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Determination of brevetoxin in recent marine sediments

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Abstract

Harmful algal blooms (HAB) of *Karenia brevis* (*K. brevis*) produce a suite of lipid soluble polyether brevetoxins, known to cause environmental, health and economic ill effects. There is evidence that *K. brevis* has increased in abundance over the past 50 years, but the dataset is incomplete. The objective of this paper was to analyze sediment from an area where *K. brevis* blooms have occurred and investigate if these compounds are incorporated into the underlying sediment, thus potentially allowing the use of brevetoxins as an indicator of past *K. Brevis* blooms.

The results from LC-ESI-MS-MS analyses of brevetoxin analogs detected in surficial sediments from three sites (Fort Meyers Beach [FMB], Big Hickory Pass [BHP] and Big Carlos Pass [BCP]) along the South-west Florida coastline with prior HAB history are promising. The analogs detected from BHP sediments were PbTx-2 and PbTx-3 with values of 0.81 and 3.1 ng g⁻¹ dry sediment, respectively. The detected PbTx-2 from BCP was 3.6 ng g⁻¹ dry sediment, while the detected PbTx-3 from BCP was 9.7 ng g⁻¹ dry sediment. PbTx-3 was only detected at the FMB site (2.7 ng g⁻¹ dry sediment). The detection of brevetoxins in recent sediments where *K. brevis* have occurred indicates brevetoxin incorporation into marine sediments.

Keywords

Brevetoxin; Harmful algal bloom; Biomarker; Sediments; Red tide; Gulf of Mexico

1. Introduction

Harmful algal blooms (HAB) are a worldwide phenomenon caused by a single dominating alga at extremely high concentrations. Highly dense algal blooms discolor surface waters that sometimes appear as red, green, yellow, and occasionally colorless depending on the algal species (Okaichi, 2004). Natural toxins from these blooms cause economic, environmental and human health problems and there is evidence that they are increasing (Hallegraef, 1993; Van Dolah 2000).

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The toxic photosynthetic dinoflagellate *Karenia brevis* (*K. brevis*) occasionally generates large blooms that kill fish and other animals (Landsberg, 2002) and cause human health problems (Van Dolah et al., 2001; Kirkpatrick et al., 2004). The blooms occur in many parts of the Gulf of Mexico but are most prevalent along the west coast of Florida (Geesey and Tester, 1993; Steidinger et al., 1998; Kusek et al., 1999). The overall distribution and abundance of *K. brevis* in space and time is not well known, both because the blooms are sporadic and the sampling has been erratic. Fish kills described by early European explorers in the fifteenth century (Kusek et al., 1999; Magaña et al., 2003) provide evidence that blooms of *K. brevis* have been occurring for many hundreds of years in the Gulf of Mexico. Brand and Compton (2007) however have examined the last 50 years of quantitative data on *K. brevis* and concluded it has increased roughly 15-fold along the west Florida coast. The sampling, however, was quite sporadic in both space and time. There are no quantitative data on *K. brevis* before around 50 years ago because *K. brevis* was not known to be the causative agent of the observed fish kills at that time.

The toxins that are associated with *K. brevis* blooms are broadly defined as belonging to a lipid soluble group of cyclic polyether compounds with a molecular weight around 900 amu (Fig. 1). Many analogs of brevetoxin have been identified in cultures of *K. brevis*, sea water blooms as well as metabolites in shellfish and other marine life (e.g. Abraham et al., 2006). Most of these analogs were reputed to be biosynthetic products of either PbTx-1 or PbTx-2 as precursors (Baden et al., 2005). Typically in marine waters the dominant brevetoxin produced by concentration is PbTx-2 with lesser amounts of PbTx-1 and PbTx-3, whereas in marine aerosols PbTx-3 dominates (Pierce et al., 2005). Recent work by Tester et al. (2008) has shown though as a *K. brevis* bloom ages there is a relative increase in the PbTx-3 compared to the PbTx-2 homologues. The mechanism is not entirely understood but is believed to be enzymatic (Poli et al., 1986).

The incorporation of the major brevetoxin analogs (PbTx-2 and 3) into underlying sediments remains unknown as of yet and potentially may be a long-term sink for these potent toxins. From the work of Pierce et al. (2004) brevetoxins do flocculate with clays thus indicating the likelihood absorption onto sediments may take place. Therefore, the hypothesis we wish to test is whether brevetoxins produced by large *K. brevis* blooms are incorporated in underlying sediments. This may serve as a potential mechanism of transfer of the toxin up the food chain to higher organisms such as manatees (Trainer and Baden, 1999) as well as a potential marker for the paleo-reconstruction.

2. Materials and methods

2.1. Sediment collection

Surficial sediment samples from Big Carlos Pass (BCP), Big Hickory Pass (BHP) and Fort Myers Beach (FMB) were collected by grab sampling the upper 10 cm in December of 2006 (Fig. 2). A red tide bloom had been in the collection area five months prior to sampling. The sediment samples were placed in solvent rinsed glass jars and transported to the laboratory on ice. Samples were frozen immediately upon returning to the University of Miami and freeze-dried before analysis.

2.2. Extraction

The extraction of brevetoxin from sediment samples was done by sonication with a 1:1 mixture of dichloromethane and acetone (e.g. Plakas et al., 2002). The sediment weight varied between 1.5 to 2.0 g and the total volume of solvent was 50 mL. Once completed, the solvent and sediment were separated by centrifugation at 1000 rpm for 10 min. The upper dichloromethane:acetone supernatant layer was transferred to solvent pre-rinsed glass vials

using a Pasteur pipette. This process was repeated for a total of three times and the supernatants were pooled into one sample. Once complete the sample was evaporated to dryness under a gentle stream of ultra high purity N₂ gas. Samples were stored at -20 °C until ready for analysis. Before HPLC analysis, the dried residue was re-solubilized in 500 µL of methanol:water (85:15 v v⁻¹) by sonicating for 30 s. Dichloromethane, methanol and acetone GC² grade solvents were purchased from Burdick and Jackson, Corp. (Muskegon, MI) while the ultrapure HPLC grade water was purchased from Alfa Aesar (Ward Hill, MA).

In order to test the sonication times, a series of experiments that manipulated the sonication time were employed. The sonication extraction times were tested by weighing 2.0 g of freeze-dried sediment sample from BHP. Before extraction, 10 µL of PbTx-9 (100 µg mL⁻¹) was added to the sediment and thoroughly mixed. Analysis was performed by HPLC-DAD using the method of Hua et al. (1996).

2.3. HPLC analysis

The HPLC-DAD analysis was performed using an Agilent 1100 LC series following the method of Hua et al. (1996). A narrow bore reversed phase (C₁₈) Eclipse XDB (Agilent Technologies, CA, USA) 3.5 µm pore size, 2.1 × 150 mm LC column was employed to separate the PbTx mixture. The mobile phase reservoirs were (A) HPLC grade water and (B) methanol run with 15% and 85% ratio, respectively. Injected volume was 5 IL using a high pressure injection valve (Rheodyne 7520, Cotati, CA, USA) with a 5 IL loop. Total acquired run time was 20 min. The flow rate was set at 0.1 mL min⁻¹ and the column temperature was at ambient room temperature (25 °C). Absorbance of the brevetoxins was read at 215 nm using a diode array detector. The peak identified as PbTx-9 from the extraction experiment was determined against external standard calibration curves with five calibration points from 0.05 to 0.5 µg mL⁻¹. (Plot not shown; PbTx-9, $r^2 = 0.999$). The limit of detection was 3.3 ng. These brevetoxin standards were purchased from CalBioChem (La Jolla, CA, USA). Procedural and instrumental blanks were routinely analyzed and no contamination was found.

The LC-ESI-MS-MS analyses conditions were modified after Cheng et al., 2005. The samples were separated by an Agilent 1100 LC series using a reverse phase (C₁₈) Phenomenex, LUNA, 3 µm pore size, 2.0 × 50 mm column. The mobile phase was 50:50 acetonitrile:water (0.3% acetic acid) for the first 40 min, then changed to 5:95 acetonitrile:water for 2 min and finally back to 50:50 acetonitrile: water for 8 min. The flow rate was set at 0.2 mL min⁻¹. Structural identification, detection and quantification was carried out using a Thermo Finnigan TSQ Quantum triple quadrupole mass spectrometer, equipped with the electrospray ionization source (Thermo Fisher Scientific, Inc., USA). Nitrogen gas was used as both drying gas and nebulizing gas. Structural information was determined in selective reaction monitoring (SRM) mode matching product ions and parent mass from standards to sedimentary brevetoxins. Optimized collision induced decomposition MS/MS spectra for each of the PbTx transitions were acquired at 4 V (PbTx-2), 18 V (PbTx-3) using argon as the collision gas at a pressure of 1.0 mTorr. The SRM transitions of the two types of toxins were set at 895.5 m/z to 877.5 m/z (PbTx-2) and 897.5 m/z to 725.3 m/z (PbTx-3). Scan events of product ions were set at scan width of 1.0 s with scan time of 0.25 s. Q1 and Q3 peak widths were set at 0.70 s. Total acquired segment time ran for 40 min. An external calibration curve was used to quantify the concentration of brevetoxins in the sediment and cell extracts with a limit of detection of 3 ng mL⁻¹ (0.75 ng g⁻¹). The sediment and cell culture extracts were analyzed in duplicate and the concentrations are reported as mean values.

2.4. Cultures

Laboratory maintained *K. brevis* cultures were analyzed as well from locations that were near the sediment sampling locations. These cultures are maintained by Dr. Larry Brand at the

University of Miami. The 200 mL of the culture was directly filtered on preconditioned (with methanol, 20 mL) C₁₈ SEPAK cartridge (Waters Co., Milford, MA). The cartridge was treated with HPLC grade distilled water; to lyse the cells and to remove residual salts (Pierce et al., 1992). The brevetoxin was immediately eluted with 2 mL methanol three times. The extract was evaporated to dryness with ultra-high purity N₂ gas, then kept frozen (−20 °C) until analysis.

3. Results and discussion

3.1. Extraction recovery

The extraction recovery of PbTx-9 brevetoxin analog from sediment previously exposed to *K. brevis* did not vary from the set sonication time of 5, 10, 30 min since each sonication time recovered ~40% of the spiked brevetoxin (Fig. 3). The standard deviation and the controls for each sonication time suggest that the time element is not a function of the extraction recovery of the brevetoxin from the sediment using the extraction method. The extent of binding of brevetoxins in marine sediments may be a factor implicated for the modest recovery of spiked PbTx-9 in the sediment even with increase in sonication time.

The sonication method of extraction was employed, since it is a widely used sample preparation for sedimentary organic studies (e.g. Mead and Goni, 2006). Other than being inexpensive, it also requires short time for extraction compared to the Soxhlet extraction with relatively higher recovery efficiency (Guerin, 1999). In our experiment, we used ambient room temperature and chose the 10 min sonication rather than the 5 min to increase contact time with the sediment and potentially could enhance the recovery in the subsequent sediment sample extraction. The 30 min sonication was not employed to decrease or eliminate unwanted side reactions (e.g. Guerin, 1999).

3.2. Culture and sedimentary concentrations

The brevetoxin extract from cultured *K. brevis* collected from the same area from where the sediment was collected was shown to contain 12.0 and 8.2 µg mL^{−1} of PbTx-2 and PbTx-3, respectively (Fig. 4 and Table 1). PbTx-2 was detected in sediments collected at BCP and BHP with values of 3.6 and 0.8 ng g^{−1} dry sediment, respectively, while PbTx-3 was detected from sediments collected at BCP, BHP and FMB with mean values 9.7, 3.2 and 2.7 ng g^{−1} dry sediment, respectively (Fig. 4 and Table 1). This may be attributed to the hydrodynamic variation affecting the harmful algal bloom dynamics at those two separate sites (Donaghay and Osborn, 1997; Steidinger and Haddad, 1981). The sheltered condition in the passes enhances particle deposition in contrast to the beach area, where sediments are continuously reworked and disturbed. This has been observed for other molecular markers in highly energetic environments (e.g. Gordon and Goni, 2004).

The stability and subsequent diagenetic fate of the brevetoxin analogs once deposited in the sediment remains poorly understood. There have been studies under extreme conditions which suggest the brevetoxins break down. For example, Baden (1989) found brevetoxins to be stable up to 300 °C and only degraded when the pH was either below 2 or above 10. In addition, a degradation experiment was conducted by Schneider et al. (2003) of brevetoxins utilizing ozonated seawater and found that despite large doses of ozone treatment of brevetoxins some of the original brevetoxins were still recovered. These two studies point to the harsh conditions required to degrade brevetoxins and are unlikely in typical recent surficial sediments. More recently, the fate of the brevetoxins under more environmentally relevant conditions has been reported. Abraham et al. (2006) have identified ring A opened PbTx-2 and 3 in aerosols and the water column during *K. brevis* blooms while Myers et al. (2008) reported phytoplankton

competitors able to degrade PbTx-2 in the water column. Given this information, this study suggests that incorporation into the underlying sediment is possible.

4. Conclusions

Since it appears that the brevetoxins are incorporated into underlying sediments, there is potential utility of the PbTx-2 and PbTx-3 to be used as a sediment molecular marker for *K. brevis* blooms in the major areas affected in the Gulf of Mexico. The detection of brevetoxins in surficial sediments has to be approached with caution as molecular marker since underlying environmental and biological factors responsible for its deposition could play a role in its preservation. Even with this caveat, using brevetoxin as a molecular marker of *K. brevis* blooms would provide a more continuous and long-term record of the organism's presence and abundance. Other proxies have been used as an indicator of dinoflagellate's presence. For example, the sterol dinosterol has been used in sediments but does not indicate individual species (e.g. Boon et al., 1979; Mouradian et al., 2007). Fossilized cysts in sediment can also provide a long-term record of the presence and abundance of an individual species (Marret and Zonneveld, 2003). *K. brevis* is a naked dinoflagellate, however, thus has no thick cell wall to fossilize therefore the brevetoxins are the only means to chemically fingerprint the occurrence of the organism. In order to validate this idea, controlled degradation experiments need to be carried out under differing regimes (e.g. sedimentary oxic and anoxic conditions, photochemical degradation in the water column) to characterize the brevetoxin breakdown products.

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Type-B Brevetoxins

PbTx-2 : $R = \text{CH}_2\text{C}(=\text{CH}_2)\text{CHO}$

PbTx-3 : $R = \text{CH}_2\text{C}(=\text{CH}_2)\text{CH}_2\text{OH}$

PbTx-8: $R = \text{CH}_2\text{COCH}_2\text{Cl}$

PbTx-9: $R = \text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{OH}$

PbTx-5: $R = \text{the K-ring acetate of PbTx-2}$

PbTx-6: $R = \text{the H-ring epoxide of PbTx-2}$

Type-A Brevetoxins:

PbTx-1 : $R = \text{CH}_2\text{C}(=\text{CH}_2)\text{CHO}$

PbTx-7 : $R = \text{CH}_2\text{C}(=\text{CH}_2)\text{CH}_2\text{OH}$

PbTx-10 : $R = \text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{OH}$

Fig. 1.
Structures of the two types of brevetoxin and their respective analogs.

Fig. 2.

Sources of sediment samples from the southwest coast of Florida were at (1) Fort Myers Beach (FMB), (2) Big Carlos Pass (BCP), and (4) Big Hickory Pass (BHP).

Fig. 3. Percentage of PbTx-9 recovered after brevetoxin was spiked into BHP sediment. Sonication times employed were 5, 10 and 30 min ($n = 2$).

Fig. 4. Reconstructed ion chromatograms of PbTx-2 and PbTx-3 from HPLC-ESI-MS-MS analysis of sediments and cultured cells. The instrument conditions are detailed in Section 2.

Table 1

Concentration from duplicate injections of PbTx-2 and PbTx-3 in sediments and cultured *K. brevis* as determined by LC-ESI-MS-MS

Sample	PbTx-2	PbTx-3
BCP (ng g ⁻¹)	3.6 ± 0.01	9.7 ± 2.0
BHP (ng g ⁻¹)	0.81 ± 0.08	3.15 ± 0.21
FMB (ng g ⁻¹)	N/D	2.7 ± 0.28
Cells (µg mL ⁻¹)	11.9 ± 0.07	8.2 ± 0.07

Station codes are the same as in Fig. 2 (mean ± SD; *n* = 2)