessed February 25, 2020. Available at: https://www.fisheries.noaa.gov/resource/document/fisheries-united-states-2017-report.

National Oceanic and Atmospheric Administration. 2004. Action plan for harmful algal blooms and the Gulf of Mexico coastal ocean observing system: results from a regional workshop. NOAA/CSC/20516-PUB. Charleston, SC: NOAA Coastal Services Center. Accessed February 25, 2020. Available at: https://tidesandcurrents.noaa.gov/hab/hab_publication/HAB_GCOOS_report.pdf.

National Research Council of the National Academies. 2011. Guide for the care and use of laboratory animals, 8th edition. Washington, DC: The National Academies Press. 220 pp.

National Shellfish Sanitation Program. 2017. Guide for the control of molluscan shellfish: 2017 revision. Washington, DC: U.S. Food and Drug Administration and Interstate Shellfish Sanitation Conference. 478 pp.

Pierce, R. H. & M. S. Henry. 2008. Harmful algal toxins of the Florida red tide (*Karenia brevis*): natural chemical stressors in South Florida coastal ecosystems. *Ecotoxicology* 17:623–631.

Plakas, S. M. & R. W. Dickey. 2010. Advances in monitoring and toxicity assessment of brevetoxins in molluscan shellfish. *Toxicon* 56:137–149.

Plakas, S. M., E. L. Jester, K. R. El Said, H. R. Granade, A. Abraham, R. W. Dickey, P. S. Scott, L. J. Flewelling, M. Henry, P. Blum & R. Pierce. 2008. Monitoring of brevetoxins in the *Karenia brevis* bloom-exposed eastern oyster (*Crassostrea virginica*). *Toxicon* 52:32–38.

Plakas, S. M., Z. Wang, K. R. El-Said, E. L. E. Jester, H. R. Granade, L. Flewelling, P. Scott & R. W. Dickey. 2004. Brevetoxin metabolism and elimination in the eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. *Toxicon* 44:677–685.

Sebaugh, J. L. & P. D. McCray. 2003. Defining the linear portion of a sigmoid-shaped curve: bend points. *Pharm. Stat*. 2:167–174.

Steidinger, K. A. & M. E. Meave del Castillo. 2018. Guide to the identification of harmful microalgae in the Gulf of Mexico, (Vols I and II). St. Petersburg, FL: DiggyPOD, Inc. 570 pp.

Steidinger, K. A., G. A. Vargo, P. A. Tester & C. R. Tomas. 1998. Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico. In: Anderson, D. M., A. D. Cembella & G. M. Hallegraeff, editors. Physiological ecology of harmful algal blooms. Heidelberg, Germany: Springer-Verlag. pp. 133–153.

Tester, P. A. & K. A. Steidinger. 1997. *Gymnodinium breve* red tide blooms: initiation, transport, and consequences of surface circulation. *Limnol. Oceanogr*. 42:1039–1051.

Thyng, K., R. Hetland, M. Ogle, X. Zhang, F. Chen & L. Campbell. 2013. Origins of *Karenia brevis* harmful algal blooms along the Texas coast. *Limnol. Oceanogr. Fluids Environ*. 3:269–278.

Tunnell, J. W., Jr. 2017. Shellfish of the Gulf of Mexico. In: Ward, C. H., editor. Habitats and biota of the Gulf of Mexico: before the deepwater horizon oil spill. New York, NY: Springer. pp. 769–839.

Turner, A. D., C. Higgins, K. Davidson, A. Veszelovszki, D. Payne, J. Hungerford & W. Higman. 2015. Potential threats posed by new or emerging marine biotoxins in UK waters and examination of detection methodology used in their control: brevetoxins. *Mar. Drugs* 13:1224–1254.

U.S. Food and Drug Administration. 2019. Fish and fishery products hazards and controls, 4th edition. Accessed February 25, 2020. Available at: https://www.fda.gov/food/seafood-guidance-documents-regulatory-information/fish-and-fishery-products-hazards-and-controls.

Van Dolah, F. M. 2000. Diversity of marine and freshwater algal toxins. In: Botana, L. M., editor. Seafood and freshwater toxins: pharmacology, physiology, and detection. New York, NY: Marcel Dekker, Inc. pp. 19–43.

Wang, Z., S. M. Plakas, K. R. E. Said, E. L. E. Jester, H. R. Granade & R. W. Dickey. 2004. LC/MS analysis of brevetoxin metabolites in the eastern oyster (*Crassostrea virginica*). *Toxicon* 43:455–465.

Watkins, S. M., A. Reich, L. E. Fleming & R. Hammond. 2008. Neurotoxic shellfish poisoning. *Mar. Drugs* 6:431–455.