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Bacteriocidal effects of brevetoxin on natural microbial communities

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ABSTRACT

The sensitivity of bacteria to the marine neurotoxins, brevetoxins, produced by the dinoflagellate *Karenia brevis* and raphidophytes *Chattonella* spp. remains an open question. We investigated the bacteriocidal effects of brevetoxin (PbTx-2) on the abundance and community composition of natural microbial communities by adding it to microbes from three coastal marine locations that have varying degrees of historical brevetoxin exposure: (1) Great Bay, New Jersey, (2) Rehoboth Bay, Delaware and (3) Sarasota Bay, Florida. The populations with limited or no documented exposure were more susceptible to the effects of PbTx-2 than the Gulf of Mexico populations which are frequently exposed to brevetoxins. The community with no prior documented exposure to brevetoxins showed significant ($p = 0.03$) changes in bacterial abundance occurring with additions greater than 2.5 μg PbTx-2 L^{-1}. Brevetoxin concentrations during *K. brevis* blooms range from ~2.5 to nearly 100 μg L^{-1} with typical concentrations of ~10–30 μg L^{-1}. In contrast to the unexposed populations, there was no significant decrease in bacterial cell number for the microbial community that was frequently exposed to brevetoxins, which implies variable sensitivity in natural communities. The diversity in the bacterial communities that were sensitive to PbTx-2 declined upon exposure. This suggests that the PbTx-2 was selecting for or against specific species. Mortality was much higher in the 200 μg PbTx-2 L^{-1} treatment after 48 h and >37% of the species disappeared in the bacterial communities with no documented exposure. These results suggest that toxic red tides may play a role in structuring bacterial communities.

1. Introduction

Brevetoxins are potent neurotoxins naturally produced by the dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*) and several species of the raphidophyte *Chattonella* (reviewed in Landsberg, 2002; Brand et al., 2012; Imai and Yamaguchi, 2012). Blooms of these species and their toxins have been linked to fish, shellfish, marine mammal, marine invertebrate and sea bird mortalities, as well as negative human health effects including neurotoxic shellfish poisoning (NSP) and respiratory distress (Kirkpatrick et al., 2004; Landsberg et al., 2009; Fleming et al., 2011). Chemically, brevetoxins are a suite of hydrophobic, polycyclic ether compounds (Baden, 1989; Nicolaou et al., 1998; Vilotijevic and Jamison, 2007). There are 2 structural types and at least 9 natural forms of brevetoxin with brevetoxin 2 (PbTx-2) being the most abundant in nature (Baden, 1989; Pierce et al., 2008; Brand et al., 2012). These compounds are known to negatively affect organisms by activating sodium channels leading to depolarization and alteration of the cell membrane (Baden, 1983; Purkerson et al., 1999; Kirkpatrick et al., 2004). Brevetoxins have also been shown to affect cellular calcium channels and likely impact other yet unidentified metabolic enzymes (Dravid et al., 2004; Kitchen, 2010). No matter what the pathway, the series of reactions that occur to a cell during brevetoxin exposure are complex and have been observed even at the gene expression level (Murrell and Gibson, 2011). The end result of brevetoxin exposure is inflammation of the cell and cell death (Purkerson et al., 1999; Murrell and Gibson, 2011) .

Toxic *K. brevis* blooms frequently disrupt the marine communities along the West Florida shelf and occasionally along the South Atlantic Bight with a range between Jacksonville, FL and Beaufort,

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NC (Buck and Pierce, 1989; Geesey and Tester, 1993; Tester and Steidinger, 1997). In 2000, brevetoxin production associated with a *Chattonella cf. verruculosa sensu* bloom appeared in Rehoboth Bay, DE and its tributaries (Bourdelais et al., 2002). These brevetoxin producing species exist in very different aquatic niches. *K. brevis* blooms nearly annually in the tropical and subtropical oligotrophic waters of the Gulf of Mexico, while *C. cf. verruculosa*, like many *Chattonella* spp. (Imai and Yamaguchi, 2012), is often observed in inland waters including shallow eutrophic canals and bays. This wide geographic range suggests that brevetoxin may impact a large number of diverse estuarine and coastal ecosystems. While extensive research has been done on the effects of brevetoxins on species ranging from zooplankton, marine mammals, humans (Landsberg, 2002; Fleming et al., 2011; Brand et al., 2012), and co-occurring phytoplankton (Kubanek et al., 2005; Prince et al., 2008, 2010), no studies have been conducted on the direct effect of brevetoxins on bacteria or bacterial community composition.

The relationship between bacteria and phytoplankton, especially many harmful algal bloom species, is complex including known symbiotic, antagonistic, predatory and competitive associations (Cole, 1982; Doucette, 1995; Kodama et al., 2006). Still, the role that many marine toxins, including brevetoxins, play in modulating microbial community structure remains enigmatic. Although algicidal bacteria active against *K. brevis* have been identified in culture (Doucette et al., 1999; Mayali and Doucette, 2002; Roth et al., 2008), it is not understood how these relationships translate to natural microbial assemblages. Some algicidal species even enhance brevetoxin release (Roth et al., 2007). Field observations have thus far provided contrasting evidence of the relationship between bacteria and the toxic species *K. brevis*. There is evidence that bacterial biomass as non-algal particles decrease with increasing *K. brevis* biomass, indicating that *K. brevis* may negatively affect natural bacterial populations (Cannizzaro, 2004; Schofield et al., 2006). Thus it has been suggested that decreased bacterial abundance during *K. brevis* blooms may be linked to brevetoxin production (Schofield et al., 2006). However, there are also examples of increased bacterial abundance and productivity within *K. brevis* blooms compared to surrounding waters (Evans, 1973; Heil et al., 2004; Jones et al., 2010) and yet other studies suggest that there is no relationship between bacteria and *K. brevis* abundances (Buck and Pierce, 1989). These results imply a variable response between bulk bacteria communities and the presence of *K. brevis*.

Like *K. brevis*, much of the research on the relationship between bacteria and *Chattonella* spp. has focused on identifying algicidal bacteria (e.g. Lovejoy et al., 1998; Furuki and Kobayashi, 1991; Liu et al., 2008a). There are also studies that have explored bloom promoting bacteria (Liu et al., 2008b). Although great advancements have been made in these areas, the role that the toxins produced by *Chattonella* spp., specifically brevetoxins, play in structuring the microbial community is relatively unexplored.

Field observations of saxitoxin, produced by *Alexandrium* spp. blooms, show shifts in bacterial species dominance when toxin levels are high (Pomati et al., 2003; Jasti et al., 2005), indicating that some species may be more or less tolerant to saxitoxin than others. Additionally, it is a commonly held belief among those who culture *K. brevis* that axenic growth is rarely, if ever, achieved. So there are likely some bacteria that are critical to the ecology of *K. brevis*, the role of the associated bacteria, and the mechanisms that control competition in the marine environment.

Under nutrient depleted conditions some harmful algal species are more likely to produce allelopathic chemicals/toxins than in nutrient replete conditions (Granéli, 2006). It has been hypothesized that this strategy aids in the reduction of nutrient stress by not only eliminating competitors for limiting nutrients but also gaining nutrients provided by their remineralization (Granéli, 2006). Bacterial cell lysis would provide a significant source of dissolved organic matter (DOM; Middelboe and Lyck, 2002; Kawasaki and Benner, 2006) in low nutrient systems. It is possible that toxins play a role in altering the microbial community composition thereby controlling competition and nutrient regulation in addition to deterring grazing (Granéli and Turner, 2006; Granéli, 2006). The ability to take full advantage of a variety of nutrient resources may give *K. brevis* a competitive edge over other bloom forming phytoplankton (Liu et al., 2001; Bronk et al., 2004; Sipler et al., 2013).

This study investigated the effects of PbTx-2 on the bacterial abundance and community composition of natural bacterial populations from 3 geographically separated regions (NJ, DE, and FL). The range of exposures spanned from no documented exposure (NJ) to rare occurrences (DE) to frequent reports of brevetoxin exposure (FL). The goal of this study was to determine if PbTx-2 negatively impacts natural microbial communities and if geographically separated communities respond similarly to brevetoxins.

2. Methods

Brevetoxin (PbTx-2) was added to three different coastal microbial populations to investigate its affect on the bacterial community abundance and composition. Locations were chosen based on available data of brevetoxin exposure. The three locations, Great Bay, NJ (39°32′22″N, 74°23′13″W), Rehoboth Bay, DE (38°39′45″N, 75°07′53″W) and Sarasota Bay, FL (27°19′54″N, 82°34′34″W), represent communities ranging from no documented exposure to brevetoxins, to occasional (Bourdelais et al., 2002) and frequent exposures to bevetoxins (Fire et al., 2007; Pierce and Henry, 2008; Gannon et al., 2009) respectively (Fig. 1). While a lack of documented brevetoxin presence does not necessarily indicate that it has never occurred in these northern areas, the lack of fish kills attributed to brevetoxins, and the lack of high density blooms of brevetoxin producing species does indicate that if present the abundances/brevetoxin concentrations have not been high enough and frequent enough to warrant observation in these highly populated areas. The bacterial community composition samples collected from each of the three sites were compared to the bacterial communities associated with *K. brevis* cultures and a natural *K. brevis* bloom.

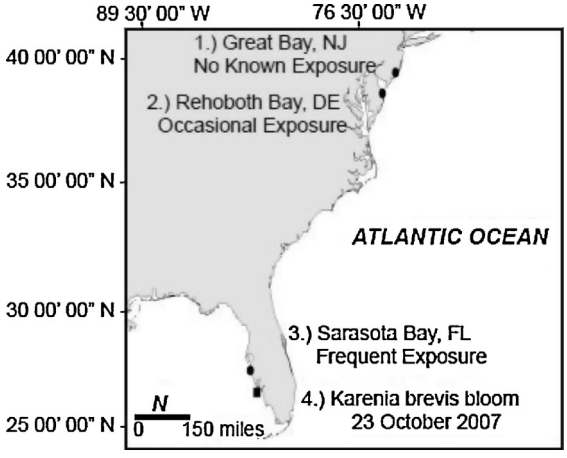

Fig. 1. Map of sample sites. Site 1 is Great Bay, NJ (NJ); site 2 is Rehoboth Bay, DE (DE); site 3 is Sarasota Bay, FL (FL); site 4 is the location of the October 2007 *Karenia brevis* bloom.

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2.1. PbTx-2 additions

Bay water samples were collected via bucket at the surface and were gently filtered through a 3 μm polycarbonate inline filter to remove phytoplankton and zooplankton. The bay water was then divided into 32 mL aliquots and pipetted into 16 acid washed (12% H_2SO_4) and combusted (500 °C) 50 mL glass culture tubes fitted with acid washed and autoclaved (121 °C) caps. The bay water samples were spiked with 0, 2.5, 5, 10, 25, 50, 100, and 200 μg L^{-1} PbTx-2 standard reference material (Calbiochem, San Diego, CA, USA).

Due to brevetoxin's hydrophobic nature, 1 mL of 99.9% pure methanol (MeOH) was used to bring 100 μg of the solid PbTx-2 reference material into solution. The PbTx-2 solution was then diluted with 10 mL deionized water (DIW). All samples were normalized with 10:1 DIW:MeOH to ensure that all contained the same amount of methanol. The final MeOH addition per sample was 0.002% v/v (MeOH: DIW+ field sample). The control treatment (0 μgμPbTx-2μL^{-1}) contained no brevetoxins but an equal amount of methanol required to bring the PbTx-2 into solution. All treatments were run in duplicate. No nutrients were added. Ambient temperatures at the time of collection ranged between 25.5 and 31.5 °C at all 3 locations. Samples were incubated at the appropriate corresponding temperature. Each treatment was sampled at time 0, 24, and 48 h for bacterial abundance and community composition. Bacterial community composition was analyzed for the 0, 25 and 200 μg L^{-1} PbTx-2 treatments at all three bay locations. There appears to be a linear relationship between cell abundance and brevetoxin concentration (Tester et al., 2008). Bloom observations along the West Florida shelf routinely show brevetoxin concentrations between~2.5 and 100 μg brevetoxin L^{-1} (Landsberg, 2002), the range of PbTx-2 concentrations explored by this study. The thresholds of response described in this work should be considered conservative estimates because recent studies have shown that waterborne brevetoxin concentrations specifically PbTx-1 and PbTx-2 are reduced in the presence proteins produced by live or lysing cells (Redshaw et al., 2010, 2011).

2.2. Karenia brevis cultures

Bacteria communities associated with two *K. brevis* cultures maintained at Mote Marine Laboratory (Sarasota, FL) were compared to the bay and bloom communities. Both cultures used were the Wilson strain of *K. brevis* which was initially isolated in 1953 (Wilson and Collier, 1955). Based on the number of years in culture, the bacteria associated with this strain likely represent those species tolerant to brevetoxins. The two cultures of Wilson strain differ in the amount of toxin produced, one producing lower levels of toxin than the other (Pierce, unpublished data). These cultures were selected for this study because they represent bacterial communities with constant yet variable brevetoxin exposure. For this study, the two strains will be designated Wilson high toxin and Wilson low toxin.

2.3. Natural K. brevis bloom transect

K. brevis cell counts and bacterial community composition samples were taken from transects through a natural *K. brevis* bloom in the Gulf of Mexico on 23 October 2007. The *K. brevis* bloom was identified through ship board observations near Sanibel Island, FL in the southeastern portion of the Gulf of Mexico. The bloom transect was based upon *K. brevis* cell counts and samples were collected at 26° 25.9335′ N, 82° 11.520′W, 26° 23.268′ N, 82° 10.864′W, and 26° 26.708′ N, 82° 12.718′W (Fig. 1). Bloom water was collected with a bucket from the water surface. Duplicate phytoplankton cell count samples were taken per transect site and were preserved in Lugols iodine solution. Unfortunately, samples collected during the bloom event for brevetoxin assessments degraded prior to analysis.

2.4. Analyses

Direct bacterial cell counts were performed using a Live/Dead BacLight Bacterial Viability stains (Invitrogen L-7012) to monitor the bacterial population through cell membrane viability. The SYTO 9 nucleic acid stain stains live cells while cells with compromised membranes that are dead or dying are stained by propidium iodide (Sachidanandham et al., 2005). This method allowed the determination of what proportion of the bacterial population was compromised but not yet lysed. Stains were added simultaneously (3 μL each) to triplicate 1 mL bioassay subsamples in 2.5 mL microcentrifuge tubes. Samples were vortexed and incubated in the dark for 15 min. After their incubation, samples were filtered under 26 cmHg vacuum onto 0.2 μm 25 mm Whatman black polycarbonate filters. Once the sample had been filtered, a 2 mL 2% gluteraldehyde wash was applied to the filter to preserve the sample. The filters were then mounted on microscope slides and counted using an epifluorescent microscope (Hobbie et al., 1977). *K. brevis* cells were enumerated using an inverted microscope on a bright field setting and were identified based on cell morphology (Steidinger et al., 2008).

2.5. Bacterial community composition

Bacterial biomass samples were collected by filtering samples onto 0.2 μm Supor-membrane filters (Pall Corporation Ann Arbor, MI) and stored at <−80 °C until analysis and were analyzed using 16S rRNA genes and Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis. A modified phenol chloroform DNA extraction method (McGuinness et al., 2006) was used to isolate DNA and a cesium chloride density gradient was run for further purification. Briefly, 0.54 g cesium was added to a total volume of 500 μl H_2O containing 2 μl of 1% ethidium bromide, 100 ng of sample DNA, and 300 ng of *Halobacterium salinerium* carrier DNA to assist in visualization. The gradients were spun for 24 h at 250,000 × g in a Beckman Optima TL Ultracentrifuge and the DNA bands were drop-dialyzed on Millipore filters (VSWP 02500) against 10 mM Tris 8.0 for 45 min. Small subunit ribosomal RNA genes were amplified via PCR using the universal primers 27 forward (5′ AGA GTT TGA TCC TGG CTC AG 3′) and the bacterial specific primer 1100 reverse (5′ GGG TTG CGC TCG TTG 3′). The amplification reactions containing 10 ng of template and 20 pmol of primers, 2 units of Taq polymerase, and dNTP's in 50 μl volumes were treated as follows: 94 °C for 5 min., followed by 25 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1.1 min and a final extension period of 72 °C for 10 min.

For TRFLP profiling, all forward primers were labeled with 6-carboxylfluorescein (6-FAM; Applied Biosystems, Foster City, CA). These fluorescently labeled amplicons were run on a 1% agarose gel for quantification via image analysis and 15 ng were digested with the endonuclease *Mnl*I (New England Biolab, Beverly, MA) at 37 °C for 6 h. The 20 μL digestion reactions were then precipitated using 2.3 μL mixture of 0.75 M sodium acetate, 5 μg of glycogen, and 37 μl of 95% ethanol. The reactions were dried briefly, re-suspended in 19.7 μL of deionized formamide and 0.3 μL of ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 min and denatured at 94 °C for 2 min before analysis. TRFLP profiling was carried out using Genescan software and an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). TRFLP peak detection was set at 50 arbitrary fluorescent units. All detected peaks were downloaded into spreadsheets and those peaks

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representing <0.1% of the total peak area were discarded. The remaining peaks were parsed and normalized to a uniform total profile area to adjust for small loading differences between samples. Peaks represent operational taxonomic units (OTUs) which in turn represent at least one bacterial species.

Clone libraries of 16S SSU genes were created from unlabeled PCR product using the TA cloning kit as per the manufacturer's instruction. Recombinant clones were screened by TRFLP to associate nearly full-length clones with specific peaks (Babcock et al., 2007). Sequencing was performed using dye terminator kits (ABI) and phylogenetic tree re-construction utilized the Geneious program and maximum likelihood methods.

2.6. Statistical analyses

A standard paired *t*-test was used to compare changes in bacterial number in each brevetoxin treatment (0, 2.5, 5, 10, 25, 50, 100, and 200 μg PbTx-2 L^{-1} as well as the peak areas of different TRFLP samples. One way ANOVAs (bacteria live, dead, and total as a function of concentrations) were run with Tukey's HSD post hoc test ($p < 0.05$) for each site. Two way ANOVAs was used to compare the affect of both site and PbTx-2 concentration on bacterial abundance (live, dead and total bacterial cell number). Those comparisons with *p*-values < 0.05 were considered to be significantly different. All relationships with *p*-values > 0.05 are henceforth referred to as not significant. A Sorensen similarity index and Simpson's index of diversity were used to assess the relationship between PbTx-2 concentration and the changes in bacterial community composition. The Sorensen similarity index is calculated as twice the number of OTUs shared between two samples divided by the sum of the total number of OTUs detected in each sample. The Simpson's index of diversity is similar to the Sorensen similarity index but also evaluates the relative abundance of each species (OTU) detected. The Simpson's index of diversity is presented here as 1-diversity allowing both tests to have the same interpretational range of 0–1 with 1 indicating the greatest degree of similarity or diversity, respectively (Magurran, 2004).

3. Results

3.1. PbTx-2 addition experiment

All natural community samples were collected between 17 June 2008 and 25 August 2008. The salinities and temperatures were 28.5 and 25.5 °C at the NJ site, 25.5 and 29.7 °C at the DE site, and 31.0 and 31.5 °C at the FL site. Initial live bacterial cell numbers were $1.5 \pm 0.86 \times 10^5$ at the NJ site, $3.4 \pm 1.5 \times 10^5$ at the DE site and $1.3 \pm 0.37 \times 10^5$ at the FL site.

The sample site was a factor determining the community response to the PbTx-2 additions. While differences in both total and live cell number were observed after 24 h this difference became more pronounced after 48 h. A dose response was observed in both the NJ and DE communities with a larger decrease in live cell number occurring after 48 h in the 200 μg PbTx-2 L^{-1} addition compared to the control (0 μg PbTx-2 L^{-1}). There was a gradual decline in live cell number in the NJ treatment with increasing PbTx-2 concentration additions above 2.5 μg PbTx-2 L^{-1} (Fig. 2). This gradual trend resulted in a significant difference in live cell number occurring between the 200 μg PbTx-2 L^{-1} treatment and the control ($p = 0.017$) and the 2.5 μg PbTx-2 L^{-1} ($p = 0.33$).

The DE community was more tolerant with changes occurring with PbTx-2 additions of 50 μg L^{-1} or more, although this change in live cell number was not significant. There was no significant decrease in live bacterial cell number within the FL community at any concentration. This indicates that the overall FL community remained resistant to the bacteriocidal effects of PbTx-2.

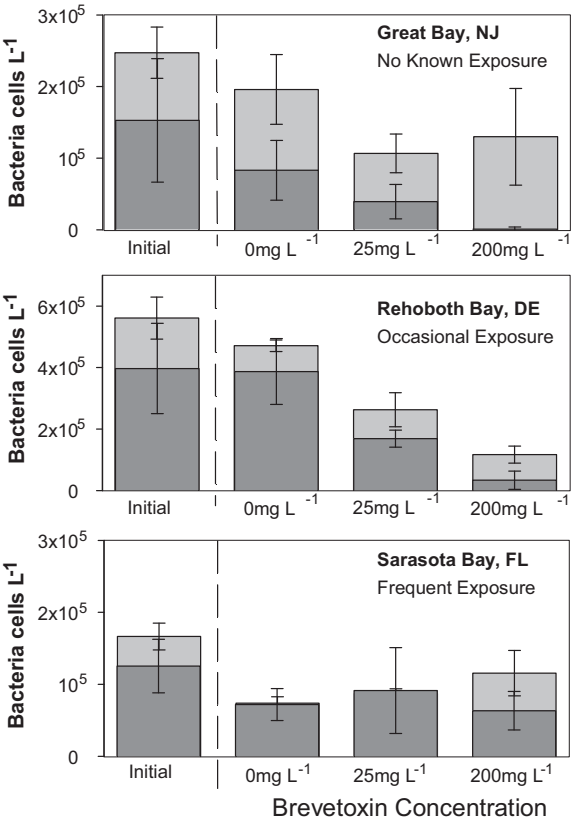

Fig. 2. Bacteria cell number of both live (dark gray) and dead (light gray) fractions ± S.D. for initial samples and those bacteria remaining after a 48-h incubation at 0, 25 and 200 μg L^{-1} brevetoxin 2.

While the number of live cells in the NJ treatments decreased with increasing brevetoxin, there were no significant differences in the number of cells with compromised membranes (dead/dying cells) in any of the PbTx-2 additions within the NJ treatments. However, within the DE community, more compromised cells were observed with PbTx-2 additions >50 μg L^{-1}. More compromised cells were also observed in the FL 200 μg PbTx-2 L^{-1} treatment than the control, although this difference was not significant.

Changes in the bacterial community, induced by PbTx-2 exposure, were assayed by TRFLP profiling of 16S rRNA genes. Changes in bacterial community composition were observed in all treatments (Fig. 3). Overall, there were 32, 38, and 48 peaks (operational taxonomic units-OTUs) detected in the initial NJ, DE and FL fingerprints, respectively. The microbial community within the NJ and DE incubations had a shared similarity index of 0.76, with the FL community being less similar to either the NJ (0.54) or DE (0.50) communities. The major OTUs detected in all sites (88, 102, 107, 127, 131, 206, 208, 240, 248, and 250 bp) were dominant (had the greatest peak area) during exposure to PbTx-2 (Fig. 3). Many of these OTUs (26 OTUs in NJ and 20 OTUs in DE) decreased in peak area when exposed to PbTx-2 and some OTUs fell below the detection limit in the 200 μg PbTx-2 L^{-1} treatments (11 in NJ and 14 in DE). The differences in OTUs between the control (0 μg L^{-1}) and PbTx-2 additions were significant in the 200 μg L^{-1} treatment for the NJ ($p = 0.004$) and DE ($p = 0.042$) communities. There was no observed difference between the species detected in the FL 200 μg L^{-1} treatment and the control.

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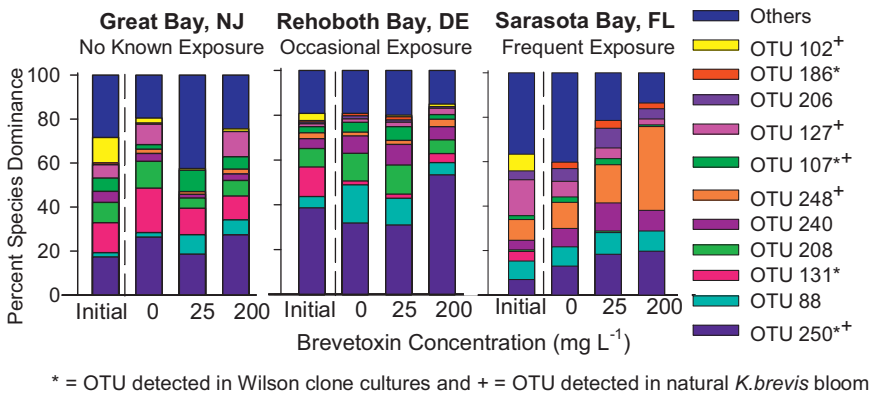

Fig. 3. Changes in bacterial community composition as percent dominance associated with changes in cell number in three treatments 0, 25 and 200 μg L^{-1} brevetoxin 2 within a 48-h incubation.

The NJ and DE bacterial communities became more similar with increasing PbTx-2 concentrations. The similarity index values of the NJ and DE initial population, 0, 25 and 200 μg PbTx-2 L^{-1} additions were 0.57, 0.63, 0.64 and 0.75, respectively. The similarities between the FL and DE or NJ sites did not change with PbTx-2 additions. The diversity in the bacterial communities that were sensitive to PbTx-2 declined upon exposure. All changes in relative diversity were ≤0.1 except in the DE 200 μg PbTx-2 L^{-1} additions which after 48 h had a diversity index of 0.62 compared to the control after 48 h or the initial (T0) populations which had diversity indexes of 0.82 and 0.86, respectively. Although there was a significant reduction in the number of live bacterial cells in the NJ treatments there was only a relatively small difference in diversity between the control and the 200 μg PbTx-2 L^{-1} additions (0.93 and 0.86, respectively). There was also a similar, small difference in diversity between the control and 200 μg PbTx-2 L^{--1} treatments in the FL community (0.88 and 0.84, respectively).

Clonal library analysis (Fig. 4) indicated that *Cyanobacterial* species (OTU 102, 240), *Actinobacterium*-like species (OTU 240 and 206), some *Alphaproteobacteria* species (OTU 122, 127, 131) and OTU 206 were negatively affected by PbTx-2 additions. The Sar 11-like clone (107) and *Pseudoalteromonas* sp. (OTU 88) were among the OTUs initially increasing then diminishing with PbTx-2 additions. OTUs 248 and 250 represented a group of *Roseobacter* like *Alphaproteobacteria* that increased in relative abundance, especially in the DE and FL incubations.

3.2. Bacterial communities associated with a natural K. brevis bloom

A transect of a natural *K. brevis* bloom revealed the bacterial communities associated with $3.8 \pm 0.3 \times 10^6$, $9.7 \pm 0.7 \times 10^5$, and $4.1 \pm 0.2 \times 10^4$ *K. brevis* cells L^{-1} (Fig. 5). Brevetoxin concentrations were not assessed due to sample degradation prior to analysis, however dead fish were observed at the surface at the site with 3.8×10^6 *K. brevis* cells L^{-1}. The *K. brevis* bloom of 3.8×10^6 cells L^{-1} had a total of 40 associated OTUs, the *K. brevis* bloom of 9.7×10^5cells L^{-1} had a total of 38 associated OTUs and a bloom of 4.1×10^4 cells L^{-1} had a total of 44 associated OTUs. Twenty of the observed OTUs were common among all bloom samples. Thirty OTUs were present in both the 9.7×10^5 and 4.1×10^4 *K. brevis* cells L^{-1} samples. Twenty-three OTUs were common among the 3.8×10^6 and 9.7×10^5 *K. brevis* cells L^{-1} samples. Based on OTUs detected, the mid density (9.7×10^5 *K. brevis* cells L^{-1}) and low density (4.1×10^4 *K. brevis* cells L^{-1}) samples had a similarity index of 0.73. These samples were less similar to the high density (3.8×10^6 *K. brevis* cells L^{-1}) bloom site with similarity index values of 0.52 (mid density) and 0.53 (low density), respectively.

3.3. Bacterial communities associated with K. brevis cultures

A total of 16 OTUs were detected associated with the Wilson high toxin strain and 25 OTUs were detected associated with the Wilson low toxin strain. Fourteen of the OTUs were shared by both the high and low toxin *K. brevis* cultures. These shared OTUs represent between 97% and 99% of the total bacterial community. Only 1 of the unique OTUs represented more than 1% of the population, OTU 279 represented 1.6% of the community in the low toxin Wilson strain.

3.4. Comparison of bacterial community composition

Thirteen of the OTUs detected at all three PbTx-2 addition sites were also detected in the natural *K. brevis* bloom. Six of these shared OTUs (102, 107, 127, 131, 248, and 250) were within the 12 most dominant OTUs in the PbTx-2 treatments. The OTUs displaying an increase in dominance with increasing PbTx-2 in the three bay communities also increased in dominance with increasing *K. brevis* concentration within the natural bloom. OTUs 107, 131, 248, and 250 were not only consistently detected in the PbTx-2 addition treatments and blooms but were also detected in both *K. brevis* cultures. OTUs 248 and 250 were identified as *Alphaproteobacteria*, closely related to *Roseobacter*-like species. This corresponds with previous findings that *Alphaproteobacteria* represent the most dominant group of bacteria associated with *K. brevis* blooms (Jones et al., 2010). The OTUs unique to the low toxin *K. brevis* culture (OTU 279 and 461) were detected in all 3 bay communities, however, they represented less than 1% of the community in any given sample and thus no definite trends could be determined and species identifications could not be made. All TRFLP signatures from replicate bottle incubations had good reproducibility and were found to be between 82% and 92% similar in OTUs detected and their relative abundances.

4. Discussion

PbTx-2 can affect both bacterial abundance and bacterial community composition. Bacterial community sensitivity was variable largely based on geographic location. The more northern populations with limited or no documented exposure were more susceptible to the effects of PbTx-2 than those populations frequently exposed to brevetoxins. The NJ community, with no documented exposure to brevetoxins, was the most sensitive to PbTx-2, with decreases in live cell number occurring at concentrations greater than 2.5 μg PbTx-2 L^{-1}. The DE community was more tolerant with changes in dead/compromised bacterial cell number occurring with ≥50 μg PbTx-2 L^{-1} brevetoxin. The population

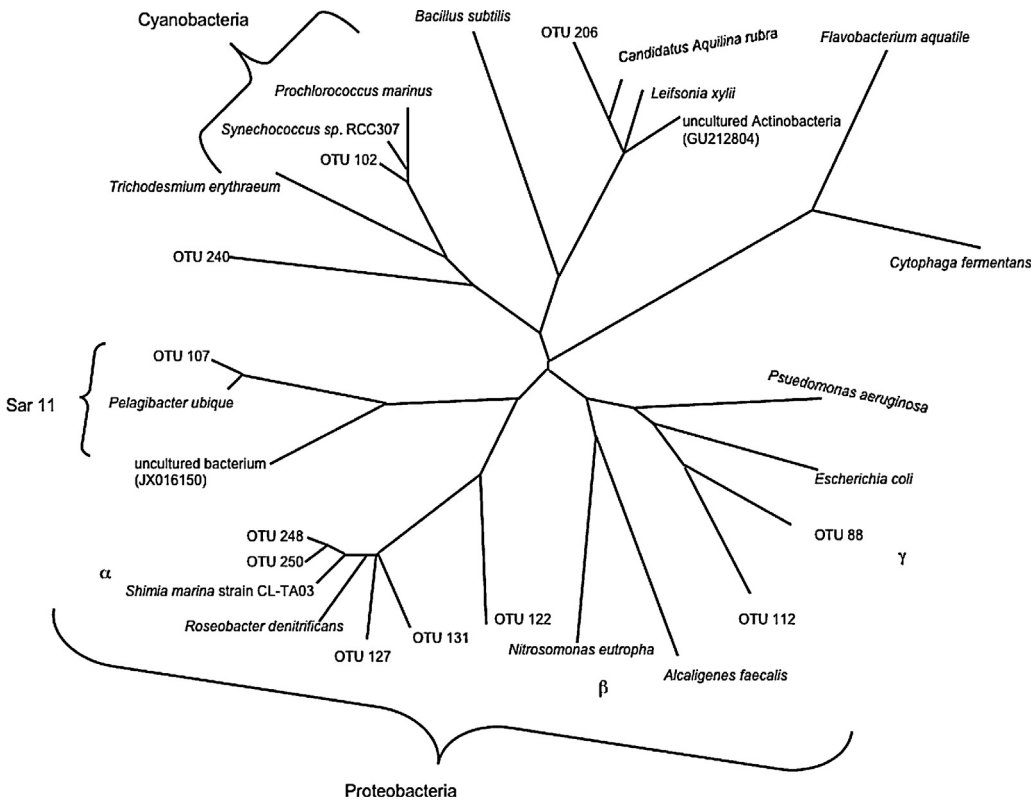

Fig. 4. Phylogenetic tree showing the relationship between dominant species detected in one or all brevetoxin addition experiments and common bacterial clones or classes.

with the highest historical exposure to brevetoxins was the most tolerant with no decreases in bacterial cell number, live or dead, occurring at any PbTx-2 concentration.

Bacterial number does not appear to differ between *K. brevis* bloom and non-bloom years near Sarasota, FL (Buck and Pierce, 1989), however, there is evidence that bacterial numbers may be elevated in samples containing >500 *K. brevis* cells mL^{-1} (Jones et al., 2010). In contrast to observations in the Gulf of Mexico, bacterial counts taken during a rare *K. brevis* bloom near Beaufort, NC showed that bacterial abundance was reduced compared to non-bloom seasons (Buck and Pierce, 1989). While there are few studies on bacterial abundance associated with the more rare northern blooms, the Beaufort study does support the current investigation in that decreased bacterial abundance was only observed at the sites with limited previous exposure to brevetoxins. The stage of these blooms when the samples were collected may also be an important factor contributing to the variability in these observations. Although the presence of brevetoxins is likely only one of several possible causes for the reduced bacterial abundance, the potential for a bacteriocidal response to brevetoxins needs to be explored.

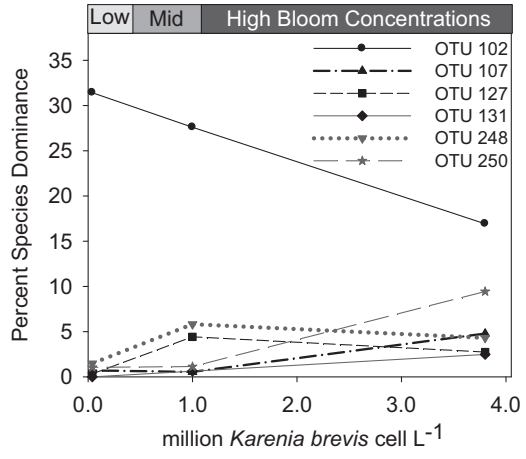

Fig. 5. Percent species dominance of OTUs present in transect through a natural *Karenia brevis* bloom. The gradient is represented as a change in *K. brevis* cells L^{-1}.

Previous studies of the allelopathic capabilities of *K. brevis* on phytoplankton cultures have suggested a minimized role of brevetoxins as allelopathic compounds (Kubanek et al., 2005; Prince et al., 2008; 2010). In one of these studies, brevetoxin purified from *K. brevis* cultures was added at 8.8 $\mu g\ L^{-1}$ final addition to cultures of different phytoplankton species (Kubanek et al., 2005). No significant change in the abundance of the tested phytoplankton cultures was observed with that addition. Although these studies investigated phytoplankton and not bacteria, according to our findings based on natural bacterial communities the 8.8 $\mu g\ L^{-1}$ addition would have been too low to observe large changes in the microbial population compared to the control.

Given the impact of PbTx-2 on bacteria shown in this work, it may suggest that over time the community (phytoplankton and bacteria) should reflect species or strains of species that are more resistant to brevetoxins specifically PbTx-2. This hypothesis is supported by laboratory manipulations where different species of cultured phytoplankton, that had no previous exposure to *K. brevis* or its exudates, reacted negatively to exudate additions while natural Gulf of Mexico phytoplankton populations, which contained some of the same species as were tested in culture, showed no negative impacts (Poulson et al., 2010). Although, the phytoplankton species present in both the cultures and field samples were genetically similar, they were in fact functionally different in the way that they were affected by *K. brevis* exudates. Therefore, the natural Gulf of Mexico community appears to be more resistant to *K. brevis* exudates than the previously unexposed isolates. The concept of evolved resistance has also been

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documented for other well studied marine toxins, for example, several studies have shown that populations of zooplankton *Acartia hudsonica* historically exposed to saxitoxin are less susceptible to its effects than previously unexposed populations (Colin and Dam, 2002, 2004, 2007; Dam and Haley, 2011). Populations of clams frequently exposed to saxitoxin have also become more resistant likely through an evolved mutation of their sodium channels (Bricelj et al., 2005, 2010; MacQuarrie and Bricelj, 2008).

The amount of time required for a community to become resistant to a toxin is unknown. Most studies, like this one, have investigated the rate of elimination and have not assessed the rate of acclimation of co-occurring species (reviewed in Macías et al., 2008). Studies of aquatic microbial resistance are generally focused on identifying resistant species in an area of high toxin. Some fundamental issues in studying resistance rates in aquatic systems include: the constant mixing and flowing of water diluting toxin concentrations, the complexity of the diverse and largely uncultured aquatic microbial communities and the potential for reintroduction of species from adjacent unaffected water masses (Macías et al., 2008).

Ecotoxilogical studies have suggested that aquatic communities will respond and recover differently to intermittent doses based on the concentration of toxin, duration of exposure and recovery time between exposures (Newman and Clements, 2008; Zhoa and Newman, 2007). Concentrations of 2.5×10^6 *K. brevis* cells L^{-1} have been observed to cause fish mortality (Quick and Henderson, 1974); however, longer exposure times at lower brevetoxin concentrations can also result in mortality (Landsberg, 2002). Therefore, longer, low brevetoxin exposures like those observed during bloom initiation may also play a role in altering microbial communities.

Proximity as a proxy for increased brevetoxin concentration may be another important factor. Although the majority of brevetoxin is often in the particulate rather than the dissolved form (Tester et al., 2008), the concentrations of brevetoxin will be greater closer to the source, i.e. the cells producing the toxin. This may be especially true if bacteria are in close proximity to *K. brevis* cells, which has been hypothesized to be required for allelopathic affects to be observed between *K. brevis* and other phytoplankton cells (Kubanek et al., 2005). At low cell/dissolved brevetoxin concentrations, brevetoxin production may be a mechanism by which *K. brevis* eliminates cell surface bacteria and those bacteria and phytoplankton in close proximity. If the toxin is too dilute than no significant response in adjacent bacterial cell number would be observed. This suggests that waterborne brevetoxin concentrations must be high in well mixed conditions or *K. brevis* cells must be in close proximity to competitors (bacteria or phytoplankton) at time of release under stratified/stable conditions. Other mechanisms for brevetoxin release include algicidal bacteria which through the rupturing of *K. brevis* cells can increase dissolved brevetoxin concentrations by >50 μg L^{-1} (Roth et al., 2007). In this scenario, the largest microbial shifts may occur during bloom senescence.

Brevetoxins may also play a role in nutrient acquisition. Bacteria are thought to indirectly promote phytoplankton growth through the remineralization of DOM (Azam, 1998). When nutrient concentrations are low, bacteria compete with phytoplankton for the limiting available nutrients (Caron, 1994). Brevetoxins may be the vector by which toxin producing species control their environment and nutrient supply. Since *K. brevis* has the ability to use a variety of organic nutrients including several forms of dissolved organic nitrogen (Baden and Mende, 1979; Bronk et al., 2004; Sipler et al., 2013), eliminating competitors (bacteria or phytoplankton) during low nutrient conditions would be a beneficial growth strategy.

If one can eliminate competition and benefit through the release of nutrients via their elimination, cell lysis, then producing toxin may be crucial to the survival of these oligotrophic organisms (Granéli, 2006). This may be the case with the relationship between *K. brevis* and cyanobacteria *Synechococcus sp.* Several studies have suggested that *K. brevis* may be phagotrophically grazing on bacteria, specifically *Synechococcus* sp. (Jeong et al., 2005; Glibert et al., 2009). Grazing rates as high as 83.8 *Synechococcuss sp.* cell *K. brevis* $cell^{-1}$ h^{-1} appear elevated due at least to the sheer size comparison of the *Synechococcus* sp. (1–2 μm) and *K. brevis* (20–28 μm) clones (Glibert et al., 2009). However, if *K. brevis* is able to rupture *Synechococcus* cells extracellularly using brevetoxins and take advantage of the nutrients released, these removal rates become more reasonable.

Although this investigation provides a clear trend showing a decrease in live bacterial cell number with increasing PbTx-2 concentrations, the mechanism by which brevetoxins attacks bacterial cells is unknown. In eukaryotic cells, brevetoxins have been shown to act as a sodium or calcium channel activator causing depolarization and compromising the membranes of affected cells (Kirkpatrick et al., 2004; Kitchen, 2010). However, this may be a gross over simplification of an organism's response to an individual marine toxin (Anderson et al., 2012). While bacteria undoubtedly possess a completely different suite of essential biomolecules than animals or even phytoplankton, one might expect some of these bacterial biomolecules to interact with brevetoxins. To further investigate the likelihood of brevetoxins affecting bacterial cell membranes, compromised cell number was assessed. None of the PbTx-2 concentrations elicited an increase in the number of compromised cells within the NJ community; however, there was an increase in the number of compromised cells observed in the DE and FL communities. It is possible that an increase in compromised cells was not detected in the NJ community because with less tolerance to the toxin, affected cells lysed at a faster rate or that the population may contain species more sensitive to rupture. The DE samples, which showed an increase in compromised cells, also showed a decrease in live cells. This supports the hypothesis that bacterial cell membranes may be compromised by brevetoxins which leads to the observed decrease in bacterial abundance and changes in community composition.

In an investigation of changes in bacterial community composition associated with the addition of DOM from *Trichodesmium* supplied to a natural population of *K. brevis*, OTU 250 was found to increase in dominance as *K. brevis* concentrations increased (Sipler, 2009). This is in agreement with this investigation in that OTU 250, an *Alphaproteobacteria*, was persistent throughout all PbTx-2 additions, was found in both high and low toxin *K. brevis* cultures and increased in dominance in the natural bloom with increasing concentrations of *K. brevis*. This is not surprising as *Alphaproteobacteria* represent the most abundant group of bacteria associated with *K. brevis* blooms (Jones et al., 2010). Since the response of the microbial populations from different aquatic systems varied based on previous exposure and several of the resilient species within these samples also displayed the same dynamics in a natural *K. brevis* bloom, the results of this study suggest that brevetoxin production may be directly linked to changes in bacterial community composition.

Due to the diverse ecological niches that sustain blooms of *K. brevis* and *Chattonella* spp. understanding brevetoxin's affect on previously unexposed populations is critical to understanding bloom dynamics and community composition of affected areas. This is especially important as there is evidence that the *Karenia* and *Chattonella* species have expanded in range and abundance along the east coast of the United States over the past decade (Verity, 2010). Both of the communities with limited (DE) or no (NJ) previous exposures to brevetoxins became more similar with

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increasing PbTx-2 concentrations indicating that brevetoxin was selecting for specific species or groups of species and against others. Based on the number of OTUs that decreased in dominance to below detection limits in the treatments compared to those in the control, as much as 37% of the species present in a system could be lost through the introduction of brevetoxin, specifically PbTx-2, to a previously unexposed community. Therefore, if the presence of brevetoxins increases in frequency and concentration in systems similar to Rehoboth Bay, DE and Great Bay, NJ, decreases in bacterial number and changes in community composition would be expected.

5. Conclusions

The goal of this study was to determine if PbTx-2 negatively impacted natural microbial communities and if geographically separated communities respond similarly to toxin exposure. Through this work it is now known that brevetoxin has the potential to negatively impact some species of bacteria as well as bacterial communities as a whole. *Alphaproteobacteria* were least affected by PbTx-2 and were the most abundant species overall as they persisted throughout all PbTx-2 addition experiments, were found in both *K. brevis* cultures and increased in dominance in the natural bloom with increasing concentrations of *K. brevis*. *Cyanobacteria and Actinobacterium*-like species were the most negatively impacted by PbTx-2. Through brevetoxin (PbTx-2), as much as 37% of bacterial species could be lost with its introduction into less tolerant, previously unexposed systems. However, more research is needed to evaluate the prevalence of brevetoxins in more northerly coastal areas and the extent to which brevetoxins may impact these communities. The work presented here was not a broad investigation of the microbial community response but was a first assessment of brevetoxin's potential to alter microbial dynamics.

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