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Compositional changes in neurotoxins and their oxidative derivatives from the dinoflagellate, *Karenia brevis*, in seawater and marine aerosol

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The harmful alga, *Karenia brevis,* produces a suite of polyether neurotoxins, brevetoxins or PbTx, that cause marine animal mortality and neurotoxic shellfish poisoning (NSP). A characteristic of *K. brevis* blooms is associated airborne toxins that result in severe respiratory problems. This study was undertaken to determine the composition of aerosolized brevetoxins and oxidative derivatives to which beachgoers are exposed during a *K. brevis* bloom. The suite of brevetoxins and derivatives in seawater is comprised of intra-cellular (IC) and extra-cellular (EC) compounds. We hypothesized that aerosolized compounds are generated primarily from EC, hydrophobic compounds in seawater by bubble-mediated transport. Thus the composition of aerosolized brevetoxins and derivatives, to which beachgoers are exposed, would reflect the EC composition of the source matrix (the local surf zone). Brevetoxins were extracted from water collected along the shore and from marine aerosols along Siesta Beach and Lido Beach in Sarasota, FL, USA, during *K. brevis* blooms. Water samples were further processed into IC and EC components. The primary brevetoxins observed in water and air included PbTx-1, -2, -3, -PbTx-2-carboxylic acid, and brevenal. Oxidation and/or hydrolysis products of PbTx-1, -2, -3 and -7 were also found in EC water and in aerosol, but not IC.

KEYWORDS: *Karenia brevis*; harmful algal blooms; brevetoxins; neurotoxins; aerosolized toxins

INTRODUCTION

The harmful alga, *Karenia brevis* (formerly, *Gymnodinium breve*, Davis) (Duagbjerg *et al.*, 2001) is responsible for the most prevalent harmful algal blooms (HABs) in the Gulf of Mexico as well as periodic blooms along the US Atlantic Coast (Tester and Steidinger, 1997; Landsberg, 1997). This dinoflagellate produces a suite of polyether neurotoxins called brevetoxins (designated as PbTx-1, -2, -3 etc.) that cause fish kills, marine mammal, sea turtle and sea bird deaths and neurotoxic shellfish poisoning (Baden *et al.*, 2005; Flewelling *et al.*, 2005; Pierce *et al.*, 2008). The brevetoxins include two similar, yet distinct polycyclic ether backbones, PbTx-1 (Type A) and PbTx-2 (Type B). The rest of the brevetoxins are thought to be metabolic derivatives of these two parent compounds, including Type A (PbTx-7) and

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Type B (PbTx-3, - 5, -6, -9, -10) (Poli, *et al.*, 1986; Landsberg, 2002; Bourdelais, *et al.*, 2005; Abraham, *et al.*, 2006). A rare characteristic among HABs is the associated airborne (aerosolized) toxin component of *K. brevis* (Pierce *et al.*, 1990, 2005). Toxins are released into the water as cells rupture increasing the amount of extracellular (EC) toxins as a bloom progresses (Pierce *et al.*, 2001). Bubbles from breaking waves transport toxins to the sea surface where they are ejected into the air as jet and film drops from the bursting bubbles (Blanchard, 1975; Pierce *et al.*, 1990). Onshore winds carry the toxin-containing aerosols on to the beach causing respiratory irritation to humans and other mammals along the shore (Cheng *et al.*, 2005; Fleming *et al.*, 2005; Pierce *et al.*, 2005; Kirkpatrick *et al.*, 2006).

The most abundant brevetoxins observed in whole water samples collected during a *K. brevis* bloom have been reported as PbTx-1, 2, -3, and PbTx-2-carboxylic acid (CA) (Pierce *et al.*, 2005). PbTx-2 was found to be the most abundant brevetoxin in water. However, aerosol samples contained about the same and in some instances greater amounts of PbTx-3 and PbTx-2-CA relative to PbTx-2, indicating changes in the relative composition of brevetoxins from water to aerosol. Previous evidence of hydrolysis and oxidation derivatives resulting in opening the A ring of the brevetoxin molecule has been reported in *K. brevis* culture (Plakas *et al.*, 2004; Wang *et al.*, 2004) and in *K. brevis* blooms (Abraham *et al.*, 2006). Similar synthetic brevetoxin derivatives were shown to exhibit varying physiological effects (Baden *et al.*, 2005) suggesting the need for the identification of major brevetoxin analogs present in *K. brevis* blooms and resulting aerosols, in order to assess human exposure and toxicity.

This study was undertaken to determine the concentration and composition of brevetoxins and major derivatives in marine aerosol and surf-zone water to determine the type and amount of toxins to which subjects are exposed during a coastal *K. brevis* red tide bloom. In addition to the brevetoxins, samples were analyzed for oxidative products of brevetoxins (Abraham *et al.*, 2006; Plakas *et al.*, 2008) and the antagonist, brevenal, that has been shown to inhibit the effects of brevetoxins in laboratory studies (Bourdelais *et al.*, 2005). Concentrations of *K. brevis* cells were monitored in the surf water, and the concentration and composition of brevetoxins and derivatives were monitored in the water and air, both during a *K. brevis* bloom and in the absence of a bloom along the Florida Gulf coast.

METHOD

Water and aerosol samples were monitored for brevetoxins during and in the absence of *K. brevis* red tide blooms in 2005 at Lido and Siesta Beach and 2006 at Siesta Beach, Sarasota, Florida. A separate set of water and aerosol samples was monitored during an intensive bloom in 2005 at Lido Beach, Sarasota, Florida. Water samples from the 2005 Lido Beach and Siesta Beach studies were separated into intra-cellular (IC) and EC fractions prior to toxin analysis. Siesta Beach water from the 2006 study was analyzed as combined IC and EC components. Oxidative, open-ring derivatives of brevetoxins were analyzed in water from all samples and in aerosol from the 2005 Lido Beach and 2006 Siesta Beach studies. At Siesta Beach, a total of six high-volume samplers were used; three were placed near the surf zone approximately 100 m apart, each adjacent to a lifeguard stand, and a second row of three was located approximately 50 m inland from the first row to provide an assessment of aerosolized toxin concentrations over time and space along the beach. A set of three water and three air samples were collected at Lido Beach.

Seawater samples were collected in 1 L glass bottles from the surf zone adjacent to each air sampler location. A 20 mL sub-sample was collected from each bottle and fixed with Utermohls solution (Guillard, 1973) for microscopic identification and enumeration of *K. brevis* cells. The remaining water sample was processed for brevetoxin analysis by liquid chromatography–mass spectrometry (LC–MS) as described below, and for verification by enzyme-linked immunosorbent assay, ELISA, according to the procedure of Naar *et al.* (Naar *et al.*, 2002).

Selected samples were processed to separate live *K. brevis* cells from the ambient water for subsequent analysis of IC and EC brevetoxins. *Karenia brevis* cells were collected using a high output stirred-cell concentrator (Millipore/Amicon; Billerica, MA, USA) fitted with a 0.8 μm polycarbonate filter (Osmonics; Westborough, MA, USA). The *K. brevis* cells retained above the filter contained brevetoxins and derivatives inside the cell, while the solution passing through the filter contained compounds in solution outside of the cell.

Brevetoxins were extracted from the water samples and from the IC and EC fractions by passing the seawater through a C-18 solid-phase extraction disk under vacuum (Ansys Technologies Inc., Lake Forest, CA, USA) according to the procedure of Pierce *et al.* (Pierce *et al.*, 2005). The C-18 disks were then rinsed with reverse osmosis water and eluted with methanol for LC–MS and ELISA analyses. Extraction efficiency was verified by recovery of standard amounts of PbTx-2 and PbTx-3 that were added to each of three seawater samples and processed for the LC–MS analysis.

Marine aerosol samples were collected using high volume air samplers (TE-5000, Tisch Environmental

Inc., Village of Cleaves, OH, USA) fitted with 20 cm × 28 cm glass fiber filters (Whatman EPM 2000, Maidstone, UK). Air samples were collected for 3–4 h period both in the morning and afternoon, sampling approximately 1.5 m^3/min. This sampling time was used to reflect changes in wind speed and direction as well as changing bloom concentrations in the water which often differ from morning to afternoon.

Brevetoxins in marine aerosol were recovered from the glass fiber filters by extraction for 12 h in acetone using a Soxhlet apparatus. The extract was evaporated and transferred to vials in methanol for the LC–MS analysis. Brevetoxin recovery was verified by the addition of standard amounts of PbTx-2 and PbTx-3 to each of three filters; filters were placed on air samplers that were run for 4 h and subsequently processed for the LC–MS analysis.

Brevetoxin analyses were performed by LC–MS using a ThermoFinnigan AqA HPLC/MS (Thermo Electron Corp., Manchester, UK). The LC consisted of a SpectraSystems, LC Pump P4000, Autosampler AS3000, and a Degasser SCM1000. Brevetoxins were detected using an AqA single quad MS system scanned from 204 to 1216 AMU with AqA Max 40 V, and a scan rate of 1.1 scans/s. All analysis was conducted using electrospray with the probe at 3 kV and 250 °C. The column was a Phenomenex Security Guard C-18 guard column with a Phenomenex Luna C-18 5 μm 250 mm × 2 mm Analytical Column. The solvent gradient was composed of acidified (0.3% acetic acid) ACN/H_2O with initial 50:50 ACN/H_2O to 95:5 ACN/H_2O over 40 min. The instrument was calibrated with a standard brevetoxin mix containing PbTx-2 and PbTx-3, obtained from the Center for Marine Science, UNC Wilmington, NC, USA.

Verification of PbTx compounds was provided by comparison of LC–MS results with standard brevetoxins provided by UNCW. Structures of brevetoxin oxidative derivatives were confirmed by LC–MS comparisons with results presented by Wang et al. (Wang *et al.*, 2004) and Abraham *et al.* (Abraham *et al.*, 2006), using MS/MS ($ESI^{+/-}$) product ion mass spectra and negative-ion MS mode.

RESULTS

Lido Beach, 28 February 2005

A comparison of brevetoxin composition in aerosol and water IC and EC is given in Fig. 1a. The cell count of $1.4 \times 10^6 \pm 1.7 \times 10^5$ cells/L was representative of a moderate-to-high intensity *K. brevis* bloom. The most

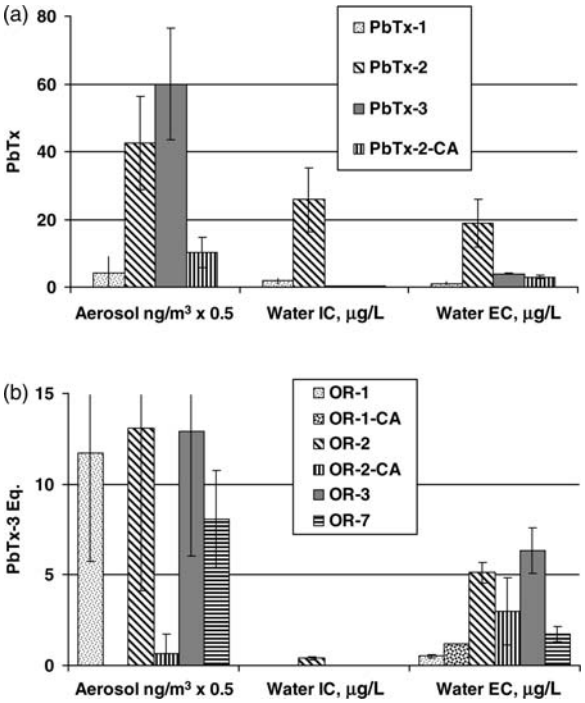

Fig. 1. (**a**) Lido PbTx Air + Water IC–EC, 28 February 2005; (**b**) Lido OR Air + Water IC–EC, 28 February 2005.

abundant IC brevetoxin was PbTx-2, with only a trace amount of PbTx-1 as the only other brevetoxin observed above the detection limit of 0.03 μg/L seawater. The most abundant EC toxin also was PbTx-2, followed by PbTx-3 and PbTx-2-CA at about 20% the concentration of PbTx-2. A trace of PbTx-1 was also observed. These results support previous observations of PbTx-1 and -2 as the primary brevetoxins produced inside the living cell, with the apparent metabolites, PbTx-3 and -2-CA, produced as the cells are lysed (Pierce *et al.*, 2001, 2005). A significant shift in the relative abundance of the toxins was observed in the aerosol samples. PbTx-3 was the most abundant toxin, followed by PbTx-2 > PbTx-2-CA > PbTx-1. No other brevetoxins were above the lower limit of detection.

Composition of brevetoxin compounds that were oxidized with an open A ring is given in Fig. 1b. The nomenclature used to identify the open ring (OR) compounds in given in Table I. The only IC toxin observed was the OR derivative of PbTx-2 (OR-2). All other OR compounds were present in the EC fraction. OR-3 was the most abundant, followed by OR-2 > OR-2-CA > OR-7 > OR-1-CA > OR-1. As with the closed ring brevetoxins, the aerosol composition of OR derivatives also exhibited a significant change in relative composition from water to aerosol. OR-1, OR-2 and OR-3 were the most abundant and all about the same

Table I: Compound nomenclature designations

PbTx	MH^+ *m/z*	Use in figures
PbTx-1	866	PbTx-1
PbTx-1-CA	883	PbTx-1-CA
PbTx-1-OR	885	OR-1
PbTx-1-OR-CA	901	OR-1-CA
PbTx-7	868	PbTx-7
PbTx-7-OR	887	OR-7
PbTx-2	894	PbTx-2
PbTx-2-CA	911	PbTx-2-CA
PbTx-2-OR	913	OR-2
PbTx-2-OR-CA	929	OR-2-CA
PbTx-3	896	PbTx-3
PbTx-3-OR	915	OR-3

CA, carboxylic acid; OR, open ring.

concentration in aerosol, followed by OR-7, and a much smaller amount of OR-2-CA. A comparison of the relative concentrations of closed ring brevetoxins to their OR derivatives revealed OR concentrations to be about 25% of the closed ring parent compounds in aerosol and EC water, Fig. 1a and b.

Siesta Beach, 12 March 2005

To observe differences in *K. Brevis* toxin composition from different locations and times, a replicate series of IC and EC samples was collected at Siesta Beach (about 12 miles) south of Lido Beach along the Florida Gulf coast. A comparison of total brevetoxins in water and aerosol is given in Fig. 2a. The *K. brevis* cell count for these samples was 1.65×10^5 cells/L, representing a low intensity red tide bloom. Again, PbTx-2 was the most abundant IC toxin, with none of the toxin metabolites above the lower limit of detection.

EC toxins were PbTx-2, -3 and -2-CA, all in about the same concentration. PbTx-3 was the most abundant toxin in aerosol, followed by PbTx-2 > PbTc-2-CA > PbTx-1, reflecting a similar compositional shift that was observed along Lido beach during the higher concentration red tide bloom 1 month earlier.

The composition of OR compounds in IC and EC water is given in ×Fig. 2b. Although in much lower abundance, the relative composition of OR compounds in IC and EC water samples from the low intensity red tide bloom at Siesta Beach was remarkably similar to that from the medium-to-high intensity bloom previously sampled at Lido Beach. Although the OR compounds were not analyzed in this set of Siesta Beach aerosol samples, these results confirm the abundance of OR PbTx derivatives in the EC water, with only the OR derivative of PbTx-2 observed as IC.

Siesta Beach, 23 and 24 September 2006

A second series of water born and aerosolized OR derivatives was obtained along Siesta Beach during a higher intensity bloom of $2.6 \times 10^6 \pm 9.2 \times 10^5$ cells/L in September 2006. A comparison of total brevetoxins in water and aerosol is given in Fig. 3a. As with the 2005 study, PbTx-2 was the most abundant brevetoxin

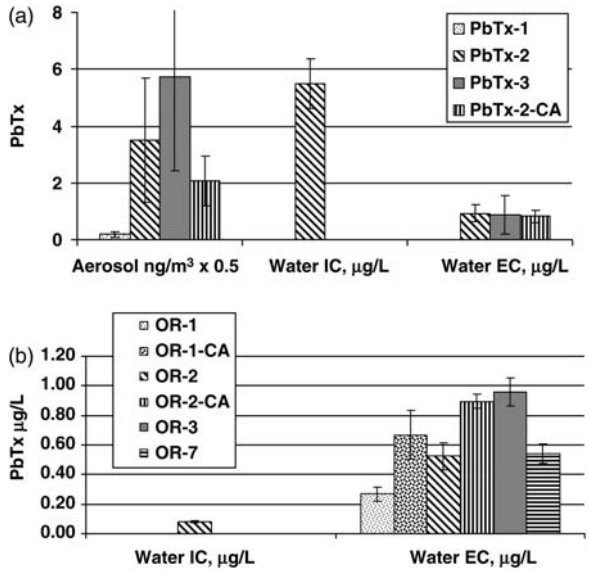

Fig. 2. (**a**) Siesta PbTx Air + Water IC–EC, 12 March 2005; (**b**) Siesta OR PbTx Water IC–EC, 12 March 2005.

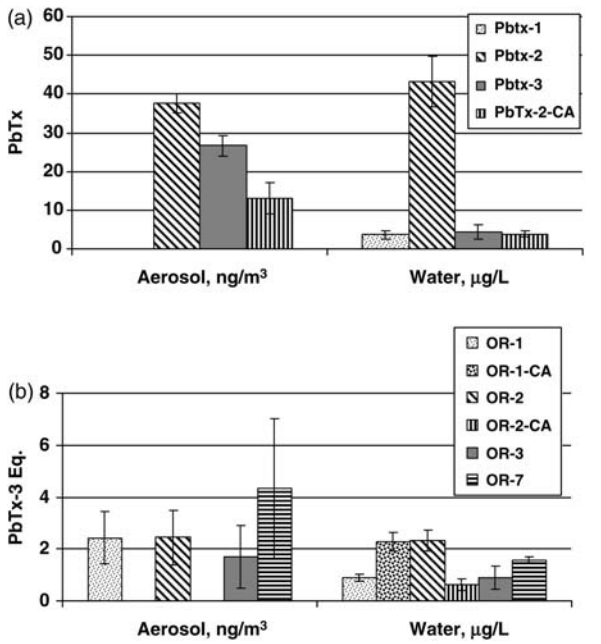

Fig. 3. (**a**) Siesta PbTx Air + Water, 24 September 2006; (**b**) Siesta OR PbTx Air + Water, 24 September 2006.

in total water samples, with lesser amounts of PbTx-1, -2 and -2-CA. The antagonist, brevenal, was observed in the water samples at concentrations similar to PbTx-1 (data not shown).

Again, the relative composition of brevetoxins in aerosol (Fig. 3b) changed significantly from that in water. Most dramatic was the increase in PbTx-3 relative to PbTx-2. PbTx-2-CA increased moderately relative to PbTx-2. Neither PbTx-1 nor brevenal were detected in aerosol.

DISCUSSION

The composition of brevetoxins and derivatives in seawater and marine aerosol has been observed to vary due to the presence of different strains of *K. brevis*, intensity and growth stage of *K. brevis* blooms and differing environmental conditions (Baden and Tomas, 1988; Baden *et al.*, 2005). Similar variations have been observed for blooms of the toxic haptophyte, *Prymnesium parvum*, that experience different environmental conditions of nutrients, solar radiation and flow dynamics (Grover, *et al.*, 2011; James *et al.*, 2011; Schwierzke-Wade *et al.*, 2011). Even so, the results of this study show that the composition of brevetoxins and their derivatives was found to be consistent, within expected natural variations, over time and space and within different *K. brevis* red tide blooms. The two parent brevetoxins, PbTx-1 (Type A) and PbTx-2 (Type B), were the most prevalent brevetoxins observed inside the *K. brevis* cells, while the metabolites (PbTx-3, -2CA and -7) were observed in the EC matrix and in marine aerosol, along with PbTx-1 and -2.

The oxidized, OR derivatives of the above brevetoxins also exhibited consistency among different samples. As with the closed ring brevetoxin compounds, only the parent PbTx-2 open ring (OR-2) compound was detected as IC. All of the remaining OR derivatives were observed in the EC matrix. Analysis of the OR composition of marine aerosol revealed the presence of all oxidized, OR compounds of the major brevetoxins (OR-1, OR-2, OR-3 and OR-7) that were present as EC compounds in the source water for aerosol formation (surf area adjacent to the beach). Obviously absent from the aerosol were the CA derivatives of both closed and OR brevetoxin derivatives. Their absence could be explained by their greater solubility, reducing their hydrophobic attraction to bubble surfaces, thus inhibiting bubble-mediated concentration and transport to the sea surface. This would reduce the amount of CA compounds subsequently ejected in associated with minute droplets in marine aerosol propelled by the energy of bursting bubbles (Pierce *et al.*, 1990).

Using the *in vitro* cytotoxicity assay, in comparison to the toxicity of PbTx-3, the intrinsic toxicity of Pbtx-1 was five times higher and the toxicity of Pbtx-2 was five times lower than PbTx-3 (Ramsdell, 2008; Plakas and Dickey, 2010). The *in vivo* (ip) toxicity (24-h LC50) of PbTx-2 and PbTx-3 was reported as 200 and 170 μg/kg body wt, respectively (Baden and Mende, 1982). The toxicity of various cysteine and fatty acid conjugates of PbTx-2 have been reported to be one to two times higher (less toxic) than the parent PbTx-2 (Plakas and Dickey, 2010). However, toxicity of the OR oxidative products has not been established.

Results from the above analyses of Type A and Type B brevetoxins and oxidative derivatives in marine aerosol and water from the adjacent surf zone support the hypothesis that EC brevetoxins and brevetoxin derivatives are the source for the aerosolized compounds to which beach goers are exposed during a red tide bloom.

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