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Bioaccumulation and depuration of brevetoxins in the eastern oyster (*Crassostrea virginica*) and the northern quahog (= hard clam, *Mercenaria mercenaria*)

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ABSTRACT

The eastern oyster (*Crassostrea virginica*) and northern quahog (= hard clam, *Mercenaria mercenaria*) are two species of economic and ecological significance in east coast waters of the United States and the Gulf of Mexico. Commercial industries for these species, especially within the state of Florida, are significant. The current study was undertaken to build upon the already established body of knowledge surrounding effects of the toxic dinoflagellate *Karenia brevis* on shellfish, to provide an understanding of the kinetics of brevetoxins within shellfish tissues, and to provide an estimate of brevetoxin retention times in these shellfish after a bloom event.

Individual clams and oysters were exposed to the toxic dinoflagellate, *K. brevis* at a bloom concentration of 5×10^5 cells·L^{-1} for eight days and then transferred to filtered water for depuration. Individuals were sampled periodically to determine depuration rates. Concentrations of brevetoxins (and/or their metabolites measured as PbTx-3 equivalent) in tissues were determined using an Enzyme Linked Immunosorbent Assay (ELISA). After five days of exposure, brevetoxin levels in tissues of both species reached concentrations well above the regulatory limit of 800 ng g^{-1} (Pb-TX3 equivalent). Averaged concentration of brevetoxins in clams was 1000 ng g^{-1}, while the oysters averaged 1986 ng g^{-1}. After two weeks of depuration, tissue concentrations in both species were below regulatory levels with clams averaging ~204 ng g^{-1} and oysters averaging ~437 ng g^{-1}. Toxins (or their metabolities) remained detectable in both clams (139 days) and oysters (82 days) for the duration of the experiment.

1. Introduction

Worldwide, there are approximately 300 species of microalgae that have adverse impacts within marine ecosystems (Hallegraeff et al., 1995; Smayda, 1997). Species-specific impacts vary, but range from sub-lethal effects on marine fauna, wide spread finfish and shellfish kills, and marine mammal and bird mortalities (Shumway and Cucci, 1987; Shumway, 1990; Landsberg, 2002; Shumway et al., 2003; Flewelling et al., 2005; Cohen et al., 2007). Over the past several decades bloom events of increasing intensity, frequency, and geographic distribution have been noted (Hallegraeff, 1993), due unquestionably to a number of factors including increased awareness, advances in detection techniques, the increased use of coastal areas for aquaculture, anthropogenic influences on nutrient loading and climate change, and possible introduction of resting cysts in the ballast water of transport ships (Hallegraeff, 1993; Hallegraeff et al., 1995; Hégaret et al., 2009).

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The harmful impacts of these algae are manifested in a variety of ways: 1) direct contact with associated toxins, i.e., the uptake of intact cells through ingestion, contact with whole cells, and exposure to extracellular toxins after lysis; 2) mechanical or physical damage to gill tissues of finfish or shellfish; and 3) anoxic conditions in the water column following dense blooms (Shumway, 1990; Hallegraeff et al., 1995; Landsberg, 2002). Vertebrates, including humans, may be exposed via inhalation of aerosolized toxins, e.g., toxins associated with *Karenia brevis* (brevetoxins) and *Pfiesteria* spp. are known to become aerosolized and subsequently inhaled by marine mammals and other air breathing organisms, causing respiratory distress (Pierce et al., 2005; Pierce and Henry, 2008; Glasgow et al., 1995). Brevetoxins can also become concentrated within the tissues of bivalve molluscs, rendering them toxic to humans and other vertebrate consumers, resulting in various forms of shellfish poisoning. Brevetoxins result in Neurotoxic Shellfish Poisoning (NSP) (Steidinger et al., 1998; Poli et al., 2000; Shumway, 1990; Hégaret et al., 2009) with symptoms ranging from mild gastrointestinal distress to bronchoconstriction posing a significant threat to both human health and aquaculture industries (Shumway, 1990; Hallegraeff et al., 1995; Matsuyama and Shumway, 2009).

Although several species of harmful algae exist naturally within the waters of the Gulf of Mexico, the most common species is the dinoflagellate *K. brevis* (Steidinger et al., 1999). While it can be present in other areas of the Gulf such as Louisiana, Mississippi, and Texas, it occurs most often on the west coast of Florida (Tester and Steidinger, 1997; Brand and Compton, 2007). There is some evidence that the intensity and/or duration of blooms within this region has been increasing, with documented blooms lasting as long as 13 months (Heil et al., 2006; Brand and Compton, 2007; Brand et al., 2012). Other reports of *Karenia* spp. have been documented in waters of the Caribbean, New Zealand, and the English Channel (Lackey, 1956; Nozawa et al., 2003; Llewellyn et al., 2005).

Toxins produced by *K. brevis* affect a wide range of organisms including zooplankton, bivalve molluscs, marine mammals, and humans (Shumway, 1990; Landsberg, 2002; Flewelling et al., 2005; Leverone et al., 2006; Cohen et al., 2007; Brand et al., 2012). The potential harm to shellfish aquaculture includes both economic and human health related impacts (Shumway, 1990; Matsuyama and Shumway, 2009). Shellfish species, via the consumption of *K. brevis*, become toxic to human consumers which in turn can have negative economic impacts on shellfish aquaculture (Cummins et al., 1971; McFarren et al., 1965). There is a large body of knowledge addressing the effects of *K. brevis* on marine organisms, yet we know little about the kinetics of brevetoxins within the tissues of bivalve molluscs.

Shumway et al. (1990) compiled data relating to toxin retention within species of shellfish and performed field experiments concerning the retention of toxins within species of oysters (*Crassostrea virginica* and *Ostrea edulis*) and mussels (*Mytilus edulis*). The depuration of shellfish toxins to below regulatory levels is dependent on a variety of factors, i.e., species of shellfish, time of exposure, and environmental factors (Shumway et al., 1990; Matsuyama and Shumway, 2009). Understanding the depuration of these toxins is of critical importance for management of monitoring programs and aquaculture facilities. Knowing when shellfish are close to quarantine levels may make regulatory monitoring more efficient (=less expensive) after intense bloom events and will provide information to the public as to when the harvesting of certain species of shellfish is safe.

Information regarding the retention of brevetoxins in shellfish and how long they pose a threat to human consumers is lacking. This research addresses the following questions: How long does it take for *Mercenaria mercenaria* and *C. virginica* to accumulate brevetoxins to levels in their tissues that pose a threat to human consumers? After intoxification, how long does it take for toxin concentrations within shellfish tissues to fall below safe limits for human consumption? Finally, how long will these toxins remain in tissues at concentrations below regulatory limits? The objective of this study is to examine the persistence of brevetoxins in oysters and clam tissues exposed to *K. brevis*. Results will have direct impacts on the regulatory practices regarding the closures of shellfish beds, provide regulators with information on how long different species of shellfish may remain toxic after exposure to *K. brevis*, and help to decrease monetary losses to local shellfish growers.

2. Materials and methods

2.1. Bivalve collection and maintenance

Adult eastern oysters (*C. virginica*) (average length 82.9 ± 3.1 mm) were collected in early June from Estero Bay, FL, with all epibionts removed. Northern quahogs (= hard clam, *M. mercenaria*) (average length 40.5 ± 3.1 mm, average width 45.7 ± 3.1 mm) were obtained from Cutthroat Clams, an aquaculture facility on Pine Island, FL. Both clams and oysters were kept in 25 L tanks with 25 individuals per tank in sterilized raw seawater at a salinity of 30. Each tank received 0.1 g Shellfish Diet© individual^{-1}day^{-1} (Chu and Volety, 1997).

2.2. Maintenance of cultures

The toxic dinoflagellate, *K. brevis* (CCMP #2229) was cultured in 8 L carboys in L1 medium (minus Si) at a salinity of 30 and at a temperature of 22–24 °C. Cultures were maintained on a 12:12 light dark cycle. Artificial seawater was passed through a 1 μm filter then autoclaved for 45 five minutes, cooled overnight, after which medium was added prior to inoculation. All cultures used for exposure were approximately 10–14 days old. Cell concentrations were determined by taking an aliquot from the main cultures, making ten-fold dilutions, and then counting them under a light microscope. Triplicate counts were made and averaged prior to dosing exposure tanks.

2.3. Experimental design

Twenty five individuals of each species were maintained in each tank with 25 L of raw sterilized seawater ($N = 5$ replicate tanks for each species and treatment) for

Table 1
Retention times of neurotoxic shellfish toxins in different species of shellfish.

Species of shellfish	Species of algae	Associated toxins	Regulatory limits	Time to regulatory limits	References
Crassostrea virginica (eastern oyster)	*Karenia brevis* (formerly *Gymndodinium breve*)	Neurotoxic shellfish toxins	80 μg 100 g^{-1}	2–6 weeks	Morton and Burklew, 1969
Crassostrea gigas (Pacific oyster)	*Karenia brevis* (formerly *Gymnodinium breve*)	Neurotoxic shellfish toxins	80 μg 100 g^{-1}	>5 days	Fletcher et al., 1998
Meretrix casta (grey clam)	*Pyrodinium bahamense* (not specified)	Paralytic shellfish toxins	80 μg 100 g^{-1}	<1 month	Karunasagar et al., 1984
Chione cancellata (crossed-bar venus clam)	*Karenia brevis* (formerly *Gymnodinium breve*)	Neurotoxic shellfish toxins	80 μg 100 g^{-1}	7 months	Steidinger et al., 1998
Mercenaria campechiensis (southern quahog)	*Karenia brevis* (formerly *Gymnodinium breve*)	Neurotoxic shellfish toxins	80 μg 100 g^{-1}	5 months	Steidinger et al., 1998

both exposed and control treatments. Exposed shellfish were fed 5×10^5 cells·L^{-1} *K. brevis* along with Shellfish Diet© (0.1 g individual^{-1} day^{-1}), dosed daily for eight consecutive days. Control treatment groups were fed only Shellfish Diet© (Donaghy and Volety, 2011). Individuals from each tank were intensively sampled every 2–3 days for brevetoxin determination up to Day 30, and then every 5–7 days from day 30 till the end of the experiment. Samples were stored at −80 °C until further brevetoxin analysis.

2.4. Tissue analysis

Single individual tissues ($N = 5$ replicates for each species and treatment) were analyzed for brevetoxins using a competitive ELISA (Naar et al., 2002). Brevetoxins were extracted from shellfish tissues according to methods outlined by Dr. Leanne Flewelling (Personal Communication, Florida Wildlife Research Institute; Naar et al., 2002). Shellfish were shucked and homogenized using a tissue homogenizer. Two gram aliquots were taken and mixed with 80% methanol. Samples were then incubated in a water bath for 20 min at a temperature of 60 °C and cooled in an ice bath for 10 min. Tissues were then centrifuged for 10 min at 3000 rcf and the supernatant poured off and stored. The process was then repeated twice for each sample. Extracts were then washed in hexane and stored at −20 °C until analysis.

Toxin assays were performed in a 96 well microplate. Dilutions were prepared in cluster tubes and added accordingly to the plate in duplicate. Brevetoxins linked to Bovine Serum Albumin were then added to each well (100 μl/well) of the microplate (Nunc Maxisorp Plates©) and incubated on a plate shaker for 1 h. Plates were then washed with Phosphate Buffered Saline (PBS). Blocking buffer (Superblock dry Blend©) was added to each well (200 μl/well) and incubated for ½ hour. Plates were then washed with a PBS-Tween solution, samples and positive controls were added along with primary antibodies to each well (100 μl/well), and the plates were incubated on a plate shaker for 1 h. After incubation, plates were washed again using PBS-Tween. Secondary antibodies labeled with horseradish peroxidase were then added to each well and incubated on a plate shaker for 1 h. Plates were washed using PBS-Tween and bound antibodies were visualized by the addition of tetramethylbenzidine (TMB) (100 μl/well). The colorimetric reaction was stopped by the addition of 0.5 M Sulfuric Acid (100 μl/well). Absorbances were read at a wavelength of 450 nm on a plate reader (Dynex Technologies©). Concentrations of brevetoxins (ng/g) are expressed as ng/g PbTX-3 equivalents.

2.5. Statistical analysis

A repeated measures analysis (sphericity assumed) was used to detect differences in mean brevetoxin concentration in shellfish tissues over time (Tables 2 and 3). Results were deemed significant at $P < 0.05$.

3. Results

3.1. Oysters

After five days of exposure to *K. brevis*, toxin concentrations in oysters were above the regulatory limit (800 ng g^{-1}) at 1986 ± 185 ng g^{-1} SE. At the end of the exposure period, the average toxin concentration was 1126 ± 185 ng g^{-1} SE. After two weeks of depuration, tissue concentrations were below 800 ng g^{-1} at 437 ± 185 ng g^{-1} SE. The tissue concentration at the end of the experiment (82 days) in the last surviving oyster was 117 ng g^{-1}. Some control samples contained very low amounts of brevetoxins (or their metabolites) throughout the experiment (60 ± 37 ng g^{-1} SE; Fig. 1). Significant differences in brevetoxin tissue concentrations within species were noted between sampling day and species ($P < 0.05$). In oysters, significant differences were found throughout the period of exposure. Tissue concentrations were significantly higher between days five through eighteen compared to the other sampling days ($P < 0.05$). Overall, oysters generally had a higher body burden of brevetoxins than clams ($P < 0.05$).

Table 2
Repeated measures analyses of brevetoxin concentrations in oysters during the experimental period.

Source		Type III sum of squares	df	Mean Square	*F*	Sig
Days	Spherecity Assumed	13890797.46	11	1262799.769	3.098	0.004
Error (Days)	Sphericity Assumed	17936612.04	44	407650.274		

Table 3
Repeated measures analysis of brevetoxin concentrations in clams during the experimental period.

Source		Type III sum of squares	df	Mean square	*F*	Sig.
Days	Sphericity assumed	1.158E7	23	503371.207	9.892	.000
Error (days)	Sphericity assumed	4681679.872	92	50887.825		

3.2. Clams

Toxins rapidly accumulated in clam tissues, reaching concentrations above the regulatory limit. After the first three days of exposure, toxin concentrations in exposed clams averaged 551 ± 185 ng g^{-1} SE (Fig. 2). After five days of exposure, concentrations averaged 1000 ± 185 ng g^{-1} SE. Peak concentrations after nine days of exposure were 1002 ± 185 ng g^{-1} SE, but decreased rapidly during depuration. After two weeks of depuration, average tissue concentrations were well below the 800 ng g^{-1} limit, averaging 204 ± 185 ng g^{-1} SE. Toxin concentrations continued to decrease with time and remained present for the duration of the experiment (<100 ng g^{-1}; 139 days). Final toxin concentrations were 58 ± 239 ng g^{-1} SE. Brevetoxins were not present in any of the control samples. While there were differences between days, in general tissue concentrations between days five through nine were significantly higher than the rest ($P < 0.05$).

3.3. Mortality

There was mortality in oyster tanks with 30 dead individuals during the exposure (8 days) compared to only two individuals in control tanks. Thereafter (10 weeks), there were another 22 deaths in exposed oyster tanks while there were 30 deaths in control tanks giving a cumulative mortality of 41% and 19% for exposed and control oysters respectively. Samples were continually taken until there were no remaining oysters. No significant mortality was encountered in clam tanks during exposure. After seven weeks of depuration, mortality in exposed clam tanks was 1.6% (two individuals) while there were no losses in control tanks. Samples were taken until there were no remaining live clams.

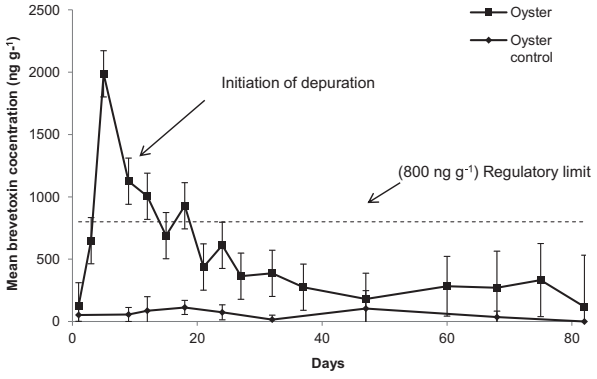

Fig. 1. Exposed and control oyster (*C. virginica*) brevetoxin tissue concentrations throughout the course of the experiment (error bars indicate standard error).

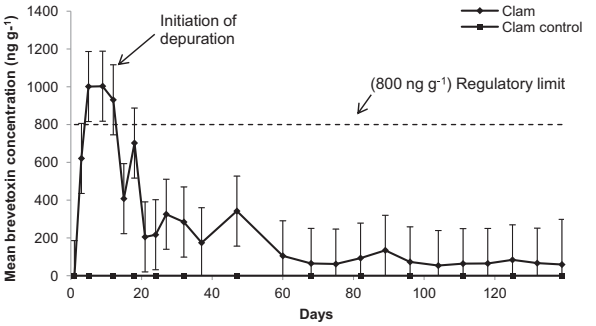

Fig. 2. Exposed and control clam (*M. mercenaria*) tissue concentrations of brevetoxins throughout course of experiment (error bars indicate standard error).

4. Discussion

To date, detection of brevetoxins in a variety of matrices (e.g., seawater, blood, urine, and various tissues of marine organisms) is accomplished by using mouse bioassay, neuroblastoma cell assay, high performance liquid chromatography mass spectroscopy (HPLC/MS), receptor binding assay, a competitive electrochemiluminescence based immuno-assay (ECL), and a competitive Enzyme Linked Immunosorbent Assay (ELISA). Each method has its advantages and potential drawbacks, including cost, handling of potentially harmful reagents, and sensitivity (Dickey et al., 1999; Naar et al., 2002; Poli et al., 2007; Plakas et al., 2008). Given that the mouse bioassay is being phased out, demonstration of the efficacy of other methods is essential.

For the purposes of this research the ELISA assay was used due to its high sensitivity, ease of use, relatively low cost, and short time for analysis (Naar et al., 2002). Sample preparation time such as collection, weighing, shucking, and homogenization still remain in this method as with other methods, but one ELISA assay can be run in as few as 6 h (Naar et al., 2002). Potential drawbacks to this particular method are that the antibodies themselves detect the brevetoxin backbone or metabolites. Therefore, it is not possible to determine the composition of the individual toxins or their toxicity using this assay. In multiple laboratory studies, however, ELISA results have correlated well with the mouse bioassay (which detects toxicity) and other methods indicating that it is a good alternative (Dickey et al., 2004; Poli et al., 2007; Plakas et al., 2008; Plakas and Dickey, 2010).

In the current study, oysters and clams readily accumulated brevetoxins to concentrations above the regulatory limit of 800 ng g^{-1} (PBTx-3 equivalent) even at a relatively modest dose (Figs. 1 and 2). Although individual shellfish were exposed to 5×10^5 cells·L^{-1} in the current study, bloom conditions occurring in the field can be significantly more concentrated reaching $>10 \times 10^6$ cells·L^{-1}. Exposed oysters were toxic after four days. Previous studies have shown oysters exposed to blooms

become toxic after only 24 h (Shumway et al., 1990; Plakas et al., 2002; Plakas and Dickey, 2010). In the current study, peak concentrations were observed by day five. While concentrations at day nine appear lower than at day five, there was no significant difference in toxin concentration, most likely due to high individual variability. Toxin concentrations were below 800 ng g^{-1} after two weeks of depuration. Brevetoxins, as determined by the ELISA assay, also remained present in tissue, at low concentrations, for extended periods of time (>82 days) as has been shown in previous studies where brevetoxins were still detected even after six months of depuration (Plakas et al., 2002, 2008).

Individual variability of toxin accumulation in oysters was extremely high at each sampling time and was especially pronounced during the exposure period (Fig. 1). Such variability is not unexpected or uncommon. White et al. (1993) showed high variability between several species of shellfish as well as between individuals of the same species, all collected from the same sample on the Georges Bank. In the present study, tissue concentration of brevetoxins in individual samples from replicate tanks ranged from approximately 500 ng g^{-1} to nearly 4500 ng g^{-1} (Fig. 2). Factors influencing individual ranges include feeding variability, factors that influence how species of shellfish feed such as salinity, temperature, and availability of diverse food sources (White et al., 1993; Bricelj and Shumway, 1998; Ward and Shumway, 2004). Although physical conditions in the experimental tanks during the experiment were identical, high individual variability was still present. Given the low concentrations of toxins in shellfish tissue by the end of the experiment, individual variability was correspondingly low.

Results of this experiment corroborate existing knowledge regarding how quickly oysters depurate other shellfish toxins. Generally, oysters are considered to be fast to moderate detoxifiers (Shumway et al., 1990; Bricelj and Shumway, 1998). The retention of brevetoxins in *C. virginica* in the current study was relatively short-lived. The retention of PSP toxins in *Crassostrea echninata* tissue has been observed to last for periods from three weeks to four months (Maclean, 1975; Worth et al., 1975). These results also compare well with previous research monitoring brevetoxins in *C. virginica*, where samples were toxic from two to six weeks after a bloom of *K. brevis* (Morton and Burklew, 1969). When compared to retention of brevetoxins in other species of shellfish, retention time for the toxins in this study was very short. Previous studies have found brevetoxins present in the clam (*Crassostrea cancellata*) for as long as seven months and in the quahog (*Mercenaria campechiensis*) for five months (Steidinger et al., 1998); however, there may have been more than one toxic episode in those cases, and differences are also expected between species.

Tissue concentrations in clams (*M. mercenaria*) were above the 800 ng g^{-1} limit after five days of exposure to a bloom concentration (Fig. 2). Peak concentrations of brevetoxins in clam tissues were not statistically different compared to oysters ($P < 0.05$). During the course of exposure, tissue concentrations steadily increased until day nine when depuration was initiated. Tissue concentrations were below 800 ng g^{-1} by day 21 of the experiment (~two week post depuration) and continued to remain at low concentrations over the duration of the experiment. As with oysters, brevetoxins were present at low concentrations in clams for the duration of the experiment, even after 130 days of depuration (Fig. 2). There was considerable variability in toxin concentrations between individual clams, but it was less pronounced compared to oysters.

Retention of brevetoxins in clams was very similar to oysters in this study. As mentioned above, *M. mercenaria* and *M. campechiensis* have been shown to retain toxins for considerably longer periods of time, i.e., up to several months (Steidinger et al., 1998; see Table 1). Differences between the current study and those completed in the past may be due to differences in the sampling designs in each project. The past research involved field collection of individuals from various sites along the west coast of Florida which may have been exposed to exceedingly dense bloom concentrations for extended periods. Individuals in the current study were exposed to relatively modest doses and kept in controlled conditions.

4.1. Mortality

During the course of the experiment there was mortality in oyster tanks, while very few clams perished. One possible explanation is the stress associated with the holding of oysters under static conditions for relatively long periods of time. It is also possible that oysters may be more susceptible to brevetoxins than clams (see review by Shumway et al., 1990) or that oysters were suffering from Dermo disease (see below). Assessment of these factors is beyond the scope of this study.

The protozoan parasite, *Perkinsus marinus* the causative agent for Dermo disease, is a well-studied oyster pathogen which in the past has decimated oyster populations in coastal areas of both the Atlantic and Gulf of Mexico (Burreson and Ragone-Calvo, 1996; Soniat, 1996). *P. marinus* is endemic and naturally present in oysters from Maine to Mexico including southwest Florida estuaries. Previous studies have shown that both salinity and temperature are strong factors influencing the progression of infection with high levels being preferred (Chu and Volety, 1997). Infection intensity is commonly described using a modified Mackin Scale where 0 = no infection, 1 = light, 2 = light-moderate, 3 = moderate, 4 = moderate-heavy, 5 = heavy (Ray, 1954; Mackin, 1962; Volety, 2008). Oysters used in this experiment were collected in early summer (June 2009) from Estero Bay, FL during a period of high salinity coupled with warm temperatures; conditions suitable for Dermo infection. Oysters samples collected nearby in the Estero Bay at the same time were classified as light to moderate on the Mackin Scale (=2.13). Thus, infection with *P. marinus* may have been a contributing factor in oyster mortality.

4.2. Implications

While more work is clearly needed, the current study provides insight regarding the retention of brevetoxins within two commercially important species of shellfish.

Further research could lead to species-specific management of shellfish beds in local areas (see Shumway et al., 1988), enabling certain species of shellfish to be harvested legally during toxin outbreaks based upon their known rates of uptake and depuration of shellfish toxins. A better knowledge of how these organisms depurate brevetoxins could lead to more efficient management of local shellfish beds.

While previous studies have investigated the negative effects of brevetoxins in various species of shellfish, very little research has been conducted regarding the retention of brevetoxins in shellfish tissues. Thus, it is critical to understand the kinetics of brevetoxins within tissue as it may have profound effects on spawning, larvae survival, survival of recently set spat, and overall health of the population. Results of the present study show that brevetoxins or their metabolites may be present even though no *Karenia* is observed, and can remain present in tissue for extended periods of time (>15 weeks). A clearer understanding of the effects brevetoxins at these concentrations is required for the successful restoration and maintenance of local shellfish populations.

Because environmental factors (i.e. salinity, temperature, diversity of food source) have an impact on how certain species of shellfish feed, more experiments need to be performed to determine how these factors may influence the accumulation and depuration of brevetoxins. Dose response experiments may, in combination with other experiments, eventually lead to a model depicting the kinetics of brevetoxins within the tissues of species of shellfish. A better understanding of the kinetics of brevetoxins within tissues of shellfish will aide local regulators with the management of local shellfish beds, providing them with knowledge as to when samples should be collected and enable them to make informed decision regarding their opening and closures.

5. Conclusion

When exposed to bloom concentrations of the toxic dinoflagellate, *K. brevis*, both species of bivalve molluscs, *M. mercenaria* and *C. virginica*, readily accumulated brevetoxins to levels above the regulatory limits (800 ng g^{-1} PbTx-3 equivalent) in less than four days. Clam and oyster tissue concentrations were below the regulatory limit after one week of depuration. Brevetoxins were detectable in both species at the conclusion of each experiment. Oyster tissue concentrations were detectable after 82 days of sampling; toxin remained present in clam tissue for >130 days of sampling. Results of this study provide information to aid local managers of shellfish beds in designing sampling regimes and possibly reducing overall laboratory costs associated with monitoring efforts.

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Conflict of interest statement

None declared.

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