

Prevalence of brevetoxins in prey fish of bottlenose dolphins in Sarasota Bay, Florida

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ABSTRACT: Blooms of the brevetoxin-producing dinoflagellate *Karenia brevis* have been linked to high mortality of bottlenose dolphins *Tursiops truncatus* on Florida's Gulf of Mexico coast. A clear understanding of trophic transfer of brevetoxin from its algal source up the food web to top predators is needed to assess exposure of affected dolphin populations. Prey fish constitute a means of accumulating and transferring brevetoxins and are potential vectors of brevetoxin to dolphins frequently exposed to *K. brevis* blooms. Here we report results of brevetoxin analyses of the primary fish species consumed by long-term resident bottlenose dolphins inhabiting Sarasota Bay, Florida. Fish collected during *K. brevis* blooms in 2003 to 2006 were analyzed by competitive enzyme-linked immunosorbent assay (ELISA) and had brevetoxin concentrations ranging from 4 to 10 844 ng PbTx-3 eq g⁻¹ tissue. Receptor binding assay (RBA) and liquid chromatography–mass spectrometry (LC-MS) analysis confirmed toxicity and the presence of parent brevetoxins and known metabolites. Fish collected in the absence of *K. brevis* blooms tested positive for brevetoxin by ELISA and RBA, with concentrations up to 1500 ng PbTx-3 eq g⁻¹ tissue. These findings implicate prey fish exposed to *K. brevis* blooms as brevetoxin vectors for their dolphin predators and provide a critical analysis of persistent brevetoxin loads in the food web of dolphins repeatedly exposed to Florida red tides.

KEY WORDS: Brevetoxin · *Karenia brevis* · *Tursiops truncatus* · Harmful algal blooms · Algal toxins · Bottlenose dolphin · Marine biotoxins · HAB · Red tide

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INTRODUCTION

Harmful algal blooms (HABs) have apparently increased in global distribution, intensity and frequency of occurrence over the past few decades, eliciting considerable concern over the effects of algal toxins on higher organisms including marine mammals and humans (Van Dolah 2000, Hallegraeff 2003). Marine mammals are sentinel organisms that act as barometers of coastal ocean health and can provide indicators of potential negative impacts to coastal and marine ecosystems (Bossart 2006). HABs composed of

the red tide algae *Karenia brevis* affect Florida's Gulf of Mexico coast almost annually and have been linked to marine animal mortality events (Walker 1884, Gunter et al. 1948, Forrester et al. 1977, O'Shea et al. 1991, Landsberg & Steidinger 1998, Mase et al. 2000, Flewelling et al. 2005). *K. brevis* (previously known as *Gymnodinium breve* and/or *Ptychodiscus brevis*; Daugbjerg et al. 2000) is a dinoflagellate that naturally produces a suite of potent neurotoxins, called brevetoxins, that can kill and/or accumulate in a variety of organisms, ranging from microscopic zooplankton to large marine mammals (Poli et al. 1986, Shimizu et al.

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1990, Bossart et al. 1998, Tester et al. 2000, Woofter et al. 2005). Negative impacts of elevated *K. brevis* populations (blooms) include respiratory irritation ($>1 \times 10^3$ cells l^{-1}), shellfish poisoning ($>5 \times 10^3$ cells l^{-1}), fish kills ($>1 \times 10^5$ cells l^{-1}) and water discoloration ($>1 \times 10^3$ cells l^{-1}) (Steidinger et al. 1998).

Studies examining the effects of red tide blooms on bottlenose dolphins *Tursiops truncatus* have investigated the link between the presence of the toxic alga *Karenia brevis* and large-scale dolphin mortalities and the accumulation of brevetoxin in tissues of recovered dolphin carcasses (Geraci 1989, Mase et al. 2000, Flewelling et al. 2005, Fire et al. 2007). However, findings have been inconclusive or unreported in some cases as to the ultimate source of brevetoxin exposure in these animals. In a large-scale dolphin mortality event in the Florida panhandle during 1999 and 2000, the presence of a *K. brevis* bloom coincided with over 120 dolphin deaths, but carcass decomposition precluded definitive diagnosis, and brevetoxin values in stomach contents or prey items were not reported (Mase et al. 2000). Previous dolphin mortality events have implicated *K. brevis* as the causative agent, but confounding factors such as morbillivirus infection, high tissue concentrations of environmental contaminants and a lack of corresponding prey fish data have made results inconclusive (Geraci 1989). The links between dolphins as apex predators and toxic *K. brevis* blooms have not clearly been established. Bottlenose dolphins inhabiting the same distribution range as the red tide organism are mainly piscivores (Barros & Odell 1990, Barros & Wells 1998) and do not feed directly on *K. brevis*, thus accumulation of brevetoxin in these animals is not likely to be a simple correlation between ambient *K. brevis* cell densities and toxin values in sampled tissues. Dolphin exposure to brevetoxins is likely dependent on a variety of factors, including, but not limited to, varying concentrations of accumulated toxin via different prey species, each with different feeding habits and varying rates of toxin accumulation, retention and metabolism. In order to better understand the fate of brevetoxins during and following a bloom, potential vectors that can transfer brevetoxin burdens to higher trophic levels must be identified.

In HABs involving other types of toxin-producing algae, planktivorous prey fish have been shown to be algal toxin vectors to coastal marine mammals (Anderson & White 1989, Geraci et al. 1989, Lefebvre et al. 1999, Scholin et al. 2000). There is mounting evidence that prey fish act as brevetoxin vectors to Gulf of Mexico dolphins, as demonstrated in a 2004 dolphin mortality event along the Florida panhandle, in which 107 dolphin carcasses were recovered and all animals sampled had detectable levels of brevetoxin (NMFS 2004,

Flewelling et al. 2005). Although during this particular event a bloom of *Karenia brevis* was not detected in the mortality region concurrent with strandings, brevetoxin was present in various tissue types (17 to 104 $ng\ g^{-1}$ in liver, kidney and lung) and at very high levels in stomach contents (up to 6176 $ng\ g^{-1}$), which consisted mostly of menhaden *Brevoortia* spp., a planktivorous fish (Flewelling et al. 2005). Other Gulf of Mexico dolphins, sampled in Sarasota Bay, Florida from 2001 to 2003, also had elevated brevetoxin levels during *K. brevis* blooms, at concentrations reaching 78 $ng\ g^{-1}$ in tissues (liver, lung, muscle and blubber), 749 $ng\ g^{-1}$ in feces and up to 2900 $ng\ g^{-1}$ in stomach contents (Fire et al. 2007). Though previous research has investigated the accumulation of brevetoxin in bottlenose dolphins, additional research is needed to document the levels of brevetoxin available to dolphins via their primary prey items.

The feeding habits of well-studied populations of bottlenose dolphins such as those found in Florida's Sarasota Bay area (Fig. 1) have been documented (Irvine et al. 1981, Barros & Wells 1998). Sarasota Bay is a shallow (<4 m) estuarine system, semi-enclosed by barrier islands and dominated by seagrasses. The dolphins inhabiting these waters are exclusively piscivorous and feed primarily on pinfish *Lagodon rhomboides*, pigfish *Orthopristis chrysoptera*, striped mullet

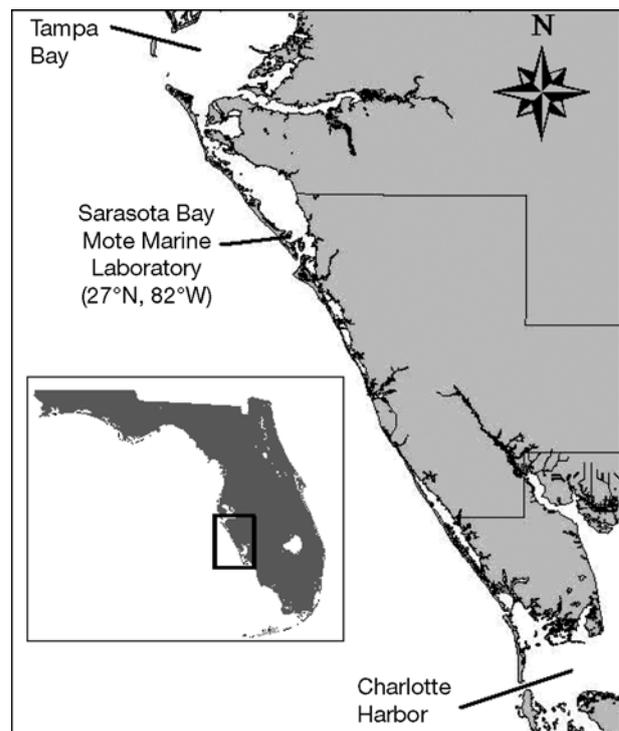


Fig. 1. Study area (Sarasota Bay and adjacent coastal waters)

Mugil cephalus and spot *Leiostomus xanthurus* (Barros & Wells 1998). Although other prey items are available to the dolphins, prey diversity in dolphin stomachs is low (1 to 3 taxa stomach⁻¹), and these 4 species of fish make up over 80% of the dolphins' diet in terms of numerical abundance (Barros & Wells 1998). As a prey group, these species of fish have widely varying feeding habits which include herbivory, carnivory, detritivory and omnivory (Collins 1985, Sutter & McIlwain 1987, Phillips et al. 1989, Motta et al. 1995). Given that (1) the consumption of toxic fish has been implicated as a major vector of brevetoxins to dolphins elsewhere along the Florida coast (Flewelling et al. 2005), (2) Sarasota Bay dolphins have been documented to accumulate brevetoxins (Fire et al. 2007), and (3) the Sarasota Bay area is routinely affected by nearly annual *Karenia brevis* blooms (Steidinger et al. 1998), it can be expected that dolphins from the present study site also have dietary brevetoxin exposure via consumption of prey fish.

To better understand this dietary exposure and the role of prey fish as vectors of brevetoxin, we collected prey fish during *Karenia brevis* blooms occurring between 2003 and 2006, as well as during non-bloom periods with background levels of *K. brevis*, and analyzed these specimens for the presence of brevetoxins. Our results present evidence that the primary prey fish species consumed by Sarasota Bay dolphins are potential brevetoxin vectors to these animals, and our data provide the first description of the persistence of brevetoxins in their primary prey and in a variety of other available prey species during non-bloom conditions.

MATERIALS AND METHODS

Sarasota Bay, Florida, was selected as the study site because it frequently experiences *Karenia brevis* blooms and supports a long-term, multi-generational, year-round resident community of bottlenose dolphins for which detailed information exists on feeding patterns (Barros & Wells 1998, Wells 2003). During the 2003 to 2006 study period, *K. brevis* cell abundance data for the Sarasota Bay region were obtained and used as an indicator of the presence, absence and/or severity of *K. brevis* blooms occurring during the fish collections described below. Samples were collected by dip bucket from the upper 10 cm of the water column, fixed with Utermöhl's solution and examined under light microscopy to determine *K. brevis* concentrations (cells l⁻¹). Daily phytoplankton monitoring data for the 2003 to 2006 period were provided by the Phytoplankton Ecology Program of Mote Marine Laboratory and were supplemented by water samples obtained during fish collections and other concurrent field studies at multiple sites in the study area (Fig. 2).

Fish and other potential prey items were collected during 5 periods between 2003 and 2006 in the Sarasota Bay region. Three of these collection periods (May to June 2003, January to May 2005 and August to September 2006) coincided with *Karenia brevis* blooms occurring in the study area (Fig. 2). Fish specimens collected during these blooms consisted of pinfish, pigfish, striped mullet and spot, and due to their predominance in the diet of Sarasota Bay dolphins (Barros & Wells 1998) will be collectively referred to as primary prey fish. Additional sets of prey items were collected

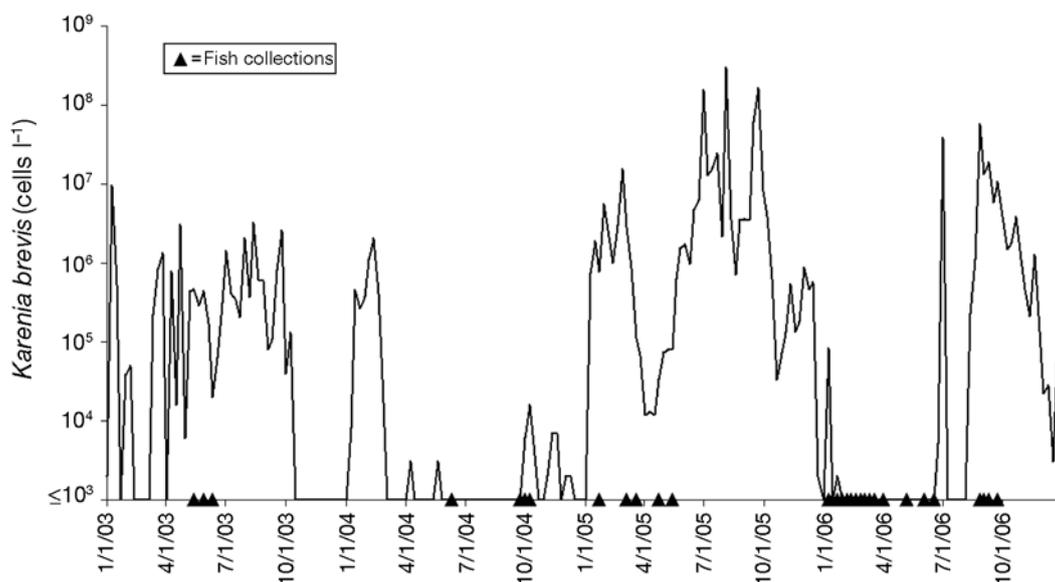


Fig. 2. *Karenia brevis* cell abundance (cells l⁻¹; log scale) and (triangle markers) prey fish collection dates (mo/d/yr) in the Sarasota Bay area 2003 to 2006

during periods when blooms of *K. brevis* were not present in Sarasota Bay (June to October 2004 and January to March 2006) and consisted of primary prey fish as well as 33 other potential prey species commonly found in Sarasota Bay. Many of these additional species are known to be consumed by bottlenose dolphins throughout the Gulf of Mexico and southeastern United States (Barros & Odell 1990) and will collectively be referred to as non-primary prey. Fish specimens were collected via purse-seine or hook and line within Sarasota Bay, and specimens were placed on wet ice until transferred to a -20°C freezer upon returning to the laboratory. Fish were identified to species and measurements of body weight and standard length (SL) were recorded.

Fish specimens were prepared for toxin extraction by dissection of target tissues or by *in toto* homogenization of whole specimens. Sample types available for brevetoxin analysis included muscle, liver, gill, stomach contents, whole viscera (all organs in intracoelomic cavity) or whole fish. For a few small fish specimens collected from the same location and time, tissues were pooled according to species to increase the amount of material available for toxin extraction. Each pooled sample is referred to hereafter as an individual fish specimen and was treated as a single sample for analytical purposes. For a subset of pinfish, pigfish and spot, total tissue mass for viscera and muscle in each specimen was recorded in order to estimate whole-fish brevetoxin burdens. Brevetoxins were extracted by homogenizing the sample material in 3 volumes of acetone, followed by sonication and centrifugation at $3000 \times g$. The supernatant was evaporated and resuspended in 85% methanol, followed by 2 solvent partitioning steps with hexane. The methanolic fraction was collected and passed through a $0.45 \mu\text{m}$ hydrophilic polypropylene syringe-driven filter (Pall Life Sciences). The filtrate was evaporated and the remaining residue was resuspended in 100% methanol. Extracts were stored at -20°C until analysis.

A competitive enzyme-linked immunosorbent assay (ELISA) was used as the primary source of brevetoxin quantification and followed protocols described by Naar et al. (2002). This type of immunological assay provides sufficient sensitivity to quantitatively detect very low brevetoxin concentrations by means of structural affinity for a target epitope and is effective as a rapid screening method (Van Dolah & Ramsdell 2001). This method utilizes polyclonal anti-brevetoxin antibodies that recognize all brevetoxin congeners and metabolites with a brevetoxin-B-type structure. ELISA results expressed as 'brevetoxins' refer to all brevetoxin congeners that respond to these antibodies, and concentrations detected are in nanograms of PbTx-3 equivalents per gram of tissue ($\text{ng PbTx-3 eq g}^{-1}$). The

ELISA limit of detection of brevetoxin in the present study was $3 \text{ ng PbTx-3 eq g}^{-1}$.

Selected samples were also analyzed by a receptor binding assay (RBA), utilizing microplate format methods outlined by Van Dolah et al. (1994). Briefly, the RBA measures binding competition between radio-labeled brevetoxin-3 and brevetoxin in samples for binding to voltage-gated sodium channels in rat brain synaptosomes, the pharmacological target of brevetoxins. In contrast to the ELISA, which quantifies brevetoxins based on structure, the RBA quantifies brevetoxins based on their affinity for a specific cell membrane receptor, in effect estimating the total toxic potency of a sample. The RBA limit of detection of brevetoxin in the present study was $70 \text{ ng PbTx-3 eq g}^{-1}$.

A subset of samples that tested positive by ELISA underwent additional analysis by 2 methods of liquid chromatography–mass spectrometry (LC-MS). In LC-MS, brevetoxin congeners are chromatographically separated and individually quantified by a mass-to-charge ratio (m/z) relative to standard reference material, and this method was utilized as a confirmatory method for ELISA and RBA data. Selected samples were initially analyzed for PbTx-3 following LC-MS methods described by Plakas et al. (2004), using a ThermoFinnigan AqA HPLC-MS equipped with an AqA single quad system, with a limit of quantification of 300 ng g^{-1} . Further analysis of additional brevetoxin congeners, including known metabolites, was performed using an Agilent 1100 LC system (Agilent Technologies) coupled to an Applied Biosystems/MDS Sciex 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a Turbo VTM ionization interface (Applied Biosystems). LC separation was performed on a Luna C8(2), $5 \mu\text{m}$, $2.0 \times 150 \text{ mm}$ column (Phenomenex). The mobile phase consisted of water and acetonitrile in a binary system, with 0.1% acetic acid as an additive. The elution gradient was 1 min of 42% acetonitrile, linear gradient to 80% acetonitrile at 22 min, 95% acetonitrile at 27 min, held for 5 min, then returned to initial conditions at 33 min and held for 7 min before next injection. The flow rate was 0.2 ml min^{-1} . Brevetoxin congeners were detected in positive ion mode using the multiple reaction monitoring (MRM) method. Twenty-two MRM transitions were monitored, which covered the following brevetoxin congeners: PbTx-1, -2, -3, -7 and -9 and hydrolysis products of PbTx-2, -3, -7 and -9 (A-ring open brevetoxin derivatives; Abraham et al. 2006), cysteine-PbTx-B/A and their sulfoxides (Wang et al. 2004) (also known as BTX-B2; Murata et al. 1998) and cysteine-PbTx sulfoxide (Plakas et al. 2002) for cysteine-B-type backbone brevetoxin sulfoxide conjugate, cysteine-PbTx (Plakas et al. 2002) and desoxy-BTX-B2 (Bottein

Table 1. Brevetoxin concentrations detected in primary prey fish species collected during and in the absence of *Karenia brevis* blooms. Concentrations (mean and range) expressed in nanograms PbTx-3 equivalents per gram sample (ng PbTx-3 eq g⁻¹). nd: not detected; –: no test made or no sample available

<i>K. brevis</i> blooms	Species	% PbTx-positive fish (n)	Muscle	Viscera	Liver	Gill	Stomach contents	Whole fish
Present	Pinfish	93 (30)	62 (11 to 197)	1313 (4 to 5188)	1378 (475 to 3759)	891 (42 to 7472)	727 (202 to 2263)	–
	Pigfish	100 (11)	57 (6 to 365)	610 (181 to 1456)	1196 (267 to 2035)	947 (77 to 2671)	812 (203 to 1424)	–
	Mullet	100 (23)	7 (5 to 9)	81 (11 to 258)	763 (147 to 1838)	44 (26 to 113)	121 (79 to 168)	–
	Spot	100 (10)	57 (21 to 149)	1982 (743 to 3282)	3981 (3117 to 4846)	869 (809 to 930)	6350 (1855 to 10844)	–
Absent	Pinfish	88 (16)	7 (3 to 14)	68 (15 to 143)	507	nd	45	456
	Pigfish	90 (10)	nd	87 (45 to 143)	293 (21 to 1047)	34	233 (202 to 263)	98
	Mullet	67 (3)	nd	156 (91 to 222)	–	–	–	–
	Spot	100 (5)	9 (5 to 12)	303 (7 to 753)	–	–	–	–

Dechraoui et al. 2007) for cysteine B-type backbone brevetoxin conjugate, glutathione-PbTx-B, glycine-cysteine-PbTx-B (Wang et al. 2004), oxidized-PbTx-2 and its hydrolysis product (Abraham et al. 2006). Nitrogen was used as the nebulization gas, turbo gas (at transmission electron microscope, TEM, 365°C), curtain gas and collision gas. The ion spray voltage was 5 kV, the declustering potential was 64 V, and the collision energy was adjusted (25 to 50 eV) as appropriate for each toxin congener. Six PbTx-3 standards (1 to 250 ng ml⁻¹), 1 PbTx-9 standard (25 ng ml⁻¹), 1 hydrolysis product of PbTx-3 standard (100 ng ml⁻¹) and 1 cysteine-PbTx-B sulfoxide standard (10 ng ml⁻¹) were used. Due to the limited availability of the standards, some toxin congeners were semi-quantified with a single standard. Concentrations of cysteine-adducts were estimated from cysteine-PbTx-B sulfoxide standard. The LC-MS analysis signal-to-noise ratio was ca. 40 for injection of 5 µl of 1 ng ml⁻¹ of PbTx-3, ca. 70 for injection of 5 µl of 25 ng ml⁻¹ of PbTx-9, ca. 700 for injection of 5 µl of 100 ng ml⁻¹ of hydrolysis product of PbTx-3 and ca. 67 for injection of 5 µl of 10 ng ml⁻¹ of cysteine-PbTx-B sulfoxide.

RESULTS

Over the course of the present study (2003 to 2006), ambient densities of *Karenia brevis* fluctuated widely in concentration, ranging from below the detection limit (<10³ cells l⁻¹) to a maximum of 2.9 × 10⁸ cells l⁻¹ (Fig. 2). This period encompassed 4 *K. brevis* blooms in

the Sarasota Bay area, occurring January to October 2003, January to February 2004, January to December 2005 and July to December 2006. Three of these blooms co-occurred with primary prey fish collections, with ambient cell densities of *K. brevis* ranging in concentration from 2.0 × 10⁴ to 5.6 × 10⁷ cells l⁻¹. SLs recorded for primary prey fish indicated that nearly all specimens collected were adults (mean SL: pinfish = 125 mm, pigfish = 122 mm, mullet = 270 mm and spot = 176 mm). These measurements fall within the size ranges reported for fish recovered from Sarasota Bay dolphin stomachs, the majority of which were also adult fish (Barros & Wells 1998). For primary and non-primary prey collected in the absence of a *K. brevis* bloom, cell concentrations were generally at or below the detection limit of 1 × 10³ cells l⁻¹.

Brevetoxins in primary prey fish collected during *Karenia brevis* blooms

Karenia brevis blooms were observed in Sarasota Bay concurrently with 3 of the primary prey fish collection periods; May to June 2003, January to May 2005 and August to September 2006. Analysis of these fish detected brevetoxins in 100% of sampled mullet (n = 23), pigfish (n = 11) and spot (n = 11) and in 93% of pinfish (n = 30) (Table 1). In total, 97% (n = 74) of all primary prey fish collected during *K. brevis* blooms were positive for brevetoxin in at least one sample/tissue type. Brevetoxins were detected in all whole viscera samples and in the majority of muscle, liver, gill and

stomach samples tested, with concentrations in PbTx-positive samples ranging between 4 to 10 844 ng PbTx-3 eq g⁻¹. All whole viscera samples (n = 49) were positive for brevetoxin, with concentrations ranging between 4 and 5188 (mean = 981) ng PbTx-3 eq g⁻¹. The majority of liver and stomach contents samples were positive for brevetoxins (92%, n = 25) and had brevetoxin concentrations comparable to whole viscera (147 to 4846 and 79 to 10 844 ng PbTx-3 eq g⁻¹, respectively). Brevetoxin was also detected frequently in gill samples (91%, n = 35) with concentrations between 26 and 7472 ng PbTx-eq g⁻¹. Brevetoxin concentrations in muscle samples were consistently lower than those detected in viscera, stomach, liver and gill samples for all species tested. Of the available muscle samples analyzed, 70% (n = 73) were positive for brevetoxin, with concentrations ranging between 5 and 365 (mean = 53) ng PbTx-3 eq g⁻¹. Brevetoxin concentrations in viscera were highest in spot, followed by pinfish, pigfish and striped mullet (mean = 1982, 1313, 610 and 81 ng PbTx-eq g⁻¹, respectively), with the

highest viscera concentration detected in pinfish (5188 ng PbTx-eq g⁻¹)

RBA analysis of ELISA-positive viscera samples detected brevetoxins in 15 of 25 samples, with detected concentrations ranging from 199 to 1566 ng PbTx-3 eq g⁻¹ (Table 2). The RBA did not detect brevetoxins in samples with a corresponding ELISA concentration of <200 ng PbTx-3 eq g⁻¹, though the detection limit for the RBA in the present study was 80 ng PbTx-3 eq g⁻¹. Although RBA measurements for these samples were generally between 30 and 60% of the corresponding ELISA brevetoxin concentration, the data obtained by RBA and ELISA were well correlated (R² = 0.830; Fig. 3).

LC-MS analyses of ELISA-positive viscera samples unambiguously confirmed the presence of parent brevetoxins (PbTx-3) as well as 6 known brevetoxin metabolites (hydrolysis products of PbTx-3 and -7, cysteine-PbTx-B/A and their sulfoxides) (Table 2). PbTx-3 was confirmed in all 8 samples tested, at concentrations ranging between 1.8 and 25 ng g⁻¹. Brevetoxin

Table 2. Comparison of ELISA, RBA and LC-MS results for brevetoxins in viscera of selected fish collected during *Karenia brevis* blooms (ng PbTx-3 eq g⁻¹ for ELISA and RBA, ng g⁻¹ for LC-MS). nd: not detected; +: qualitatively determined to be present (positive); nt: not tested

Sample	Date (mo/d/yr)	ELISA	RBA	LC-MS						
				Parent toxin PbTx-3	Brevetoxin metabolites					
				Hydrolysis product of PbTx-3	Hydrolysis product of PbTx-7	Cysteine- PbTx-B	Cysteine- PbTx-B sulfoxide	Cysteine- PbTx-A	Cysteine- PbTx-A sulfoxide	
Pinfish	5/19/03	3628	1566	25	3.2	+	291	1822	3.1	204
Spot	3/15/05	3282	1357	6.3	nd	+	612	1354	8.5	236
		2738	1222	2.3	nd	+	29	829	1.2	70
		2097	1324	7.8	nd	+	319	885	3.7	107
		2070	706	1.8	nd	+	17	281	nd	32
		2057	713	3	nd	+	232	1692	9.3	438
		1963	862	1.9	2.4	+	508	1225	8.3	63
Pinfish	5/19/03	1829	1038	3.6	4.9	nt	50	764	2.4	110
		1453	538							
Pigfish	5/28/03	1284	540							
Spot	3/15/05	905	247							
		743	230							
Pinfish	5/19/03	436	416							
Pinfish	6/11/03	261	nd							
Mullet	3/1/05	258	222							
Pigfish	5/28/03	239	nd							
		222	199							
Mullet	3/1/05	170	nd							
Pinfish	5/19/03	169	nd							
		142	nd							
Mullet	3/15/05	139	nd							
Mullet	3/1/05	131	nd							
		120	nd							
Pinfish	1/25/05	95	nd							
Mullet	3/15/05	80	nd							

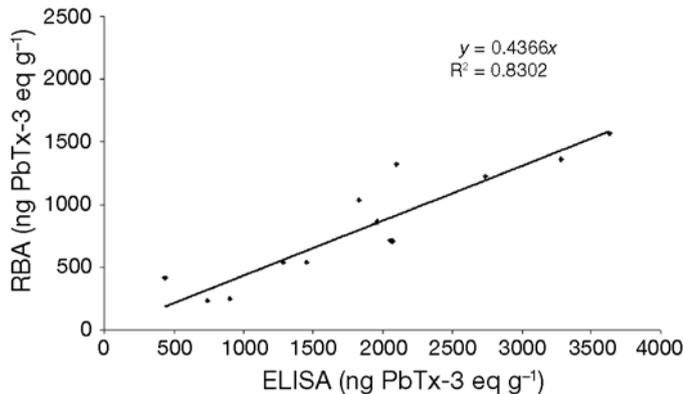


Fig. 3. Correlation of data obtained from RBA and ELISA methods for brevetoxin concentration in viscera of fish collected during *Karenia brevis* blooms

metabolites were detected in these samples at concentrations up to 1822 ng g^{-1} , with cysteine-PbTx-B sulfoxide as the most dominant congener. The total brevetoxin content for these samples (parent PbTx + quantifiable metabolites) ranged in concentration from 333 to 2375 ng g^{-1} .

Fish muscle, viscera and total body mass measurements were used to back-calculate brevetoxin concentrations on a whole-fish basis, in order to compare to allowable concentrations in seafood for human consumption and to estimate potential brevetoxin doses to dolphins during *Karenia brevis* blooms. Mean specimen weights for pinfish, pigfish and spot were 78 , 113 and 54 g , respectively, with muscle contributing between 48 and 52% and viscera contributing 6 to 8% of body mass. Based on these morphometrics and toxin data presented above, estimated brevetoxin concentrations in whole fish were greatest for pinfish (433 ng g^{-1}), followed by spot (289 ng g^{-1}) and pigfish (287 ng g^{-1}).

Brevetoxins in primary prey fish collected in the absence of *Karenia brevis* blooms

Primary prey fish (pinfish, pigfish, striped mullet and spot) collections during June to October 2004 and May to June 2005 coincided with non-bloom intervals in the Sarasota Bay region (Fig. 2). *Karenia brevis* cell concentrations during these intervals were generally at or below the detection limit of $1 \times 10^{-3} \text{ cells l}^{-1}$, indicative of background cell abundance (FWRI 2008). The majority of fish from this sample set were collected between 15 and 32 wk following the termination of the previous *K. brevis* bloom in the study area.

Analysis of these fish detected brevetoxins in the majority of specimens from each species (pinfish 88% ,

pigfish 90% , mullet 67% and spot 100%), with 88% ($n = 34$) of all primary prey fish sampled during non-bloom intervals testing positive for brevetoxin (Table 1). Brevetoxins were detected in 96% ($n = 26$) of viscera samples and in 20% of muscle samples ($n = 25$) at concentrations ranging from 7 to 753 (mean = 126) and 3 to 14 (mean = 7) ng PbTx-eq g^{-1} , respectively. Brevetoxin concentrations in liver, gill and stomach contents were varied, with concentrations of 21 to 1047 , 34 and 45 to $263 \text{ ng PbTx-eq g}^{-1}$, respectively. A subset of the ELISA-positive viscera samples (84 to $461 \text{ ng PbTx-3 eq g}^{-1}$) were analyzed by RBA, but brevetoxin was not detected above the limit of quantification of 80 ng g^{-1} . Back-calculated brevetoxin concentrations on a whole-fish basis resulted in estimated brevetoxin loads of 17 , 11 and 48 ng g^{-1} in pinfish, spot and pigfish, respectively.

Brevetoxins in non-primary prey items in the absence of a *Karenia brevis* bloom

Various species of fish, shrimp and squid were collected from Sarasota Bay during January through March 2006 and represented 33 potential and known prey species of bottlenose dolphins throughout the southeastern USA (Barros & Odell 1990). These prey items were collected between 3 and 15 wk following the termination of the July to December 2006 *Karenia brevis* bloom, and corresponding cell densities at time of collection were generally at or below the detection limit of $1 \times 10^{-3} \text{ cells l}^{-1}$. Analysis of these non-primary prey items detected brevetoxins in 65% ($n = 72$) of all specimens collected and in 76% of the species represented (Table 3). Viscera brevetoxin content in positive specimens ranged from 13 to over $1500 \text{ ng PbTx-eq g}^{-1}$. All specimens that were homogenized *in toto* were negative for brevetoxins.

DISCUSSION

Brevetoxins in primary prey fish collected during *Karenia brevis* blooms

The levels of brevetoxin detected in primary prey fish collected during *Karenia brevis* blooms (viscera, mean = $981 \text{ ng PbTx-3 eq g}^{-1}$, max. = $5188 \text{ ng PbTx-3 eq g}^{-1}$) indicate a significant amount of toxin accumulation in pinfish, pigfish, spot and striped mullet. Elevated densities of *K. brevis* cells (3.3×10^4 to $5.6 \times 10^7 \text{ cells l}^{-1}$) concurrent with collection of these fish are consistent with moderate to severe bloom conditions and implicate the *K. brevis* blooms as the causative agent for toxin accumulation in the fish. These findings

Table 3. Brevetoxin concentrations detected in non-primary prey species collected in the absence of a *Karenia brevis* bloom (January to March 2006). Concentrations expressed in nanograms PbTx-3 equivalents per gram sample (ng PbTx-3 eq g⁻¹). nd: not detected; -: no test made

Species name	Common name	n	Viscera; mean (range)	Whole fish
<i>Achirus lineatus</i>	Lined sole	2	nd	nd
<i>Anchoa hepsetus</i>	Striped anchovy	2	nd	-
<i>Ancylopsetta quadrocellata</i>	Ocellated flounder	1	nd	nd
<i>Archosargus probatocephalus</i>	Sheepshead	2	209 (207–211)	-
<i>Arius felis</i>	Hardhead catfish	2	404 (356–452)	-
<i>Bagre marinus</i>	Gafftopsail catfish	2	216 (203–228)	-
<i>Bairdiella chrysoura</i>	Silver perch	2	106 (31–180)	-
<i>Brevoortia</i> spp.	Menhaden	1	555	-
<i>Caranx hippos</i>	Crevalle jack	2	166	nd
<i>Citharichthys macrops</i>	Spotted whiff	1	nd	-
<i>Cynoscion nebulosus</i>	Spotted seatrout	2	30 (29–30)	-
<i>Elops saurus</i>	Ladyfish	2	180 (142–218)	-
<i>Gerreidae</i> spp.	Mojarra	3	373 (255–491)	nd
<i>Harengula jaguana</i>	Scaled sardine	2	190	nd
<i>Lactophrys quadricornis</i>	Scrawled cowfish	3	245 (152–393)	-
<i>Lolliguncula</i> sp.	Brief squid	4	-	nd
<i>Lutjanus griseus</i>	Mangrove snapper	2	-	nd
<i>Lutjanus synagris</i>	Lane snapper	1	-	nd
<i>Menticirrhus</i> spp.	Kingfish	2	748 (354–1141)	-
<i>Oligoplites saurus</i>	Leatherjacket	3	148 (20–352)	-
<i>Opisthonema oglinum</i>	Atlantic thread herring	3	71 (70–72)	nd
<i>Opsanus beta</i>	Gulf toadfish	4	42 (13–90)	-
<i>Paralichthys albigutta</i>	Gulf flounder	2	145	nd
<i>Peprilus alepidotus</i>	Harvestfish	2	184 (57–311)	-
<i>Pomatomus saltatrix</i>	Bluefish	2	94 (78–110)	-
<i>Prionotus scitulus</i>	Leopard searobin	2	342	nd
<i>Prionotus tribulus</i>	Bighead searobin	3	92 (88–95)	nd
<i>Sciaenops ocellatus</i>	Red drum	3	89 (76–101)	-
<i>Symphurus plagiusa</i>	Blackcheek tonguefish	1	1413	-
<i>Synodus foetens</i>	Inshore lizardfish	3	221 (210–232)	-
<i>Trachinotus falcatus</i>	Permit	2	>1500	-
<i>Urophycis floridana</i>	Southern hake	3	105 (25–185)	nd
—	Unidentified shrimp	1	-	nd

are concordant with findings of Naar et al. (2007) from near St. Joseph Bay, Florida, where 100% of the fish specimens analyzed from each species tested positive for brevetoxins in viscera samples. The detection of brevetoxins in stomach and gill samples from these fish demonstrates exposure via ingestion and respiratory pathways, respectively (Pierce et al. 2001), and brevetoxin in muscle tissue samples is an indication of bioaccumulation of the toxin (Poli et al. 1990). These results confirm that the primary prey items of Sarasota Bay area dolphins achieve elevated levels of brevetoxin during *K. brevis* blooms.

The LC-MS data show that nearly all the detectable brevetoxin present in these samples is in the form of metabolites. These metabolites can retain toxic potency and are likely to be the true cause of brevetoxin poisoning (Poli et al. 2000, Bottein-Dechraoui et al. 2007). In addition, between 35 and 63% (mean 45%) of the ELISA brevetoxin response in selected PbTx-positive viscera samples was detected by RBA

analysis (i.e. the toxic potency of these samples was approximately half that of pure PbTx-3). These complementary LC-MS and RBA data confirm that a large portion of the total brevetoxin content in these fish (as detected by ELISA) represents pharmacologically active brevetoxin molecules that have not been completely detoxified by metabolism. Thus the brevetoxin content that is present in these fish, and therefore available for consumption by dolphins, represents a toxic food source to these dolphins.

The levels of brevetoxin detected in viscera of spot and pinfish (mean = 1982 and 1313 ng PbTx-3 eq g⁻¹, respectively) are comparable to reported concentrations detected in fish caught live (>300 ng g⁻¹ in spot viscera; >400 ng g⁻¹ in pinfish viscera) from St. Joseph Bay during the 2004 dolphin mortality event (Flewelling et al. 2005) and in fish from a subsequent follow-up study in St. Joseph Bay (386 ng g⁻¹, spot; 1453 ng g⁻¹, pinfish) (Naar et al. 2007). These values are also comparable to those reported in stomach contents of

dolphins from the 2004 mortality event (2126 ng g⁻¹; Flewelling et al. 2005) as well as to those reported in Sarasota Bay dolphin stomach contents (1142 ng g⁻¹; Fire et al. 2007). These data support previous work demonstrating that the ichthyotoxic effects of brevetoxin do not preclude its accumulation in live fish (Flewelling et al. 2005, Naar et al. 2007) and suggest that fish are potential vectors of brevetoxins to upper trophic levels.

Of the 4 prey species of interest, brevetoxin loads were greatest in whole pinfish (433 ng g⁻¹). The current regulatory limit in Florida for brevetoxins in seafood for human consumption is 800 ng g⁻¹ (USFDA 2005). When considered in this context, these toxin loads are unlikely to constitute an acute dose. However, *Karenia brevis* blooms have been observed to last for 12 mo or more (Landsberg 2002), and the effects of chronic dietary exposure to strict piscivores such as Sarasota Bay dolphins are unknown. Using brevetoxin concentrations detected in prey fish, in addition to estimates of dolphin feeding rate and body mass, a total daily brevetoxin dose can be estimated for Sarasota Bay dolphins feeding on toxic fish. Previous studies estimate adult bottlenose dolphins consume 4.2 to 6.7% of their body weight daily (Young & Phillips 2002). Adult bottlenose dolphins inhabiting Sarasota Bay have body masses between 200 and 250 kg (Wells & Scott 1999). Assuming these dolphins feed exclusively on pinfish (433 ng PbTx g⁻¹ whole fish, the present study), this results in a maximum daily brevetoxin dose of 29 µg PbTx kg⁻¹ body weight. This value is similar to non-lethal levels used in oral brevetoxin dosing studies with rats (18.6 µg kg⁻¹; Cattet & Geraci 1993). However, these studies involved single-dose toxin administrations, and it is possible that repeat sub-acute brevetoxin exposures to dolphins over a period of months may have negative health effects less conspicuous than increased strandings and mortalities.

Brevetoxins in primary and potential prey species collected in the absence of *Karenia brevis* blooms

During the January to March 2006 collection, specimens from 33 non-primary prey species found in Sarasota Bay were collected within 3 to 15 wk following the termination of a particularly intense (up to 3×10^8 cells l⁻¹) and long-lasting (>11 mo) *Karenia brevis* bloom. The majority of specimens and species analyzed were positive for brevetoxins and concentrations detected (13 to 1500 ng PbTx-eq g⁻¹) were comparable to those observed in primary prey species (7 to 753 ng PbTx-eq g⁻¹) collected during non-bloom intervals. Although brevetoxins were not detected in specimens that were homogenized *in toto*, this may have been an artifact of

brevetoxins in viscera becoming diluted beyond the detection limit when combined with the more abundant muscle tissue.

Primary prey fish (pinfish, pigfish, striped mullet and spot) collected 15 to 32 wk following the termination of a *Karenia brevis* bloom displayed a high prevalence of brevetoxins (88% of specimens), although the concentrations detected were much lower than for primary prey items collected during *K. brevis* blooms. The majority (80%) of these brevetoxin-positive specimens were collected in September to October 2004 and May to June 2006. Prior to each of these collection periods the study area had remained free of *K. brevis* blooms for a period of over 6 mo. This detection of brevetoxins in fish following a 6 mo absence of *K. brevis* blooms is supported by a growing body of work demonstrating long-term persistence of brevetoxin in marine organisms and food webs. Lipophilic molecules like brevetoxin can bioaccumulate in fish (Bottein Dechraoui et al. 2007) and are generally eliminated much more slowly than other hydrophilic harmful algal toxins (Landsberg 2002). Brevetoxins are detectable in experimentally and naturally exposed shellfish and finfish several weeks to several months post-exposure (Plakas et al. 2004, Pierce et al. 2006, Hinton & Ramsdell 2008) and in some cases over 1 yr post-bloom (Naar et al. 2007). In southwest Florida, seagrasses also appear to have persistent detectable levels of brevetoxin in the absence of *K. brevis* blooms (L. J. Flewelling unpubl. data). With an estimated biological half-life of 229 h in finfish (Hinton & Ramsdell 2008), slow depuration of brevetoxin accumulated during *K. brevis* blooms may account for some of the brevetoxin detected in fish up to several days post-bloom. However, for fish collected several months after an identifiable bloom, it is more likely that brevetoxin exposure is through unidentified yet continual toxin sources, such as food matter consumed by prey fish. The presence of brevetoxins in these prey items is consistent with previous findings that Sarasota Bay dolphins sampled during non-bloom conditions had detectable levels of the toxin in their tissues (Fire et al. 2007). These results confirm that Sarasota Bay fish associated with background *K. brevis* levels have detectable brevetoxins, and the present study offers a first step in investigating the persistence of brevetoxins in the diet of Sarasota Bay dolphins.

Trophic transfer of brevetoxins

A review of feeding habits for the primary prey fish in question may begin to address the brevetoxin levels observed in the present study for fish collected during and in the absence of *Karenia brevis* blooms. A common paradigm in marine mammal exposure to harmful

algae is the trophic transfer of toxins via a short food chain, typically from toxic primary producers to end consumers mediated by a planktivorous fish vector (Anderson & White 1989, Geraci et al. 1989, Scholin et al. 2000, Flewelling et al. 2005). However, this does not seem to be the case for the Sarasota Bay dolphin community, as none of the 4 primary prey species which compose >80% of the dolphin diet are known to be planktivorous as adults. Although mullet exhibit planktivory as juveniles, mullet specimens collected for the present study were of the size (270 mm SL) corresponding to detritivory and herbivory on benthic epiphytes (Bishop & Miglarese 1978, Larson & Shanks 1996). The size class for pigfish (96 mm SL) and spot (176 mm SL) collected in the present study corresponded to a strictly carnivorous feeding strategy, utilizing infaunal and epibenthic invertebrates such as polychaetes and amphipods (Sutter & McIlwain 1987, Phillips et al. 1989). Pinfish of the size range represented here (125 mm SL) have feeding habits ranging from omnivory to strict herbivory, and their diet appears to be heavily influenced by location and local food availability (Darcy 1985). The diet of pinfish from Tampa Bay, Florida (an estuarine habitat adjacent to Sarasota Bay with abundant seagrass coverage), is dominated by filamentous algae, tunicates and seagrasses (Motta et al. 1995). Collectively, it appears that trophic transfer of brevetoxin to Sarasota dolphins occurs via an indirect benthic link rather than the direct plankton grazing link commonly observed in many HAB events. Unfortunately, brevetoxin loads in detritus and benthic invertebrates during periods following *K. brevis* blooms are not known, and future work is required to assess the impact of each benthic component on toxin accumulation in this food web. However, brevetoxin concentrations of up to 1263 ng g⁻¹ have been detected in Florida seagrasses *Thalassia testudinum* (Flewelling et al. 2005), and these are a likely post-bloom brevetoxin source to pinfish in this region, since they are primarily herbivorous and are associated with seagrasses for most of their life history (Luczkovich & Stellwag 1993, Luczkovich et al. 1995). Furthermore, since pinfish (1) had the highest whole-fish brevetoxin concentrations in the present study, (2) are the most common and most numerically abundant fish in the diet of Sarasota Bay dolphins (Barros & Wells 1998) and (3) have an abundant toxic food source, this species of fish in particular has significant potential to transfer brevetoxins to higher trophic levels and suggests that pinfish are an important brevetoxin vector to dolphins in this region.

The presence and persistence of brevetoxins in primary prey fish collected during and following *Karenia brevis* blooms suggests long-term availability of toxic food items to Sarasota Bay dolphins. Although the con-

centrations detected in the present study were likely not acute levels, persistence of brevetoxin in the dolphin diet constitutes a potential health risk, and further investigation of this food web will benefit conservation efforts. Acute exposure to dolphins during severe blooms is positively correlated to increased stranding frequency and mortality (Fauquier et al. 2005), but less is known about long-term, sublethal exposure. Chronic exposure of marine mammals to brevetoxin may result in immunological compromise which can in turn lead to other deleterious health effects (Bossart et al. 1998).

The present study demonstrates that, similar to fish from the St. Joseph Bay area (Flewelling et al. 2005, Naar et al. 2007), primary prey fish of Sarasota Bay dolphins can accumulate high concentrations of brevetoxins during *Karenia brevis* blooms. Fish collected in the absence of *K. brevis* blooms also contain detectable levels of brevetoxins, and the present study is the first to quantify non-bloom concentrations of brevetoxins in Sarasota Bay fish. Given their predominance in the diet of long-term, year-round resident Sarasota Bay dolphins (Barros & Wells 1998, Wells 2003), pinfish are a potential brevetoxin vector during *K. brevis* blooms and can accumulate brevetoxins in concentrations comparable to fish implicated as vectors in large-scale dolphin mortality events. The present study emphasizes the importance of investigating the feeding habits of animals involved in bloom-associated mortalities in order to establish specific routes of toxin trophic transfer from phytoplankton to top consumers. Understanding the capacity of the benthic component of this food web to accumulate brevetoxins is critical in predicting the exposure of Sarasota Bay dolphins to the harmful effects of *K. brevis* blooms. Additional studies determining all stages of trophic transfer of brevetoxins from *K. brevis* to top-level predators are needed, given the scarcity of available data on toxin loads in many organisms. Specifically, studies comparing toxin accumulation rates and persistence in zooplankton, detritus, fish, invertebrates and seagrass species are necessary for comparing toxin exposure between dolphin populations with different feeding habits. Brevetoxin exposure is more complex than a linear response to increased *K. brevis* cell densities and involves the interaction of various feeding strategies at multiple trophic levels. Once the fate of brevetoxins produced in a bloom is known and the distribution of toxin among the various organisms is established, researchers can more effectively determine the degree of exposure to dolphins in the context of their ranging patterns, feeding strategies and prey choice.

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