ELSEVIER

Toxicon 50 (2007) 707–723

TOXICON

www.elsevier.com/locate/toxicon

Brevetoxins, like ciguatoxins, are potent ichthyotoxic neurotoxins that accumulate in fish ☆

Jerome P. Naar[a,*], Leanne J. Flewelling[b], Allison Lenzi[a], Jay P. Abbott[b], April Granholm[b], Henry M. Jacocks[a], Damon Gannon[c], Michael Henry[c], Richard Pierce[c], Daniel G. Baden[a], Jennifer Wolny[b], Jan H. Landsberg[b]

[a] *Center for Marine Science, University of North Carolina at Wilmington, NC 28409, USA*
[b] *Florida Fish and Wildlife Conservation Commission, Fish and Wildlife Research Institute, St. Petersburg, FL 33701, USA*
[c] *Mote Marine Laboratory, Sarasota, FL, USA*

Received 23 March 2007; received in revised form 4 June 2007; accepted 5 June 2007
Available online 26 June 2007

Abstract

Brevetoxins and ciguatoxins are closely related potent marine neurotoxins. Although ciguatoxins accumulate in fish to levels that are dangerous for human consumption, live fish have not been considered as potential sources of brevetoxin exposure in humans. Here we show that, analogous to ciguatoxins, brevetoxins can accumulate in live fish by dietary transfer. We experimentally identify two pathways leading to brevetoxin-contaminated omnivorous and planktivorous fish. Fish fed with toxic shellfish and *Karenia brevis* cultures remained healthy and accumulated high brevetoxin levels in their tissues (up to 2675 ng g^{-1} in viscera and 1540 ng g^{-1} in muscle).

Repeated collections of fish from St. Joseph Bay in the Florida panhandle reveal that accumulation of brevetoxins in healthy fish occurs in the wild. We observed that levels of brevetoxins in the muscle of fish at all trophic levels rise significantly, but not to dangerous levels, during a *K. brevis* bloom. Concentrations were highest in fish liver and stomach contents, and increased during and immediately following the bloom. The persistence of brevetoxins in the fish food web was followed for 1 year after the *K. brevis* bloom.

Keywords: Brevetoxins; Ciguatoxins; *Karenia brevis*; *Gambierdiscus toxicus*; Neurotoxic Shellfish Poisoning (NSP); Ciguatera Fish Poisoning (CFP); Food web transfer; Vector; Ichthyotoxins; Human health

1. Introduction

Brevetoxins and ciguatoxins are potent marine neurotoxins. The source for the former is the planktonic red tide dinoflagellate *Karenia brevis* (McFarren et al., 1965; Baden et al., 1979) and for the latter is the epibenthic dinoflagellate *Gambierdiscus toxicus* (Yasumoto et al., 1977; Lewis et al., 2000). Both groups of toxins have similar chemical

☆ *Ethical statement*: The results presented in this manuscript are originals and are not being considered elsewhere. Regarding the use of vertebrate animals as described in the manuscript, experiments were performed according to the animal use policy (approved IUCAC protocol) at the corresponding author's institution.

*Corresponding author. Tel.: +1 910 962 2367; fax: +1 910 962 2410.
E-mail address: naarj@uncw.edu (J.P. Naar).

0041-0101/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.toxicon.2007.06.005

natures and similar biological activities. They are lipid-soluble polycyclic polyether compounds and are the only molecules known to activate voltage-sensitive sodium channels in mammals through a specific interaction with site 5 of the alpha subunit of the sodium channel (Poli et al., 1986; Lombet et al., 1987; Dechraoui et al., 1999). Both are toxic to mammals, and in humans the ingestion of brevetoxin-contaminated shellfish (Halstead, 1978; Steidinger, 1993) and of ciguatoxin-contaminated fish (Bagnis et al., 1979) results in severe forms of food poisoning—Neurotoxic Shellfish Poisoning (NSP) and Ciguatera Fish Poisoning (CFP), respectively. Because both toxins possess similar modes of action, the clinical manifestations following ingestion, although more severe, varied and longer-lasting for genuine ciguatera, are quite similar, with gastrointestinal, neurological and cardiovascular components (Bagnis et al., 1979; Baden et al., 1995; Poli et al., 2000; Kirkpatrick et al., 2004). The reversal of temperature discrimination, also known as paradoxical dysthesia, is probably the most characteristic symptom associated with ciguatera poisoning (Bagnis et al., 1979), but is also documented in severe cases of brevetoxin poisoning (Baden et al., 1995).

Despite these similarities, these toxins present obvious differences in their impacts on fish. Ciguatoxins are well known to accumulate in fish by trophic transfer in tropical fish food webs (Legrand, 1991), and have not been documented in association with fish mortalities. In contrast, *K. brevis* blooms (Florida red tides) characteristically result in massive fish kills (Steidinger et al., 1973; Landsberg, 2002). Because of the fish kills routinely observed along the west coast of Florida during red tides, it has been assumed that the ichthyotoxicity of brevetoxins precluded their vectoring or accumulation via live fish.

In 2004, a mass mortality of bottlenose dolphins (*Tursiops truncatus*) in the Florida panhandle clearly indicated that fish are not always killed by *K. brevis* red tides and that they have the potential to vector brevetoxins to higher trophic levels (Flewelling et al., 2005). Besides a coincident mortality of large redfish (*Sciaenops ocellatus*), no obvious indicators of a red tide were reported (i.e. there was no obvious bloom or other animal mortalities). However, high levels of brevetoxins were measured in multiple tissues of all bottlenose dolphins examined ($n = 36$). Tissues from six undigested menhaden (*Brevoortia* sp.) recovered from the stomach contents of some dolphins contained excessive levels of brevetoxins, with concentrations reaching 33,200 ng g^{-1} in viscera and 1500 ng g^{-1} in muscle. At least eight other species of fish collected live from the area while the mortality was ongoing also contained elevated, but much lower, concentrations of brevetoxins in their tissues (Flewelling et al., 2005). During this event, we confirmed that bottlenose dolphins are susceptible to brevetoxicosis and that planktivorous fish can vector lethal concentrations of brevetoxins to higher trophic levels.

The goals of the present study were to experimentally identify pathways by which brevetoxins may accumulate in the tissues of live fish, and to determine whether the brevetoxin accumulation that we previously observed in live fish (Flewelling et al., 2005) was an exception or alternatively, an undocumented common occurrence.

2. Materials and methods

2.1. Experimental exposure of omnivorous fish to contaminated shellfish

Hard clams (*Mercenaria* sp.) naturally contaminated with brevetoxins were collected from Charlotte Harbor, Florida during a red tide in March of 2003 and stored at −20 °C for 2 months prior to the experiments. Locally harvested nontoxic hard clams (*M. mercenaria*) were purchased in North Carolina immediately before the experiments began.

Adult pinfish (*Lagodon rhomboides*; 12–17 cm total length) and Atlantic croakers (*Micropogonias undulatus*; 15–22 cm total length) were collected using hook-and-line gear from the Intracoastal Waterway in Wilmington, NC, USA. Fish (36 per species) were maintained in separate 24-l aquaria (3 fish per aquarium, 24 aquaria) in natural filtered seawater under constant flow and aeration. Salinity of the seawater source (Intracoastal Waterway) varied between 20 and 32 ppt. Fish were acclimated to aquaria conditions for 1 week, during which they were fed shucked nontoxic clams (7–10 g each, one clam per fish) twice per day. After acclimation, fish were fed shucked toxic hard clams (7–10 g each, one clam per fish) twice daily for 14 days, and feeding was confirmed by direct observation. Fish were then fed nontoxic shucked hard clams (7–10 g each, one clam per fish) from North Carolina twice daily for another 14 days. Three pinfish and three croakers each were sampled at days 0, 7, 14, 21 and 28. Control fish were similarly fed nontoxic hard clams

throughout the experiment and were sampled in duplicate at days 0, 7, 14, 21 and 28.

2.2. Experimental exposure of planktivorous fish to K. brevis culture

Juvenile striped mullet (*Mugil cephalus*; 3–4 cm total length) were collected using a dip net from the Intracoastal Waterway in Wilmington, NC, and acclimated to synthetic seawater media (NH15) under aeration in 4-l aquaria for 48 h. Then 10 fish each were exposed to four densities of *K. brevis* cells (Wilson clone). *K. brevis* cultures were grown in NH15 media at 30 ppt. Only *K. brevis* cultures in exponential growth phase were used. Cell density of the culture was determined by enumeration of Lugol's iodine-preserved aliquots at 100 × using a Nikon light microscope. Aeration in the aquaria was stopped before addition of the culture to prevent lysis of the *K. brevis* cells. After addition of the culture, final cell densities were 500, 1000, 2000 and 4000 cells ml^{-1}, all within the range of densities observed in the Gulf of Mexico during red tides. Cell integrity was confirmed by microscopic examination. Five fish were sampled from each aquarium after 6 h and again after 24 h. Dissolved brevetoxin concentrations and total brevetoxin concentrations in the water were measured after 6 and 24 h. Control fish (juvenile striped mullet held in NH15 media and not exposed to *K. brevis*) were sampled on the same schedule.

To demonstrate brevetoxin ichthyotoxicity, identical experiments were performed, but *K. brevis* cells were lysed by sonication (15 min) before adding the culture to the aquaria. After adding the lysed *K. brevis* culture, fish were continually monitored and the time to death was recorded to the nearest minute. Juvenile striped mullet held in NH15 media and not exposed to *K. brevis* served as control fish.

2.3. Field collections

Collections of live fish were made from St. Joseph Bay in northwest Florida in February, June, September and November of 2005, and in May, August and November of 2006. Fish were collected using a 3.6-m cast net (19-mm mesh) as well as hook-and-line gear. Water samples were collected using a horizontal PVC beta bottle (3.2 l; Wildco^{TM}) from 0.5 m below the surface and from 1 m above the bottom at fish collection sites and multiple other sites (10–20) throughout St. Joseph Bay. Aliquots (500 ml) for brevetoxin analysis were stored on ice in polyethylene bottles while in the field and frozen at −20 °C immediately upon return until processed.

K. brevis cells were enumerated from Lugol's iodine-preserved aliquots. Samples were mixed by inverting (>10 ×) and 3 ml placed in a Lab-Tek chamber (Nalge Nunc # 155380). Cells were allowed to settle for at least 1 h prior to enumerating. Samples were identified and enumerated at 100 × and 400 × using a Zeiss Axiovert 25 or Olympus IX71 inverted microscope. Identifications were based on Haywood et al. (2004) and descriptions published in Steidinger et al. (in press). In addition to the water samples we obtained during each fish collection, *K. brevis* cell counts were also obtained for five fixed sites in St. Joseph Bay that were sampled every month by the Florida Department of Environmental Protection's St. Joseph Bay Aquatic Preserve staff from January 2005 through December 2006.

2.4. Sample extractions

Fish from exposure studies were euthanized by exposure to a lethal concentration of MS-222 anesthetic (tricaine methanesulfonate, 50 mg l^{-1}). Death was confirmed by absence of ventilation and absence of reaction after stimulus. From pinfish and croaker, muscle, liver and remaining viscera were extracted separately. Due to the small size of the juvenile mullet, muscle was separated from viscera and each was extracted separately. Field-caught fish were weighed and their total length was measured. Muscle, liver and stomach contents were sampled, weighed and extracted separately. Brevetoxins in fish tissues and stomach contents were extracted by homogenization in 100% acetone (4 ml g^{-1} tissue). Homogenates were centrifuged (10 min at 3200*g*), the supernatants were retained and the pellets were extracted a second time in the same manner. The supernatants were pooled and evaporated to dryness. The extracts were then re-dissolved in 80% aqueous methanol and partitioned twice with 100% hexane (1:1 v:v). The methanol fraction was evaporated to dryness, and re-dissolved in 100% methanol. For LC-MS analyses, a 1 g equivalent in a subsample of extracts was diluted to 25% methanol and applied to a pre-conditioned C18 SPE cartridge (Supelco, 500 mg, 3 ml). The column was washed with 25% methanol and toxins were eluted with 100% methanol. The methanol

extract was then evaporated to dryness and re-dissolved in 1 ml of 100% methanol.

Water samples collected from fish exposure aquaria were either gravity filtered by passing through a cotton-packed glass Pasteur pipette (dissolved brevetoxins) or sonicated for 15 min (total brevetoxins) and immediately analyzed. Total brevetoxins were extracted from field-collected seawater samples by passing 500 ml through a preconditioned C18 SPE disk (3 M Empore^{TM}). The disk was then rinsed twice with 10 ml of deionized water, and brevetoxins were eluted with 20 ml of 100% methanol. The methanol extracts were evaporated to dryness and re-dissolved in 2 ml of 100% methanol. Fish and water extracts were stored at −20 °C until analyzed.

2.5. Brevetoxin analyses

Brevetoxin concentrations were measured in fish tissues, stomach contents, shellfish and water extracts by competitive ELISA according to Naar et al. (2002). This assay has been shown to be an effective tool to assess brevetoxin and brevetoxin metabolite contamination in a wide range of biological and environmental matrices including seawater and seaspray (Cheng et al., 2005; Pierce et al., 2005), shellfish (Naar et al., 2002, 2004; Dickey et al., 2004; Plakas et al., 2004; Pierce et al., 2006), dolphin and manatee tissues and body fluids (Flewelling et al., 2005), and seagrass (Flewelling et al., 2005). The antibodies used in this assay were obtained following goat immunization with PbTx-3-KLH conjugates (Trainer and Baden, 1991). Because standards for brevetoxin metabolites are unavailable, the exact affinity of these polyclonal antibodies for the different brevetoxin metabolites is not known, but the good correlation observed between results from ELISA and HPLC-MS analyses of shellfish tissues (Dickey et al., 2004; Plakas et al., 2004; Pierce et al., 2006) indicates a similar affinity of our antibodies for the brevetoxins and their derivatives. Results are expressed as PbTx-3 equivalents and reflect the overall concentration of brevetoxins and brevetoxin-like compounds present in the sample. As performed, the limit of detection by ELISA was 5 ng g^{-1} in tissues and 0.1 μg l^{-1} in extracted seawater.

Toxicity of clams used in exposure studies was determined by mouse bioassay conducted according to the official method (APHA, 1970).

Brevetoxin confirmation and composition was determined in a subset of samples by LC-MS performed on a ThermoFinnigan AqA HPLC/MS. The AqA single quad system scanned from 204 to 1216 AMU with AqA Max 40 V, and a scan rate of 1.1 scans per second. All analyses were conducted using electrospray ionization 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 5Fm $250 \times 2\,mm^2$ 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. Parent brevetoxins (PbTx-1:867, PbTx-2:895, PbTx-3:897, PbTx-6:911, PbTx-7:869, PbTx-9:899, PbTx-10:871, Brevenal:657) and brevetoxin metabolites (Cyst-PbTx-2:1018, Ox-Cyst-PbTx-2:1034, Cyst-PbTx-1:990, Ox-Cyst-PbTx-1:1006) were monitored at indicated masses. 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.

3. Results

3.1. Experimental exposure of omnivorous fish to toxic shellfish

A subset of hard clams used in the exposure studies was analyzed by LC-MS prior to exposures and was found to contain PbTx-3 and metabolites. Brevetoxin concentration by ELISA was 1800 ng g^{-1}, and toxicity assessed by mouse bioassay was 30 mouse units (MU) per 100 g. No brevetoxins were detected by ELISA in the control clams.

While feeding toxic hard clams for 2 consecutive weeks, none of the pinfish or croakers died or exhibited any obvious signs of adverse effects. For comparison with the planktivorous fish exposures, toxin concentrations measured in liver and remaining viscera were averaged by tissue weight and are referred to as viscera. Higher concentrations of brevetoxins were measured on day 14 compared to day 7 of the exposures (Fig. 1), with the maximum average levels in pinfish (1412 ± 151 ng g^{-1} in muscle and 2233 ± 442 ng g^{-1} in viscera, $n = 3$) exceeding those measured in croakers (955 ± 73 ng g^{-1} in muscle and 1990 ± 166 ng g^{-1} in viscera, $n = 3$). The highest levels measured in an individual fish were 2675 ng g^{-1} in viscera and 1540 ng g^{-1} in muscle in a pinfish on the final day of the exposure

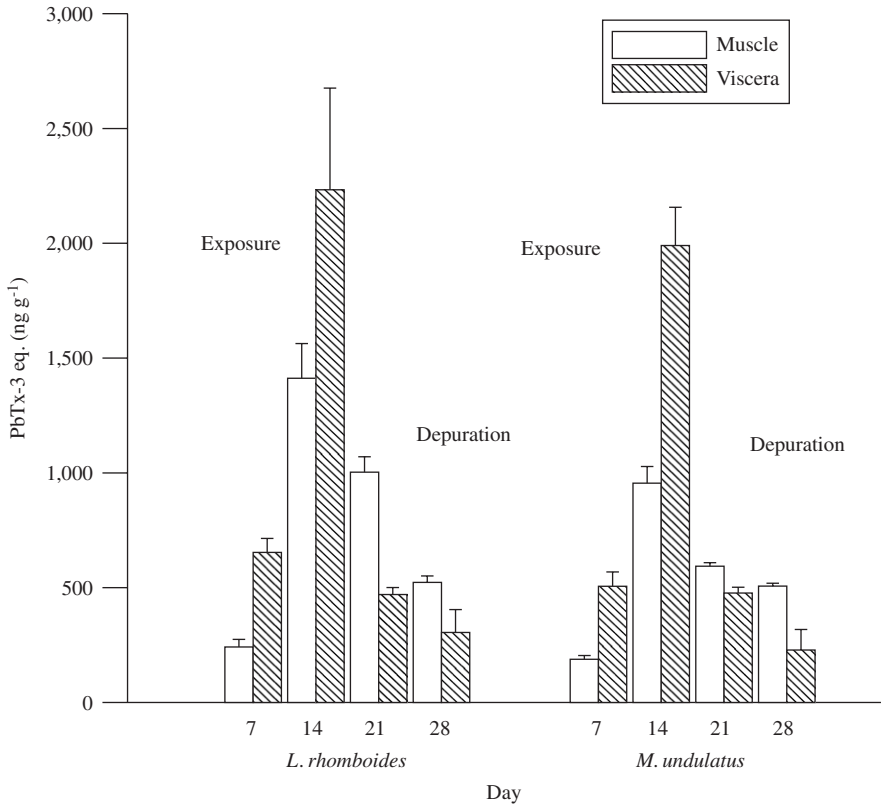

Fig. 1. Brevetoxin concentrations measured in muscle and viscera of pinfish (*L. rhomboides*) and croakers (*M. undulatus*) fed brevetoxin-contaminated clams (day 1–14) followed by noncontaminated clams (day 15–28). Error bars indicate standard deviation between individual fish ($n = 3$).

period (day 14). When fish were switched to a diet of nontoxic clams, brevetoxin concentrations dropped but remained detectable in both muscle and viscera after 2 weeks of feeding on the nontoxic diet. Brevetoxins were not detected in fish on day 0 (before exposure) or in any of the control fish.

3.2. Experimental exposure of planktivorous fish to *K. brevis* culture

During the exposure of juvenile striped mullet to *K. brevis* cultures (500–4000 cells ml^{-1}), microscopic observations of the exposure media at 6 and 24 h verified that *K. brevis* cells remained intact. ELISA analyses of the exposure media after 6 and 24 h confirmed that greater than 95% of brevetoxin measured in the water was associated with the *K. brevis* cells (Fig. 2a). Under these conditions, all fish survived and quickly accumulated brevetoxins in both muscle and viscera. After 6 h, brevetoxins were measurable in all fish with concentrations ranging from 25 to 231 ng g^{-1} in muscle and 120–335 ng g^{-1} in viscera (data not shown). Brevetoxin concentrations in fish tissues increased with increasing *K. brevis* cell densities and with the duration of exposure—reaching an average of 333 ng g^{-1} in muscle and 616 ng g^{-1} in viscera after 24 h (Fig. 2a). In viscera, this increase was linear with increasing cell concentration ($r^2 = 0.998$), while in muscle the increase was only linear up to 2000 cells ml^{-1} ($r^2 = 0.91$), with no further increase at the highest cell density. Brevetoxins were not detected in any of the control fish.

In parallel experiments, the ichthyotoxicity of dissolved brevetoxins was confirmed when *K. brevis* cells were lysed by sonication before addition to the aquaria (Fig. 2b). In this case, the fish did not accumulate toxins but died quickly (7–80 min), and the time to death correlated with the number of lysed cells ($r^2 = 0.99$). In both experiments, the total brevetoxin concentrations in the aquaria were nearly identical (ranging from 8 to 66 ng ml^{-1}) and were proportional to the initial *K. brevis* cell densities (Figs. 2a and b).

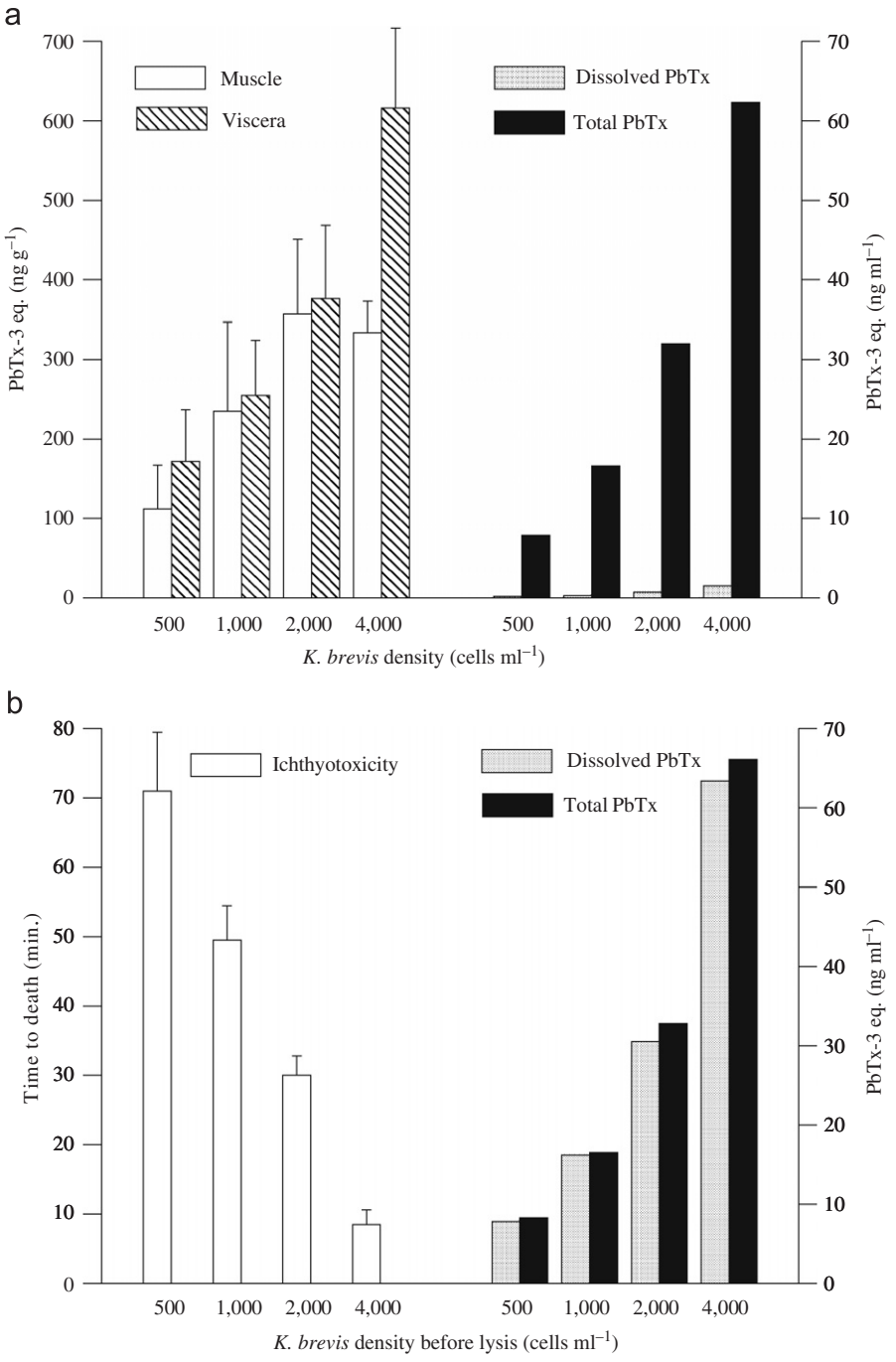

Fig. 2. (a) Brevetoxin concentrations in muscle and viscera of juvenile striped mullet after 24 h exposure to *K. brevis* cell cultures at indicated cell densities, and total and dissolved concentrations of brevetoxins measured in exposure media. (b) Time to the death of juvenile striped mullet exposed to identical densities of lysed *K. brevis* cells, and total and dissolved concentrations of brevetoxins measured in exposure media. For (a) and (b), error bars indicate standard deviation between individual fish.

3.3. Field results

Maximum *K. brevis* cell densities observed in St. Joseph Bay each month from January 2005 through December 2006 are shown in Fig. 3. No *K. brevis* cells at bloom densities were observed in samples collected in St. Joseph Bay between January and July 2005; and only background levels were present

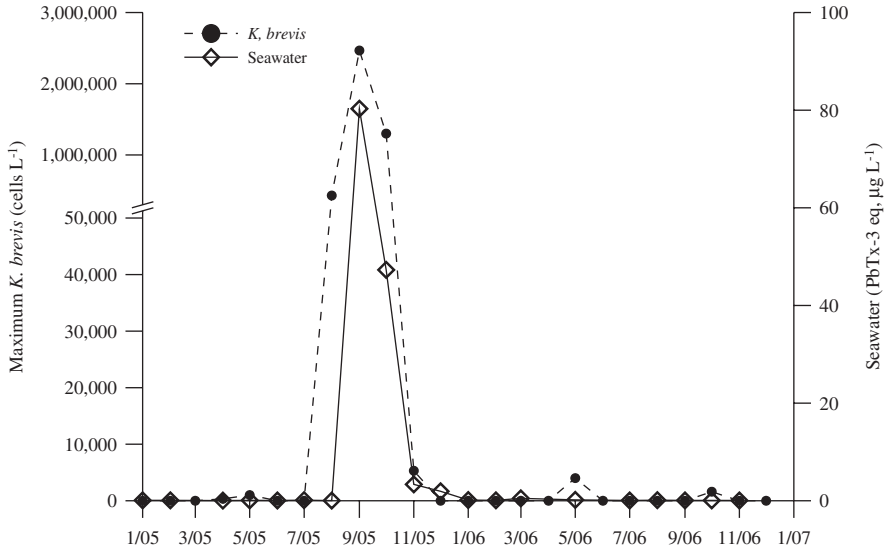

Fig. 3. Maximum *Karenia brevis* cell densities and brevetoxin concentrations measured in seawater samples collected throughout St. Joseph Bay between January 2005 and December 2006.

in a few of the samples collected in April and May 2005. In August 2005, a southwest Florida *K. brevis* bloom was transported into the Florida panhandle, and elevated cell densities were first observed in St. Joseph Bay in a sample collected on August 31, 2005. In September 2005, *K. brevis* cell concentrations in the bay reached as high as 2.5 million-cells l^{-1} (Fig. 3). *K. brevis* cell densities remained elevated in the bay until late October. In mid-November 2005, maximum densities of 5300 *K. brevis* cells l^{-1} were observed. Subsequent to this date, no *K. brevis* were found until May 2006, when very low levels (maximum of 4000 cells l^{-1}) were noted at only a few sites. Brevetoxin concentrations measured in St. Joseph Bay water samples ranged from <1 to 80 µg PbTx l^{-1} during the red tide bloom in September 2005, and were often measurable but low (typically <1 µg l^{-1}) in the absence of a *K. brevis* bloom (Fig. 3).

From February 2005 to November 2006, 184 fish representing 38 species of varying diets and trophic groups were collected from St. Joseph Bay and analyzed for brevetoxins. Species collected and maximum brevetoxins concentrations measured in muscle, liver and stomach contents are listed in Table 1, which includes 101 fish collected during the spring of 2004. Species included small planktivorous thread herring (*Opisthonema oglinum*) and sardines (*Harengula jaguana*), large piscivorous bluefish (*Pomatomus saltatrix*) and seatrout (*Cynoscion nebulosus*), as well as demersal and benthic omnivorous species such as pinfish (*L. rhomboides*) and catfish (*Ariopsis felis*).

The average brevetoxin concentrations measured by ELISA in muscle, liver and stomach contents for each collection are shown in Fig. 4, and the prevalence of brevetoxins, expressed as the percentage of brevetoxin-positive samples (by ELISA) of each tissue type, is shown in Fig. 5. Although no *K. brevis* bloom was noted in the bay until September 2005, brevetoxins were detected in more than 69% of fish collected in February and June 2005 (Fig. 5). In general, average levels were highest during and immediately following the red tide in September and November 2005, after which they slowly decreased (Fig. 4).

In February 2005, no toxin was measured in the muscle of any fish. In June, 10% of the fish tested contained low brevetoxin levels (13–20 ng g^{-1}) in the muscle. During the *K. brevis* bloom in September 2005, brevetoxins were measurable in the muscle of 96% of the fish tested, with concentrations ranging from 9 to 581 ng g^{-1}. In November 2005 after the *K. brevis* bloom, brevetoxin was found in the muscle of 88% of the fish collected ($n = 17$), with a maximum concentration of 116 ng g^{-1} measured in a gag grouper (*Mycteroperca microlepis*). By May 2006, both the proportion of positive muscle samples (64%, $n = 23$) and the concentrations measured in the muscle had decreased. With the exception of one hardhead sea catfish (*A. felis*), brevetoxin was not found in the muscle of any fish

Table 1
Maximum brevetoxin concentrations measured in fish collected from St. Joseph Bay between March 2004 and November 2006

Species	Common name	Main diet	*N*	Maximum [PbTx-3 eq. (ng g^{-1})]		
				Muscle	Liver	GI contents
Sardinella aurita[a,b]	Spanish sardine	Plankton	6	581	<ld	137
Mugil cephalues[a]	Striped mullet	Plankton	9	40	6682	393
Harengula jaguana	Scaled sardine	Plankton	13	52	2472	2839
Opisthonema oglinum	Atlantic thread herring	Plankton	21	54	473	508
Remora remora	Remora	Plankton	1	<ld	131	384
Lagodon rhomboides[a]	Pinfish	Herbivore	45	129	1453	911
Hyporhamphus meeki[a]	American halfbeak	Invertebrates	5	124	1977	188
Chilomycterus schoepfi[a]	Striped burrfish	Benthic invert.	1	27	571	138
Acanthostracion quadricornis	Scrawled cowfish	Benthic invert.	2	20	228	52
Bairdiella chrysoura	Silver perch	Benthic invert.	1	<ld	246	37
Orthopristis chrysoptera	Pigfish	Benthic invert.	3	<ld	277	105
Syngnathus scovelli	Gulf pipefish	Benthic invert.	1	<ld	<ld	<ld
Archosargus probatocephalus	Sheepshead	Benthic invert.	5	18	3709	613
Haemulon plumierii	White grunt	Benthic invert.	1	<ld	19	<ld
Lobotes surinamensis	Tripletail	Benthic invert.	1	<ld	<ld	<ld
Ariopsis felis	Hardhead sea catfish	Benthic invert.	2	12	1408	27
Bagre marinus	Gafftopsail catfish	Benthic invert.	1	<ld	1312	<ld
Leiostomus xanthurus	Spot	Benthic invert.	27	186	Viscera 386[c]	
Menticirrhus americanus	Southern kingfish	Benthic invert.	3	<ld	22	8
Menticirrhus littoralis	Gulf kingfish	Benthic invert.	2	<ld	Viscera 31[c]	
Menticirrhus saxatilis	Northern kingfish	Benthic invert.	7	<ld	Viscera 77[c]	
Micropogonias undulatus	Atlantic croaker	Benthic invert.	6	<ld	51	57
Urophycis floridana	Southern codling	Benthic invert.	4	60	Viscera 285[c]	
Prionotus tribulus	Bighead sea robin	Benthic invert.	1	<ld	<ld	<ld
Elops saurus[a]	Ladyfish	Piscivore	5	11	76	49
Cynoscion nebulosus[a]	Spotted seatrout	Piscivore	16	414	5133	3955
Lutjanus griseus[a]	Mangrove snapper	Piscivore	10	52	321	615
Mycteroperca microlepis[a]	Gag grouper	Piscivore	6	116	6034	720
Opsanus beta[a]	Gulf toadfish	Piscivore	1	69	7	400
Lutjanus campechanus[a]	Red snapper	Piscivore	3	102	16483	664
Paralichthys albigutta[a]	Gulf flounder	Piscivore	5	65	528	742
Carangoides bartholomaei	Yellow jack	Piscivore	1	<ld	218	<ld
Caranx crysos	Blue runner jack	Piscivore	1	<ld	478	<ld
Pomatomus saltatrix	Bluefish	Piscivore	18	45	190	131
Scomberomorus maculatus	Spanish mackerel	Piscivore	28	24	221	11
Strongylura marina	Atlantic needlefish	Piscivore	1	<ld	<ld	<ld
Centropristis striata	Black sea bass	Piscivore	10	<ld	52	206
Cynoscion arenarius	Sand seatrout	Piscivore	1	<ld	468	14
Lutjanus synagris	Lane snapper	Piscivore	1	<ld	<ld	16
Oligoplites saurus	Leatherjacket	Piscivore	4	<ld	109	33
Paralichthys lethostigma	Southern flounder	Piscivore	2	<ld	8	<ld
Synodus foetens	Inshore lizardfish	Piscivore	4	<ld	96	12

<ld, below level of detection.
[a]At least one specimen collected during September or November 2005 (i.e. during or just after *K. brevis* bloom).
[b]No liver or GI contents analyzed from *K. brevis* bloom period.
[c]Fish collected only in 2004, muscle and viscera (organs and GI tract) analyzed.

collected in August or November 2006. Overall, the highest brevetoxin concentration we measured in muscle (581 ng g^{-1}) was from a Spanish sardine (*Sardinella aurita*) collected during the September 2005 red tide.

Liver samples consistently yielded the greatest proportion of brevetoxin-positive results (Figs. 4 and 5). In February 2005, brevetoxin concentrations in fish livers ranged from not detectable (<5–10 ng g^{-1}) in 30% of the fish collected to

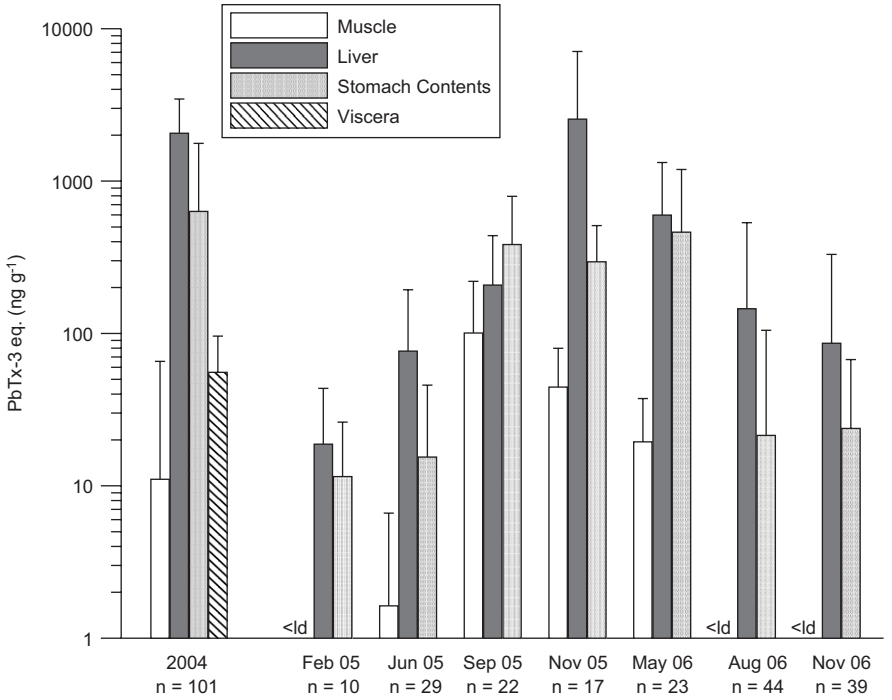

Fig. 4. Average concentrations of brevetoxins measured in fish (all species) collected throughout St. Joseph Bay between 2004 and 2006. In 2005 and 2006, fish collections were performed during 2–3 day sampling trips. Error bars indicate standard deviation.

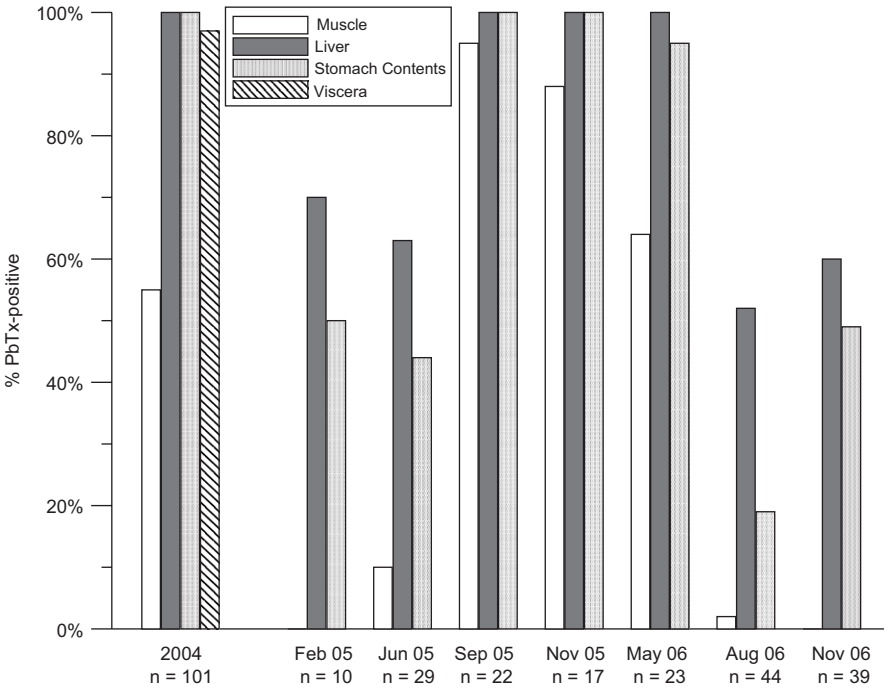

Fig. 5. Prevalence of brevetoxin contamination in fish (all species) collected throughout St. Joseph Bay between 2004 and 2006. Prevalence is expressed as the percentage of fish containing detectable levels of brevetoxins (>5–$10\,\mathrm{ng\,g^{-1}}$).

81 ng g^{-1}. Average liver brevetoxin concentrations increased in each collection reaching peak levels in November 2005, just after the red tide ended. The highest liver concentration measured was 16,483 ng g^{-1} in a red snapper (*Lutjanus campechanus*) collected in November 2005. In May 2006, the average concentration in the livers had decreased but was still high (598 ng g^{-1}), and brevetoxin was detected in 100% of the livers tested. In August and November 2006, the averages continued to decline, but brevetoxin was still found in the livers of more than half of the fish collected.

Brevetoxin concentrations in stomach contents displayed the greatest variability. In February and June 2005, brevetoxins were measured in the stomach contents of approximately half of the fish collected (Fig. 5) and at only low levels, with maximum concentrations of 43 and 144 ng g^{-1}, respectively, both measured in pinfish (*L. rhomboides*). In September and November 2005, brevetoxins were measured in all fish stomach contents. Surprisingly, the average concentrations in stomach contents in fish collected in September and November 2005 and May 2006 were not significantly different from each other (Kruskal–Wallis one-way analysis of variance by ranks followed by a Dunn's multiple comparisons test, 95% significance level). The highest level we measured in 2005 and 2006 was 2839 ng g^{-1} in the stomach of a scaled sardine (*H. jaguana*) collected in May 2006. However, the highest level reported in Table 1 (3955 ng g^{-1}) is from a spotted seatrout (*C. nebulosus*) collected in 2004.

3.4. Toxin profiles in fish

For both types of experiments (fish exposures to toxic clams and to *K. brevis* cultures), LC-MS analyses revealed that the fish had accumulated the same toxins to which they were exposed (Table 2). The toxin profile observed in the hard clams was almost identical to that observed in the liver of both pinfish and croakers, consisting principally of brevetoxin metabolites (cys-PbTx-2 and OxCys-PbTx-2) and, to a lesser extent, PbTx-3 (approximately 30% of the total amount of toxins). For planktivorous fish, the relative proportion of the toxins (PbTx-2 > PbTx-3 > PbTx-6 > PbTx-9) observed in viscera was almost identical to that observed in the *K. brevis* culture (PbTx-2 > PbTx-3,-9 > PbTx-6 > brevenal). Interestingly, brevenal, the brevetoxin antagonist produced by *K. brevis* was not detected in the viscera of these fish despite its presence in the *K. brevis* culture.

Toxin profiles determined for fish collected live from St. Joseph Bay were mainly metabolized brevetoxins (Table 2). No brevetoxins could be identified by LC-MS in extracts from four additional fish (two pinfish, *L. rhomboides;* one scaled sardine, *H. jaguana*; and one Atlantic thread herring, *O. oglinum*). These sample extracts were less concentrated (150–850 ng ml^{-1} by ELISA) than those listed in Table 2 for which profiles could be obtained. The lack of any detectable brevetoxins may be the result of insufficient sensitivity of our LC-MS method, the duration of sample storage prior to LC-MS analysis (6–30 months at −20 °C)

Table 2
Brevetoxin composition in fish compared to their food sources in experimental exposures (*K. brevis*, toxic hard clams) and in selected fish species collected from St. Joseph Bay

Species	Common name	Tissue type	Brevetoxins detected by HPLC-MS
Karenia brevis[a]	Florida red tide		PbTx-2 > PbTx-3,-9 > PbTx-6 > brevenal
Mugil cephalus[a]	Striped mullet	Viscera	PbTx-2 > PbTx-3 > PbTx-6 > PbTx-9
Mercenaria sp.[a]	Hard clam	Whole	cys-PbTx-2 > PbTx-3 > OxCys-PbTx-2
Lagodon rhomboides[a]	Pinfish	Liver	cys-PbTx-2 > PbTx-3 > OxCys-PbTx-2
Micropogonias undulatus[a]	Atlantic croaker	Liver	cys-PbTx-2 > PbTx-3 > OxCys-PbTx-2
Brevoortia sp.[b,c]	Menhaden	Muscle/viscera	PbTx-3 > PbTx-2 > cys-PbTx-2
Cynoscion nebulosus[b]	Spotted seatrout	Liver	cys-PbTx-2 > PbTx-3
Pomatomus saltatrix[b]	Bluefish	Liver	cys-PbTx-2 > PbTx-3
Ariopsis felis	Hardhead catfish	Liver	cys-PbTx-2
Hyporhamphus meeki	American halfbeak	Liver	cys-PbTx-2
Cynoscion nebulosus	Spotted seatrout	Liver	cys-PbTx-2

[a]From experimental exposures.
[b]Fish collected in 2004.
[c]Menhaden recovered from bottlenose dolphin stomach contents.

or the presence of unidentified brevetoxin metabolites.

4. Discussion

4.1. Pathways for accumulation of brevetoxins in fish

Our experimental results demonstrate that fish can accumulate ichthyotoxic brevetoxins when exposed through their diet, and that toxins can be transferred through the food web by fish feeding on toxic prey such as toxic shellfish and *K. brevis* cells. However, fish in red tide-endemic areas are exposed to brevetoxins through other prey or forage items as well, including zooplankton, fish and seagrass with associated epiphytes (Fig. 6), all of which have the potential to accumulate brevetoxins (Tester et al., 2000; Flewelling et al., 2005). The presence of small planktivorous contaminated fish (clupeids) in the stomachs of larger piscivorous fish (sea trout and hardhead sea catfish) from St. Joseph Bay pointedly illustrated the transfer of brevetoxins to additional trophic levels in the fish food web.

From our field-caught fish, the highest brevetoxin level observed in muscle was 581 ng g^{-1} in planktivorous Spanish sardines (Table 1). During our experiments, the levels of brevetoxin accumulation resulting from exposure of planktivorous striped mullet to *K. brevis* were lower than this and lower than those achieved when fish fed on toxic shellfish, but the exposure duration for the former was restricted to 24 h due to the need to prevent lysing of the *K. brevis* cells (no filtration or aeration), while the latter was 2-weeks long. While brevetoxin concentration in the viscera of experimentally exposed mullet increased linearly with cell concentration, brevetoxin concentration in muscle increased linearly only up to 2000 cells ml^{-1}, no longer increasing at the highest level. This could reflect a saturation effect or more likely a change in the kinetics of accumulation since higher levels were measured in the fish exposed to toxic shellfish as well as in field specimens. Transfer of brevetoxins into fish during similar short-term exposures to *K. brevis* cultures was also demonstrated by Woofter et al. (2005) who noted immediate uptake of brevetoxin with measurable blood levels as soon as 1 h after exposing juvenile striped mullet to *K. brevis*. Over the course of the 24-h experiment, maximum blood levels were found after 8–12 h of exposure, with a drop to 50% of maximum levels after 24 h. Tissue concentrations were not determined, but slow elimination was observed with brevetoxin measurable in blood several days after exposure.

Although problematic, longer exposure studies with planktivorous fish and *K. brevis* are required to assess to full potential of planktivorous fish to accumulate brevetoxins. Undigested menhaden recovered from the stomach contents of bottlenose dolphins during the 2004 mortality event indicate that such potential far exceeds what we observed in our experiments. These fish contained excessive levels of brevetoxins, with concentrations reaching 33,200 ng g^{-1} in viscera and 1500 ng g^{-1} in muscle (Flewelling et al., 2005). The toxins present were mainly PbTx-3 and PbTx-2 with lesser amounts of cys-PbTx-2 (Table 2; Flewelling et al., 2005).

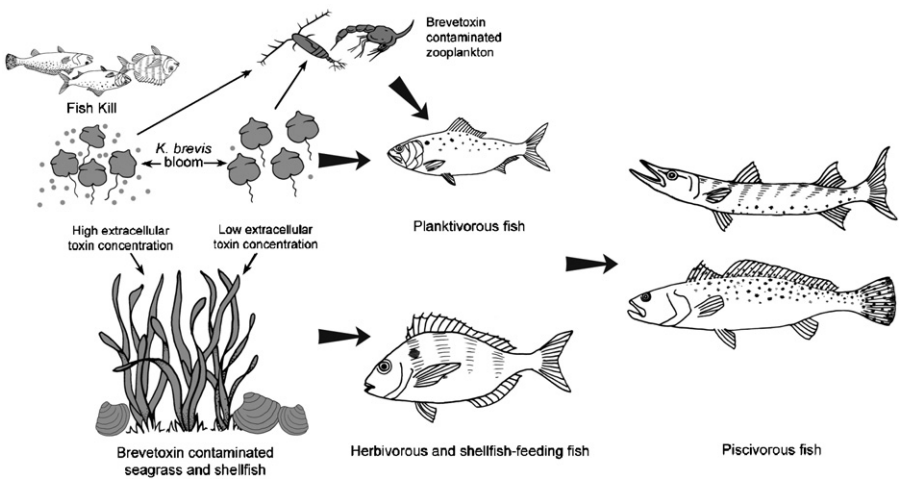

Fig. 6. Potential sources of brevetoxins and trophic transfer pathways in the fish food web.

Tester et al. (2000) clearly demonstrated vectorial transport of brevetoxins from copepods to fish in short-term experiments (2–25 h). In these experiments, they exposed three species of copepod to various concentrations of *K. brevis* cultures. Copepod mortality was low and no comments on a change in the behavior of the copepods were noted. Juvenile fish that were fed these toxic copepods accumulated PbTx-2 and PbTx-3 in both the muscle and viscera. It is noteworthy that copepod grazing experiments have reported differences in copepod response to *K. brevis* exposure, with adverse effects noted in some cases (Huntley et al., 1986, 1987; Turner and Tester, 1997), but not others (Turner and Tester, 1989; Tester et al., 2000). While these studies may have illustrated species differences amongst copepods in their response to brevetoxin exposure, dissolved vs. intracellular brevetoxin concentrations of the exposure media were not considered. The rapid recovery of copepods when transferred out of water containing *K. brevis* reported by both groups of investigators may suggest that the adverse effects that have been noted on copepods are at least partially due to exposure to dissolved toxins.

4.2. Persistence of brevetoxins in fish

After a 2-week experimental exposure to brevetoxic shellfish, pinfish and croakers were switched to a diet of nontoxic clams and depuration was further monitored for 2 weeks. Even after 2 weeks without exposure, muscle tissue and viscera still contained 40% and 15% of the maximum observed concentrations, respectively. Brevetoxin profiles in pinfish and croaker measured after 2 weeks exposure reflected those of the toxic clams. However, conclusions regarding metabolism of brevetoxins in fish cannot be made based on these experiments since the shellfish used in the exposure experiments already contained metabolized brevetoxins as well as PbTx-3.

During the red tide from September to October 2005, there were reports of massive fish kills in St. Joseph Bay. Decomposed fish were still piled along the shores even in November 2005. Nevertheless, apparently unaffected live fish were collected from the bay during the red tide bloom in September and immediately following the bloom in November 2005. However, our sample size during and immediately following the *K. brevis* bloom (17–22 fish per collection) was approximately half the sample size of collections 1 year later (39–43 fish per collection). While the average muscle and stomach contents dropped between September and November 2005, average liver concentrations rose (Fig. 4), possibly reflecting the accumulation of brevetoxin in those fish that were not killed during the 2-month bloom.

Since it would be difficult to assess the persistence of brevetoxin contamination in fish collected from southwest Florida where *K. brevis* blooms occur almost annually and can last for several months (Steidinger et al., 1998b), this study was carried out in the Florida panhandle where red tides historically occur less frequently. Given the occurrence of only one 2-month *K. brevis* bloom during the 2 years of our collections, our data may suggest that, although the average levels of brevetoxins are higher during or just after red tide event, brevetoxins continue to be present in the livers of fish for more than a year after the cessation of a bloom. Brevetoxins are known to accumulate in shellfish where they can persist at levels dangerous for human consumers for several weeks after a *K. brevis* bloom has ended (Morton and Burklew, 1969; Pierce et al., 2006), and in some species, for several months (Steidinger et al., 1998a). Although PbTx-3 is largely eliminated from shellfish within weeks after a red tide has ended, brevetoxin metabolites (identical to those identified here in fish) have been found to persist for months (Plakas et al., 2002, 2004; Pierce et al., 2006). However, the surprising levels of brevetoxins measured in the stomach contents of the majority of fish, especially in planktivorous species (scaled sardines and Atlantic thread herring), collected in May 2006 when no red tide bloom was present in St. Joseph Bay, suggest there were undetected sources of brevetoxins. With the exception of low levels of *K. brevis* observed in two samples from the mouth of the bay (333–4000 cells l^{-1}), no other potential brevetoxin-producing organisms were seen and only very low levels of brevetoxins were measured in any St. Joseph Bay water samples. The low levels of *K. brevis* found only at the mouth of the bay may be evidence of a bloom that remained undetected outside of the bay. Additionally, chronic exposure of omnivorous and piscivorous fish to toxins in food sources is likely since brevetoxins are known to persist in the environment (e.g. in shellfish and seagrass communities) for extensive period of time after red tides. Regardless of the source, the brevetoxins in the stomach contents of these fish would certainly contribute to the persistence of

toxins we noted in the livers over the next several months.

4.3. Brevetoxins and ciguatoxins

Even before ciguatoxins and brevetoxins were identified, chemically characterized and their mode of action understood, their similarity was recognized by the symptoms they induced following ingestion (McFarren et al., 1965). However, while ciguatoxins are known to accumulate in fish by dietary transfer up the food chain (Legrand, 1991), brevetoxin-producing *K. brevis* blooms (Florida red tides) characteristically result in massive fish kills (Steidinger et al., 1973; Landsberg, 2002). The ichthyotoxicity of brevetoxins is their most obvious property; it has been recognized since 1844 (Ingersoll, 1882) and was originally used to guide the fractionation and the purification of these toxins leading to the elucidation of their structures (Baden et al., 1981). Curiously, ciguatoxins, like brevetoxins, are also extremely toxic to fish. In fact, under identical exposure conditions when toxins were dissolved in seawater, ciguatoxins were more potent to fish than brevetoxins (Lewis, 1992). A more recent study also demonstrates that ciguatoxins have a greater affinity than brevetoxins on fish sodium channels (Dechraoui et al., 2006). Nonetheless, this toxicity does not prevent the accumulation of ciguatoxins in fish tissue by dietary transfer. Our experimental exposures as well as the results obtained from fish collected live in Florida demonstrate that fish also have the potential to accumulate high levels of brevetoxins in their tissues without obvious adverse effects.

The experimental exposure of planktivorous fish to *K. brevis* cells may illustrate the importance of exposure route to the toxins and their consequences. When we exposed planktivorous mullet to intact *K. brevis* cells with only low concentrations of brevetoxins dissolved in the water, the fish survived, fed on *K. brevis* and quickly accumulated toxins in their tissues. However, when *K. brevis* cells were lysed, the release of brevetoxins caused rapid fish death, probably from acute absorption of fatal concentrations of dissolved toxins across the gills (Abbott et al., 1975; Baden, 1988). These results support the hypothesis that the apparent dissimilarity regarding the typical impact of ciguatoxins and brevetoxins on fish (accumulation vs. massive fish kills) does not result from differences in their ichthyotoxicity, but rather reflects important differences in the physiology and ecology of the two toxin-producing dinoflagellates. *G. toxicus*, the source of ciguatoxins and their precursors, is an armored epibenthic dinoflagellate (Adachi and Fukuyo, 1979) that does not typically produce planktonic blooms. This species lives in close association with macrophytes, corals, sediments and other substrates associated with coral reefs in tropical and subtropical waters (Bagnis et al., 1979; Anderson and Lobel, 1987). Ciguatoxins have been shown experimentally to adversely affect fish (Davin and Kohler, 1986; Davin et al., 1988; Kohler et al., 1989; Gonzalez et al., 1994), and were postulated in association with tropical reef fish mortalities (Landsberg, 1995), but these toxins are not typically associated with fish kills. We suggest that the concentrations of dissolved ciguatoxins that would be lethal to fish are prevented from occurring in situ due to insufficient cell densities, minimal extracellular toxin and rapid dilution of toxin in the water column. Fish are primarily exposed to ciguatoxins by food web transfer. In contrast, although less toxic than ciguatoxins, lethal ichthyotoxic brevetoxin concentrations commonly occur in seawater because *K. brevis* is an unarmored and easily lysed planktonic bloom-forming dinoflagellate (Steidinger and Baden, 1984). Blooms can cover tens of thousands of square kilometers of ocean (Vargo et al., 1987) with cells densities commonly exceeding 1 million cells l^{-1} (Steidinger et al., 1998b) and even documented up to 1.1 billion cells l^{-1} (Trebatoski, 1988). Dense blooms can result in the release of high concentrations of dissolved brevetoxins by lysed or dying cells throughout the water column, and routinely cause massive fish kills in southwest Florida (Landsberg, 2002).

As is the case for ciguatoxins, the biological mechanism allowing accumulation of brevetoxins after ingestion without toxic effects remains unidentified. It has been suggested by Lewis (1992) that the capacity of fish to accumulate ichthyotoxins without adverse effects could be restricted and may impose an upper limit to the levels of ciguatoxins carried by fish, thus contributing to the low incidence of human fatality associated with ciguatera. Although there may be an upper limit to the dosage of brevetoxin that fish can tolerate by ingestion, we could not assess that in our experiments.

While we draw parallels regarding the biological pathways that allow for the accumulation of ichthyotoxic brevetoxins and ciguatoxins in healthy

fish, the implications for human health of brevetoxin and ciguatoxin accumulation in fish are very different. Brevetoxins are far-less toxic than ciguatoxins. As a comparison, the established action limit for brevetoxin levels in shellfish is 20 MU per 100 g of shellfish meat (USFDA, 2005). Using a conversion factor of 4 μg per MU based on the LD50 (i.p.) of PbTx-2 and -3 as determined by Baden and Mende (1982), this action limit would correspond to 80 μg per 100 g or 800 ng g^{-1}. For ciguatoxins, levels as low as 0.1–1 μg kg^{-1} or 0.1–1 ng g^{-1} are believed to be toxic for humans (Lehane and Lewis, 2000). Based on the levels of brevetoxins we have measured, the risk of acute poisoning in humans following consumption of fish fillets appears to be highly unlikely.

Additionally, it has been shown that as ciguatoxins and their precursors, gambiertoxins, are transferred through the fish food web, they are bioaccumulated and metabolized into more potent compounds, resulting in higher trophic level fish presenting greater risks to human health (Lewis and Holmes, 1993; Lewis et al., 2000). It is not known how fish metabolize brevetoxins. Brevetoxin composition determined in a limited number of live-caught fish revealed mainly metabolized brevetoxins identical to those found in shellfish, but the toxicity of these metabolites is not well known. Although they are generally believed to be less toxic (Naar et al., 2004), other metabolites identified from shellfish have been clearly implicated as causative factors for NSP (Morohashi et al., 1999; Poli et al., 2000; Plakas et al., 2004; Pierce et al., 2006). Studies on the toxicity of known brevetoxin metabolites are lacking, with many brevetoxin metabolites remaining unidentified and their toxicity uncharacterized. Until more information is available on the full suite of brevetoxin metabolites, the potential health risk they present cannot be conclusively assessed.

5. Conclusions

Through experimental exposures we have demonstrated that fish have the potential to accumulate brevetoxins in their muscle and, at higher levels, their viscera when feeding on toxic prey; and our data from live-caught fish confirm that they do, in fact, accumulate brevetoxins in the wild. During a *K. brevis* bloom, brevetoxins were detected in the muscle and liver of 95% and 100% of the fish tested, respectively.

We have presented an overview of the levels of brevetoxins that we observed in fish in St. Joseph Bay during 2005 and 2006. Many of the species tested were not caught during the bloom in the fall of 2005, and in several cases only one fish per species was tested. For these reasons, our results are not sufficiently robust for a species comparison, or for drawing any conclusions regarding the ability for brevetoxins to accumulate in particular fish species. Such topics are the focus of a separate study. However, we have demonstrated that the brevetoxin accumulation in fish we observed in St. Joseph Bay during the 2004 dolphin mortality was not a unique occurrence, and that the presence of brevetoxin in fish tissues can persist for an extended period of time.

Despite the almost annual occurrence of red tide in the eastern Gulf of Mexico, and with the exception of one anecdotal case involving consumption of whole fish (NRC, 1999), human intoxications from eating fish caught during *K. brevis* red tides have not been documented. In this study, we observed levels of brevetoxins in the muscle of fish at all trophic levels rise significantly, but not to dangerous levels, during a relatively brief red tide. However, our analyses of less than 300 fish from the Florida panhandle, an area far-less impacted by *K. brevis* blooms than the southwest coast of Florida, cannot rule out the potential that this could occur. Current larger-scale monitoring of brevetoxin concentrations in fish muscle from areas in southwest Florida that are more frequently impacted by red tide will help address this issue. Nevertheless, it is clear that brevetoxin concentrations in the liver and digestive tracts of fish can become excessively high and would likely result in acute intoxication if consumed. Differing from other populations from around the world, people in the US do not typically consume whole fish or their digestive organs, thus reducing the risk of exposure to harmful levels of brevetoxin. However, our results reveal an obvious threat to marine birds and mammals that feed directly on whole live fish prey—a threat that was clearly realized during the bottlenose dolphin mortality in 2004 (NMFS, 2004; Flewelling et al., 2005).

The persistence of brevetoxins and brevetoxin metabolites in fish tissues as well as shellfish over extended periods of time suggests that marine organisms in red tide endemic areas are routinely exposed to these compounds. Little information is available on their toxicity and nothing is known

about how chronic, low-level exposure to brevetoxins may impact human or animal health. The growing number of brevetoxin metabolites that are being identified, together with the increasing evidence for widespread distribution and persistence of brevetoxins in the marine environment, supports the need for human and environmental risk assessments of chronic or long-term exposure to these toxins.

Acknowledgments

We thank Dr. Tomas Lankford and Dr. Carmelo Thomas (UNCW) for their assistance with exposure experiments and experimental *K. brevis* cell counts, Melissa Smith (UNCW) for artistic representation of brevetoxin transfer in the fish food web, Karen Atwood and Sheila O Dea (FWRI) for laboratory assistance, Earnest Truby (FWRI) for *K. brevis* cell counts and the Florida DEP St. Joseph Bay Aquatic Preserve staff for collection of monthly water samples. This work was supported by funds from the CDC and Florida Department of Health (Expanding Existing Surveillance Systems to Include Pfiesteria, Other Harmful Algal Blooms and Marine Toxins in Florida, Grant # U50-CCU423360-01), NOAA Oceans and Human Health Initiative (Grant # NA05NOS4781246), the State of Florida and the NIEHS (Grant # PO1ES010594-06A1).

References

Abbott, B.C., Siger, A., Spigelstein, M., 1975. Toxins from the blooms of *Gymnodinium breve*. In: LoCicero, V.R. (Ed.), Proceedings of the First International Conference on Toxic Dinoflagellate Blooms. Massachusetts Science and Technology Foundation, Wakefield, pp. 355–366.

Adachi, R., Fukuyo, Y., 1979. The thecal structure of a marine toxic dinoflagellate *Gambierdiscus toxicus* gen. et sp. nov. collected in a ciguatera-endemic area. Bull. Jpn. Soc. Sci. Fish. 45, 67–71.

Anderson, D.M., Lobel, P.S., 1987. The continuing enigma of ciguatera. Biol. Bull. 172, 89–107.

APHA, 1970. Subcommittee on laboratory methods for the examination of shellfish. Method for the bioassay of *Gymnodinium breve* toxin(s) in shellfish. In: Recommended Procedures for the Examination of Sea Water and Shellfish, fourth ed. American Public Health Association, Washington, DC, pp. 61–66.

Baden, D.G., 1988. Public health problems of red tides. In: Tu, A.T. (Ed.), Handbook of Natural Toxins, vol. 3: Marine Toxins and Venoms. Marcel Dekker, Inc., New York, pp. 259–277.

Baden, D.G., Mende, T.J., 1982. Toxicity of two toxins from the Florida red tide marine dinoflagellate, *Ptychodiscus brevis*. Toxicon 20, 457–461.

Baden, D.G., Mende, T.J., Block, R.E., 1979. Two similar toxins isolated from *Gymnodinium breve*. In: Taylor, D.L., Seliger, H.H. (Eds.), Toxic Dinoflagellate Blooms. Elsevier, New York, pp. 327–334.

Baden, D.G., Mende, T.J., Lichter, W., Wellham, L., 1981. Crystallization and toxicology of T34: a major toxin from Florida's red tide organism (*Ptychodiscus brevis*). Toxicon 19, 455–462.

Baden, D.G., Fleming, L.E., Bean, J.A., 1995. Marine toxins. In: DeWolf, F.A. (Ed.), Handbook of Clinical Neurology: Intoxications of the Nervous System. Part H. Natural Toxins and Drugs. Elsevier Press, Amsterdam, pp. 141–175.

Bagnis, R., Kuberski, T., Laugier, S., 1979. Clinical observation on 3,009 cases of ciguatera (fish poisoning) in the South Pacific. Am. J. Trop. Med. Hyg. 28, 1067–1073.

Cheng, Y.S., Zhou, Y., Irvin, C.M., Pierce, R., Naar, J., Backer, L.C., Fleming, L.E., Kirkpatrick, B., Baden, D.G., 2005. Characterization of marine aerosol for assessment of human exposure to brevetoxins. Environ. Health Perspect. 113, 638–643.

Davin Jr., W.T., Kohler, C.C., 1986. Effects of ciguatera toxins on the bluehead. Trans. Am. Fish. Soc. 115, 908–912.

Davin Jr., W.T., Kohler, C.C., Tindall, D.R., 1988. Ciguatera toxins adversely affect piscivorous fishes. Trans. Am. Fish. Soc. 117, 374–384.

Dechraoui, M.-Y., Naar, J., Pauillac, S., Legrand, A.-M., 1999. Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. Toxicon 37, 125–143.

Dechraoui, M.Y., Wacksman, J.J., Ramsdell, J.S., 2006. Species selective resistance of cardiac muscle voltage gated sodium channels: characterization of brevetoxin and ciguatoxin binding sites in rats and fish. Toxicon 48, 702–712.

Dickey, R.W., Plakas, S.M., Jester, E.L.E., El Said, K.R., Johannessen, J.N., Flewelling, L.J., Scott, P., Hammond, D.G., Dolah, F.M.V., Leighfield, T.A., Dachraoui, M.-Y.B., Ramsdell, J.S., Pierce, R.H., Henry, M.S., Poli, M.A., Walker, C., Kurtz, J., Naar, J., Baden, D.G., Musser, S.M., White, K.D., Truman, P., Miller, A., Hawryluk, T.P., Wekell, M.M., Stirling, D., Quilliam, M.A., Lee, J.K., 2004. Multilaboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, St. Petersburg, FL, USA, pp. 300–302.

Flewelling, L.J., Naar, J.P., Abbott, J.P., Baden, D.G., Barros, N.B., Bossart, G.D., Bottein, M.-Y.D., Hammond, D.G., Haubold, E.M., Heil, C.A., Henry, M.S., Jacocks, H.M., Leighfield, T.A., Pierce, R.H., Pitchford, T.D., Rommel, S.A., Scott, P.S., Steidinger, K.A., Truby, E.W., Dolah, F.M.V., Landsberg, J.H., 2005. Red tides and marine mammal mortalities. Nature 435, 755–756.

Gonzalez, G., Brusle, J., Crespo, S., 1994. Ultrastructural alterations of cabrilla sea bass *Serranus cabrill*a liver related to experimental *Gambierdiscus toxicus* (dinoflagellate) ingestion. Dis. Aquat. Organ. 18, 187–193.

Halstead, B.W., 1978. Poisonous and Venomous Marine Animals of the World. Darwin Press, Princeton.

Haywood, A.J., Steidinger, K.A., Truby, E.W., Bergquist, P.R., Bergquist, P.L., Adamson, J., Mackenzie, L., 2004. Comparative morphology and molecular phylogenetic analysis of

three new species of the genus *Karenia* (Dinophyceae) from New Zealand. J. Phycol. 40, 165–179.

Huntley, M.E., Sykes, P.F., Rohan, S., Marin, V., 1986. Chemically-mediated rejection of dinoflagellate prey by the copepod *Calanus pacificus* and *Paracalanus parvus*: mechanism, occurrence and significance. Mar. Ecol. Prog. Ser. 28, 105–120.

Huntley, M.E., Ciminiello, P., Lopez, M.D.G., 1987. Importance of food quality in determining development and survival of *Calanus pacificus* (Copepoda: Calanoida). Mar. Biol. 95, 103–113.

Ingersoll, E., 1882. On the fish mortality in the Gulf of Mexico. Proc. US Natl. Mus. 4, 74–80.

Kirkpatrick, B., Fleming, L.E., Squicciarini, D., Backer, L.C., Clark, R., Abraham, W., Benson, J., Cheng, Y.S., Johnson, D., Pierce, R., Zaias, J., Bossart, G.D., Baden, D.G., 2004. Literature review of Florida red tide: implications for human health effects. Harmful Algae 3, 99–115.

Kohler, C.C., Paleudis, G.A., Tindall, D.R., 1989. Behavioral abnormalities displayed by ocean surgeon following consumption of ciguatoxigenic dinoflagellates. Proc. Assoc. Island Mar. Labs. Carrib. 22, 34.

Landsberg, J.H., 1995. Tropical reef-fish disease outbreaks and mass mortalities in Florida, USA: what is the role of dietary biological toxins? Dis. Aquat. Organ. 22, 83–100.

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 10, 113–390.

Legrand, A.M., 1991. Les toxines de la ciguatera. In: Fremy, J.M. (Ed.), Proceedings of the Symposium on Marine Biotoxins, CNEVA, Godin, Paris, France, pp. 53–59.

Lehane, L., Lewis, R.J., 2000. Ciguatera: recent advances but the risk remains. Int. J. Food Microbiol. 61, 91–125.

Lewis, R.J., 1992. Ciguatoxins are potent ichthyotoxins. Toxicon 30, 207–211.

Lewis, R.J., Holmes, M.J., 1993. Origin and transfer of toxins involved in ciguatera. Comp. Biochem. Physiol. C 106, 615–628.

Lewis, R.J., Molgó, J., Adams, D.J., 2000. Ciguatera toxins: pharmacology of toxins involved in ciguatera and related fish poisonings. In: Botana, L.M. (Ed.), Seafood and Freshwater Toxins: Pharmacology, Physiology, and Detection. Marcel Dekker, Inc., New York, pp. 419–447.

Lombet, A., Bidard, J.N., Lazdunski, M., 1987. Ciguatoxin and brevetoxins share a common receptor-site on the neuronal voltage-dependent Na^+ channel. FEBS Lett. 219, 355–359.

McFarren, E.F., Tanabe, H., Silva, F.J., Wilson, W.B., Campbell, J.E., Lewis, K.H., 1965. The occurrence of a ciguatera-like poison in oysters, clams, and *Gymnodinium breve* cultures. Toxicon 3, 111–123.

Morohashi, A., Satake, M., Naoki, H., Kaspar, H.F., Oshima, Y., Yasumoto, T., 1999. Brevetoxin B4 isolated from greenshell mussels *Perna canaliculus*, the major toxin involved in neurotoxic shellfish poisoning in New Zealand. Nat. Toxins 7, 45–48.

Morton, R.A., Burklew, M.A., 1969. Florida Shellfish Toxicity Following Blooms of the Dinoflagellate *Gymnodinium breve*. Florida Department of Natural Resources—Marine Research Laboratory, Technical Series No. 60, St. Petersburg, p. 26.

Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P.L., Flewelling, L.J., Steidinger, K.A., Lancaster, J., Baden, D.G., 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ. Health Perspect. 110, 179–185.

Naar, J., Kubanek, J., Weidner, A.L., Flewelling, L.J., Bourdelais, A., Steidinger, K., Baden, D.G., 2004. Brevetoxin depuration in shellfish via production of non-toxic metabolites: consequences for seafood safety and the environmental fate of brevetoxins. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, St. Petersburg, FL, USA, pp. 488–490.

NMFS, 2004. Interim Report on the bottlenose dolphin (*Tursiops truncatus*) unusual mortality event along the panhandle of Florida, March–April 2004. National Oceanic and Atmospheric Administration, National Marine Fisheries Service and the Florida Fish and Wildlife Conservation Commission.

NRC, 1999. Harmful algal blooms. In: From Monsoons to Microbes: Understanding the Ocean's Role in Human Health. Ocean Studies Board; Commission on Geosciences, Environment, and Resources; National Research Council (US), National Academy Press, Washington, DC, pp. 59–70.

Pierce, R.H., Henry, M.S., Blum, P.C., Hamel, S.L., Kirkpatrick, B., Cheng, Y.S., Zhou, Y., Irvin, C.M., Naar, J., Weidner, A., Fleming, L.E., Backer, L.C., Baden, D.G., 2005. Brevetoxin composition in water and marine aerosol along a Florida beach: assessing potential human exposure to marine biotoxins. Harmful Algae 4, 965–972.

Pierce, R.H., Henry, M.S., Blum, P.C., Plakas, S.M., Granade, H.R., Jester, E.L.E., Said, K.R.E., Dickey, R.W., Steidinger, K.A., Scott, P.S., Flewelling, L.J., Wright, J.L.C., 2006. Comparison of methods for determination of brevetoxins and their metabolites in NSP-toxic bivalved molluscs. In: Henshilwood, K., Deegan, B., McMahon, T., Cusack, C., Keaveney, S., Silke, J., O'Cinneide, M., Lyons, D., Hess, P. (Eds.), Proceedings of the Fifth International Conference on Molluscan Shellfish Safety, Galway Ireland, June 14–18, 2004. The Marine Institute, Rinville, Oranmore, Galway, Ireland, pp. 37–42.

Plakas, S.M., el-Said, K.R., Jester, E.L., Granade, H.R., Musser, S.M., Dickey, R.W., 2002. Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. Toxicon 40, 721–729.

Plakas, S.M., Wang, Z., Said, K.R.E., Jester, E.L.E., Granade, H.R., Flewelling, L., Scott, P., Dickey, R.W., 2004. Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*. Toxicon 44, 677–685.

Poli, M.A., Mende, T.J., Baden, D.G., 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol. Pharmacol. 30, 129–135.

Poli, M.A., Musser, S.M., Dickey, R.W., Eilers, P.P., Hall, S., 2000. Neurotoxic shellfish poisoning and brevetoxin metabolites: a case study from Florida. Toxicon 38, 981–993.

Steidinger, K.A., 1993. Some taxonomic and biologic aspects of toxic dinoflagellates. In: Falconer, I. (Ed.), Algal Toxins in Seafood and Drinking Water. Academic Press Limited, London, pp. 1–28.

Steidinger, K.A., Baden, D.G., 1984. Toxic marine dinoflagellates. In: Spector, D. (Ed.), Dinoflagellates. Academic Press, Orlando, pp. 201–261.

Steidinger, K.A., Burklew, M.A., Ingle, R.M., 1973. The effects of *Gymnodinium breve* toxin on estuarine animals. In: Martin, D.F., Padilla, G.M. (Eds.), Marine Pharmacognosy. Academic Press, New York, pp. 179–202.

Steidinger, K.A., Carlson, P., Baden, D., Rodriguez, C., Seagle, J., 1998a. Neurotoxic shellfish poisoning due to toxin retention in the clam *Chione cancellata*. In: Reguera, B., Blanco, J., Fernandez, M.L., Wyatt, T. (Eds.), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, pp. 457–458.

Steidinger, K.A., Vargo, G.A., Tester, P.A., Tomas, C.R., 1998b. Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer, Heidelberg, pp. 133–153.

Steidinger, K.A., Wolny, J.L., Haywood, A.J., in press. Identification of Kareniaceae (Dinophyceae) in the Gulf of Mexico. Nova Hedwigia.

Tester, P.A., Turner, J.T., Shea, D., 2000. Vectorial transport of toxins from the dinoflagellate *Gymnodinium breve* through copepods to fish. J. Plankton Res. 22, 47–61.

Trebatoski, B., 1988. Observations on the 1986–1987 Texas red tide (*Ptychodiscus brevis*). Report 88-02, Texas Water Commission, Austin.

Trainer, V.L., Baden, D.G., 1991. An enzyme immunoassay for the detection of Florida red tide brevetoxins. Toxicon 29, 1387–1394.

Turner, J.T., Tester, P.A., 1989. Zooplankton feeding ecology: copepod grazing during an expatriate red tide. In: Cosper, E.M., Bricelj, V.M., Carpenter, E.J. (Eds.), Coastal and Estuarine Studies, 35. Novel Phytoplakton Blooms. Causes and Impacts of Recurrent Brown Tides and Other Unusual Blooms. Springer, Berlin, pp. 359–374.

Turner, J.T., Tester, P.A., 1997. Toxic marine phytoplankton, zooplankton grazers, and pelagic food webs. Limnol. Oceanogr. 42, 1203–1214.

USFDA, 2005. National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish. US Food and Drug Administration, Center for Food Safety and Applied Nutrition, Washington, DC.

Vargo, G.A., Carder, K.L., Gregg, W., Heil, E., Shanley, E., Steidinger, K.A., Haddad, K.D., 1987. The potential contribution of primary production by red tides to the west Florida shelf ecosystem. Limnol. Oceanogr. 32, 762–767.

Woofter, R.T., Brendtro, K., Ramsdell, J.S., 2005. Uptake and elimination of brevetoxin in blood of striped mullet (*Mugil cephalus*) after aqueous exposure to *Karenia brevis*. Environ. Health Perspect. 113, 11–16.

Yasumoto, T., Nakajima, I., Bagnis, R., Adachi, R., 1977. Finding of a dinoflagellate as a likely culprit of ciguatera. Bull. Jpn. Soc. Sci. Fish. 43, 1021–1026.