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Brevetoxin in two planktivorous fishes after exposure to *Karenia brevis*: implications for food-web transfer to bottlenose dolphins

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ABSTRACT: Brevetoxin uptake was analyzed in 2 common planktivorous fish that are likely food-web vectors for dolphin mortality events associated with brevetoxin-producing red tides. Fish were exposed to brevetoxin-producing *Karenia brevis* for 10 h under conditions previously reported to produce optimal uptake of toxin in blood after oral exposure. Striped mullet *Mugil cephalus* were exposed to a low dose of brevetoxin, and uptake and depuration by specific organs were evaluated over a 2 mo period. Atlantic menhaden *Brevoortia tyrannus* specimens were used to characterize a higher brevetoxin dose uptake into whole body components and evaluate depuration over 1 mo. We found a high uptake of toxin by menhaden, with a body to water ratio of 57 after a 10 h exposure and a slow elimination with a half life ($t_{½}$) of 24 d. Elimination occurred rapidly from the intestine ($t_{½}$ < 1 wk) and muscle ($t_{½}$ ≈ 1 wk) compartments and redistributed to liver which continued to accumulate body stores of toxin for 4 wk. The accumulation and elimination characteristics of the vectoring capacity of these 2 fish species are interpreted in relation to data from the Florida Panhandle dolphin mortality event of 2004. We show that due to slow elimination rate of brevetoxin in planktivorous fish, brevetoxin-related dolphin mortality events may occur without evidence of a concurrent harmful algal bloom event.

KEY WORDS: Brevetoxin · *Karenia brevis* · Red tide · Menhaden · Mullet · Dolphin

INTRODUCTION

The Gulf of Mexico coast of Florida has been affected by red tide events on a nearly annual basis, going back as far as 1530 (Taylor 1917). These red tides, more appropriately termed harmful algal blooms (HABs), are caused by the dinoflagellate *Karenia brevis* (Davis 1948), which produces a group of polycyclic ether neurotoxins of 2 backbone structures referred to as brevetoxins (Risk et al. 1979, Lin et al. 1981, Shimizu et al. 1986). Brevetoxins bind to the voltage gated sodium channel and interfere with the sensitive movements that transition the channel's closed, open and inactivated states to induce membrane depolarization which first activates and then inhibits nerve activity (Ramsdell 2007). Exposure to brevetoxins can affect a variety of organisms from filter-feeding shellfish to protected marine mammals to humans, both directly via ingestion and indirectly through trophic transfer and bioaccumulation (Landsberg 2002). Mortality events attributed to HABs along the Florida Gulf of Mexico coast have been documented for fish, manatees, dolphins and seabirds (Landsberg 2002). Over the last several years, mortality events (associated with *K. brevis* blooms) involving bottlenose dolphins *Tursiops truncatus* have become increasingly frequent, each often lasting more than several months (Van Dolah et al. 2003)

The 2004 Florida Panhandle bottlenose dolphin mortality event was unique in that a large number (n = 107) of bottlenose dolphins were stranded dead over the course of a month, coincident with the death of fish and

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invertebrates, yet without documentation of a bloom of *Karenia brevis* (Anonymous 2004). Different from previously investigated dolphin mortality events, the dolphins were found with their stomachs engorged with fish. The stomach contents of all dolphins contained high concentrations of brevetoxins. Menhaden was the dominant prey fish in 50% of the dolphin stomachs analyzed, with specimens in the 9.5 to 16 cm range class (Anonymous 2004). High concentrations of brevetoxins also occurred in the undigested menhaden collected from the dolphin stomachs. The remaining stomach contents included a mixture of omnivorous fish, silver perch, spot, kingfish, croaker or shrimp.

Woofter et al. (2005) reported a model for aqueous exposure of planktivorous fish to brevetoxin-producing cultures of *Karenia brevis*. In this model, fish accumulate toxin in blood with uptake kinetics consistent with oral raking of cells or particulates followed by intestinal absorption. Initial studies indicated that both striped mullet *Mugil cephalus* and Atlantic menhaden *Brevoortia tyrannus* performed well in this system and accumulated measurable levels of toxin in their blood over a 24 h exposure to *K. brevis* (R. Woofter & J. Ramsdell unpubl. data). Striped mullet were chosen as a model species in the first part of the present study of toxin uptake, distribution and elimination based upon their robustness in captivity. Under conditions described by Woofter et al. (2005), fish reach equilibrium with toxin content in the water after approximately 10 h, and toxin accumulation in blood was proportional to toxin content of the *K. brevis* culture. Depuration studies showed that toxin was retained in the blood with a half life ($t_{½}$) of approximately 7 d. In the second part of the present study, we used menhaden as an alternative model species that may have been particularly relevant as a food web vector in the Florida Panhandle dolphin mortality event of 2004.

We conducted 2 complementary studies, each based upon exposure of fish to *Karenia brevis* calibrated to brevetoxin content. The first study was a low brevetoxin dose exposure of striped mullet via *K. brevis* culture, followed by a 2 mo depuration. Brevetoxin content was analyzed in the major tissues using a direct competitive ELISA developed for high sensitivity analysis of brevetoxins (Maucher et al. 2007). The second study utlized a higher brevetoxin dose exposure of Atlantic menhaden via *K. brevis* culture followed by a 1 mo depuration. Brevetoxin content was analyzed in 3 isolated whole body components using a brevetoxin radioimmunoassay (Woofter et al. 2003). We utilized these data (with consideration given to the timing of retention of toxin in different tissues) to evaluate a biological mechanism involving consumption of *K. brevis* by striped mullet and Atlantic menhaden. This analysis was extended to relate the toxin content of a single or multiple exposure to a *K. brevis* bloom(s) for prediction of dolphin mortality via feeding on planktivorous fish (based upon the observations during the 2004 Florida mortality event).

MATERIALS AND METHODS

Fish collection and maintenance. Striped mullet between 10 and 20 cm total length were collected by cast netting in estuarine creeks near Charleston Harbor, South Carolina, USA. Atlantic menhaden between 9 and 14 cm total length were also collected by cast netting in Charleston Harbor. Both mullet and menhaden were transported to the laboratory in aerated coolers and held for 10 d to ensure viability. They were held in 950 l specimen tanks with constant filtration and aeration using two 20 l Eheim filtration systems. The salinity of the seawater was maintained at 20 psu. Mullet were fed Seaweed Selects Green Marine Algae (Ocean Nutrition) during the holding periods up to 24 h prior to *Karenia brevis* exposure. Menhaden were fed 30 to 60 µm mesh Plankton Supreme (Hikari) during the holding periods up to 24 h prior to *K. brevis* exposure. Fish were maintained according to NOAA/NOS/CCEHBR institutional guidelines for vertebrates.

Algal cultures. Algal exposures were performed with the Wilson isolate of *Karenia brevis*. The cells were maintained in 1 l batch cultures comprising f/2 medium (Guillard 1973) with the following modifications to the trace metal solution: ferric sequestrene was used in place of EDTA·Na_2 EDTA and $FeCl_3 \cdot 6H_2O$, and 0.01 µM selenous acid was added. *K. brevis* cell densities in culture prior to harvest were determined using a Multisizer 3 Coulter Counter (Beckman Coulter). All cultures were maintained at 25 ± 1°C on a 16:8 h light:dark cycle with autoclaved, 20 µm-filtered 36 psu seawater obtained from the seawater system at the Florida Institute of Technology field station (Vero Beach). Cool white lights (Vita-Lite) provided a photon flux density of 150 to 175 µmol m^{-2} s^{-1}. The cultures were monitored for toxin by radioimmunoassay and harvested for use in exposure experiments during the mid- to late log phase of growth (10 to 20×10^6 cells l^{-1}).

Fish exposure design. Two fish exposure designs were used. The first used mullet at a low dose exposure with depuration for 2 mo. Mullet were chosen because *Karenia brevis* exposure and depuration of toxin from blood had been established in this species (Woofter et al. 2005). Five organs were dissected and analyzed for brevetoxin in order to follow toxin transfer at the organ level. The second design used menhaden at a higher exposure with depuration for only 1 mo. Menhaden have not been used for *K. brevis* exposures except in preliminary experiments. The menhaden were dis-

sected into head, body and viscera sections that were analyzed individually for brevetoxin and then summed to determine total body burden.

Exposure of mullet and menhaden were conducted in 6 aerated, round 60 l treatment tanks each containing 7 fish. The log growth phases of *Karenia brevis* cultures were analyzed for brevetoxin content by radioimmunoassay, and cultures were selected to achieve a brevetoxin concentration of 1 to 2 µg l^{-1} for mullet and 4 to 6 µg l^{-1} for menhaden. One mullet and 2 menhaden were removed from each tank after 10 h, euthanized in ice water and stored frozen (–20 or –80°C) at which time 50 ml water samples were taken from each tank to determine brevetoxin concentration. The remaining fish from the treatment tanks were then transferred and combined in a 950 l tank containing no *K. brevis*. For mullet exposure, fish were removed from the tank at each time point (1, 2, 4, 6, and 8 wk post-exposure) and stored frozen (–20 or –80°C). For menhaden exposure, a subgroup of fish was removed from the 950 l tank and exposed to a 2–3× higher toxin concentration of toxic culture for 10 h and processed as described above. At Day 17, another subgroup of fish was removed from the 950 l tank and stored frozen. The final collection time was 31 d post-exposure.

Brevetoxin extraction from seawater. Total brevetoxin was extracted from the *Karenia brevis* cultures and seawater in the tank containing *K. brevis* cultures in a separation funnel with 1 × 10 ml, then 2 × 2.5 ml HPLC grade methylene chloride. The methylene chloride fractions were combined and dried with vacuum centrifugation using an SC210A Speedvac plus (Thermo Savant), then reconstituted in 1 ml methanol. Total brevetoxin was determined by radioimmunoassay as described below.

Tissue dissection and extraction. Striped mullet were dissected and liver, muscle, stomach, intestine and kidney were collected. The intestinal contents were not emptied (to fit the experimental design to evaluate potential for trophic transfer). Tissues were homogenized in borosilicate glass test tubes containing HPLC grade acetone. Homogenates were centrifuged at 100 × *g* and the supernatant was collected. The acetone was then filtered through 0.45 µm Acrodisc® filters (Pall) and placed in the freezer overnight at –19°C. The next day, the extract was filtered again through 0.45 µm Acrodisc filters and brought to a volume of 6 ml with HPLC grade methanol. The extract was then liquid-liquid partitioned twice with hexane 2:1 volume and the organic portion was collected. The sample was dried and reconstituted with 1 ml of methanol. This extract was transferred, dried again and resuspended in a final volume of 100 µl of methanol.

Menhaden were dissected and their head, viscera, and body cavity were collected. The head and body were homogenized in acetone using a Waring commercial blender, and the viscera were homogenized in acetone using a PRO Scientific 5 speed homogenizer, each in a volume 3 times that of the weight of the body section (head, viscera, body cavity). Extracts were transferred to borosilicate glass test tubes and centrifuged at 100 × *g*; the supernatant was collected. The acetone extract was then filtered using 0.45 µm Acrodisc filters and placed in a freezer overnight at –19°C. The next day, the extract was filtered again through Acrodisc filters and brought to a volume of 6 ml with methanol. The extract was then liquid-liquid partitioned twice with hexane 2:1 volume, and the organic portion collected. The sample was dried and reconstituted with 1 ml of methanol. It was then transferred, dried again and resuspended in a final volume of 100 µl of methanol.

Extract cleanup. Two extract cleanup methods were compared. The first method was a C18 SPE column (500 mg Varian Bond Elut with a 10 ml reservoir) preconditioned first with 4 ml methanol, then 3 ml of water. The sample was loaded with 2 ml of 35% methanol. The sample was then eluted with 4 to 6 ml of 85% methanol. The second method utilized a hexane-80% methanol partition of the extract. The organic component was part of the 80% methanol fraction. Extraction efficiency with each cleanup method was compared with liver and intestines, the 2 tissues with greatest matrix effect for brevetoxin detection. The C18 Sep-Pak protocol gave 61 and 47% recoveries for liver and intestines, respectively, while the liquid partition protocol gave 62 and 86% recoveries, respectively. The liquid partition protocol was chosen for higher extraction efficiency.

Brevetoxin ELISA. ELISA was performed with reagents produced by AgResearch (Ruakura Research Centre). 96-well Nunc Maxisorp immunoplates (Fisher Scientific) were coated overnight with anti-brevetoxin antibody in 0.05 M carbonate/bicarbonate, pH 9.6, as described in Maucher et al. (2007). All methanolic samples and standards were diluted to the desired concentration with PBS containing 138 mM NaCl, 2.7 mM KCl and 0.05% Tween-20. The plate was blocked with blocking/conjugate buffer (PBST-1% BSA [bovine serum albumin]) and allowed to incubate at 25°C for 1 h. The plate was washed 4 times with an Elx405 Automated Plate Washer (Biotek) and then the sample and standards (50 µl) were added. Brevetoxin standards ranged from 20 000 to 1.0 pg ml^{-1}. To each well, brevetoxin-horseradish peroxidase conjugate (50 µl) was added to the sample and standards, and the plate was incubated at room temperature for 1 h. The plate was washed 4 times and 100 µl of Neogen K-Blue Aqueous TMB substrate (Neogen) was added. The whole plate containing substrate was shaken for 15 min at 84 rpm in a Max Q Mini 4450 shaker and

the reaction was quenched with 0.3 M sulfuric acid (100 µl). Absorbance was measured on a Fluorstar Galaxy (BMG Lab Technologies). Analyses of brevetoxin concentrations in the standard curves and samples were done with ELISA data processing software provided by AgResearch, using non-linear sigmoidal dose-response regression curves for standards from which unknown samples were quantified.

We determined the limit of quantitation (LOQ) for each of the 5 tissues collected from mullet. One of each tissue of a control fish was analyzed by ELISA to determine the minimal dilution of each tissue to yield no effect on the Amax reading. Each of the control tissues was optimal at a dilution of 1:100, except for the liver which was optimal at a final dilution of 1:1000. The LOQ was determined to be 0.69 ng g^{-1} (stomach and muscle), 0.71 ng g^{-1} (intestines), 1.49 ng g^{-1} (kidney) and 24.86 ng g^{-1} (liver).

To better relate to food web-impact, we present our data as toxin content using units of ng or µg toxin in a given organ or body compartment normalized to a 100 g fish. Values were calculated as the product of tissue toxin concentration (ng or µg g^{-1}, wet weight), tissue weight (g, wet weight) and fish weight (g blotted dry)/100. Where needed to compare to existing data, we discuss our data as toxin concentration in units of ng or µg toxin per gram of individual tissue weight.

Radioimmunoassay. Radioimmunoassays were performed using sheep antisera prepared against a PbTx-2-fetuin conjugate (Garthwaite et al. 2001, Woofter et al. 2003). Radioimmunoassays were run in 12 × 75 borosilicate glass tubes in amended phosphate-buffered saline (PBS) containing 137 mM NaCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 0.01% Emulphor-EL (GAF). The assay tubes consisted of PbTx-3 standard or blood spot extract (50 µl), anti-PbTx antiserum (1:4000), [^{3}H]PbTx-3 (0.4 nM), in PBS (final assay volume of 500 µl). The 7 PbTx-3 standards ranged from 0.01 to 1000 ng ml^{-1}. The PbTx-3 standards and extracts were incubated at room temperature with the anti-PbTx-3 antibody for 1 h before the [^{3}H]PbTx-3 tracer was added. The tubes were placed on a Titramax 100 shaker (Heidolph Instruments) and incubated at 23°C for an additional 1 h. Sac-Cel (Alpco Diagnostics) was then added to the assay tubes and the Sac-Cel-bound antibody was collected by filtration onto 25 mm glass fiber filters; each assay tube was rinsed with PBS (3 × 2 ml) using a 48-sample harvester (Brandel). The filters were placed in scintillation vials containing 5 ml Scintiverse BD (Fisher Scientific), and the radioactivity was counted on a Tri-Carb 3100TR Liquid Scintilation Counter (Packard-PerkinElmer). All concentrations and half-maximal effective concentration (EC_{50}) values and statistical analyses were determined using Prism Graph Pad 4.0 (GraphPad Software).

RESULTS

We first conducted a low brevetoxin dose exposure of striped mullet via *Karenia brevis* culture followed by a 2 mo depuration. The amount of toxin (±SE) extracted from the exposure tank water (determined by radioimmunoassay) was 1.52 ± 0.09 µg l^{-1} PbTx-3 equivalents. After a 10 h accumulation period, the mean value for brevetoxin concentration was high in the intestine, yet the intestine showed substantial fish to fish variability, likely due to differences in unabsorbed *K. brevis* cells remaining in the gut lumen (Fig. 1). For all of the tissues, toxin concentration failed to show a linear correlation ($p > 0.05$) with size of fish (20, 21, 124 and 212 g). The brevetoxin content of tissues, standardized to a 100 g fish, is shown in Fig. 2. Among the tissues that contained absorbed toxin, muscle contained the largest concentration; there were significantly lower amounts of toxin in the liver, stomach and kidneys (Fig. 2).

The live mullet remaining after 10 h exposure to *Karenia brevis* were combined and placed in a toxin-free holding tank; the fish were then removed for analysis after one or more weeks. Brevetoxin content of the intestines and the muscle was significantly reduced by 1 wk depuration, whereas brevetoxin content in the stomach and kidneys did not change significantly during depuration (Fig. 3). By contrast, toxin content of the liver continued to increase after the animals were returned to toxin-free water, reaching a maximum 2.5-fold increase after 1 mo of depuration, and did not decrease below pre-depuration levels after 2 mo.

We next conducted a higher brevetoxin dose exposure of Atlantic menhaden via *Karenia brevis* culture

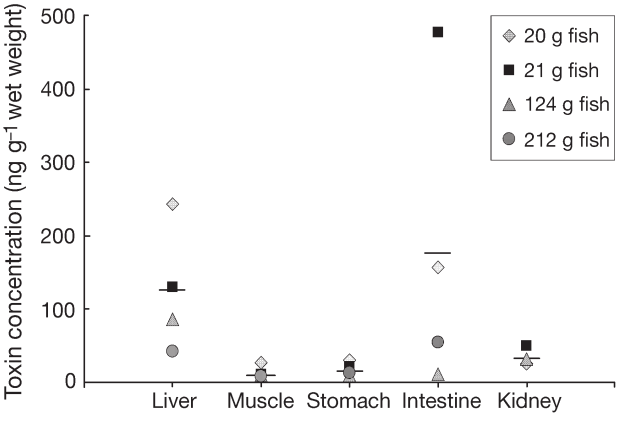

Fig. 1. *Mugil cephalus*. Brevetoxin concentration in 5 tissues of striped mullet after 10 h exposure to *Karenia brevis*. Values for intestine, muscle, liver, stomach (n = 4) and kidney (n = 3) are given for individual fish. Horizontal lines indicate means

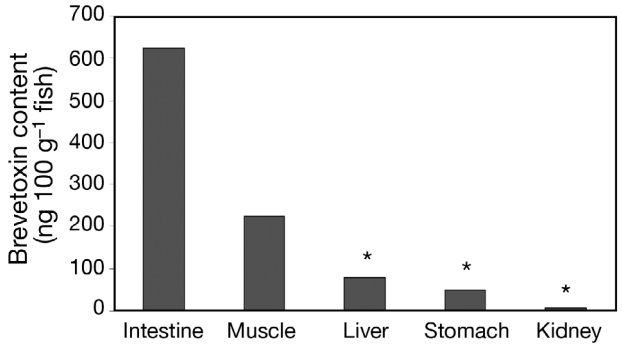

Fig. 2. *Mugil cephalus*. Mean brevetoxin content (normalized to ng 100 g^{-1} fish) in 5 tissues of striped mullet after 10 h exposure to *Karenia brevis*. *Tissue content significantly different from muscle content (Dunnett's multiple comparison test, $p < 0.05$)

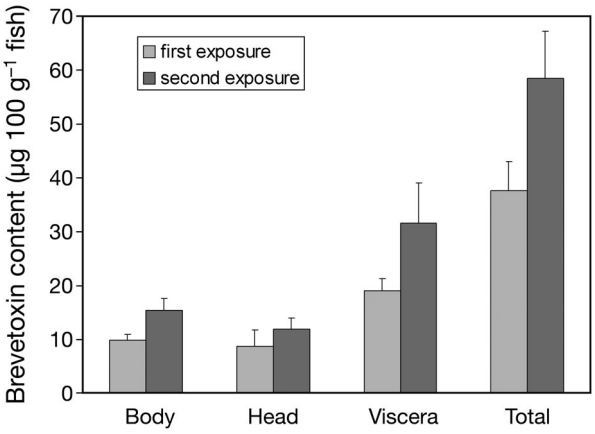

Fig. 4. *Brevoortia tyrannus*. Brevetoxin content (normalized to µg 100^{-1} g fish) in body, head and viscera of Atlantic menhaden after 1 and 2 to 10 h exposure to *Karenia brevis*. Values are means ± SE (n = 4) after 10 h exposure (first exposure) and after a second 10 h exposure following a 48 h waiting period (second exposure)

followed by a 1 mo depuration. The amount of toxin extracted from the exposure tank water was determined by radioimmunoassay to be 6.5 µg l^{-1} PbTx-3 equivalents. Menhaden whole body burden of toxin was 37 ± 5 µg 100 g^{-1} fish with approximately 50% of the content in the viscera, 25% in the body and 25% in the head (Fig. 4, first exposure bars). The viscerosomatic ratio (VSR), calculated as the toxin content of viscera/body, was 2.2 ± 0.3 (SE).

To assess repeat exposure to brevetoxins, a subset of the menhaden in the toxin-free holding tanks was removed after 48 h of depuration and exposed to *Karenia brevis* in a single exposure tank for a second 10 h exposure period. The level of toxin in the water was several-fold higher (20.5 µg l^{-1}) than in the first exposure. Analysis of the whole body components of these fish indicated that the levels of toxin in the viscera exceeded the single lower exposure by 65% and maintained a comparable VSR (2.5 ± 0.4) (Fig. 4, second exposure bars).

The menhaden remaining from the single 10 h *Karenia brevis* exposure were removed from the toxin-free depuration tanks after 17 or 31 d. Analysis of brevetoxin in the whole body components indicated that after 17 d of depuration brevetoxin content of viscera was unchanged and not eliminated until Day 31 (Fig. 5). By contrast, brevetoxin contents in the body and head were reduced by at least 50% after 17 d of depuration. Of the total toxin content in whole fish (sum of head, body and viscera), 65% was eliminated after 30 d. These data were analyzed by a 1-phase exponential decay, and the half time of complete elimination was calculated to be 24 d (Fig. 6).

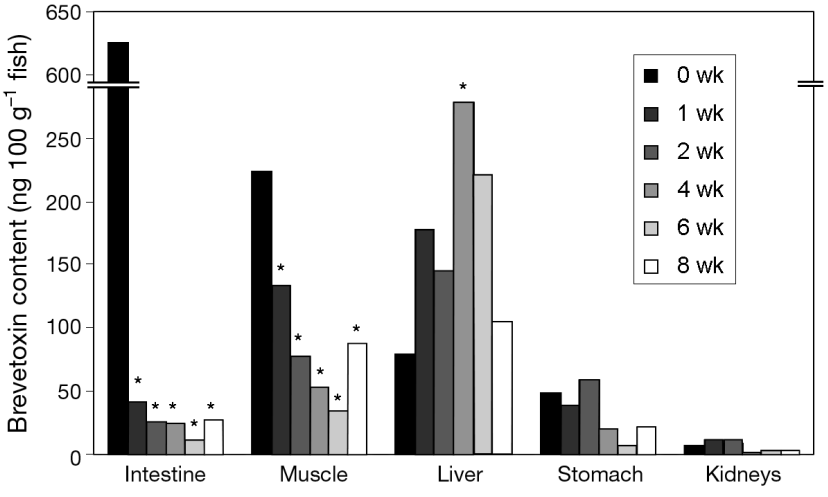

Fig. 3. *Mugil cephalus*. Brevetoxin content (normalized to ng 100 g^{-1} fish) in 5 tissues of striped mullet after 1, 2, 4, 6 and 8 wk depuration in *Karenia brevis*-free water (preceded by 10 h accumulation — see Fig. 2). *Tissue content significantly different from that at time zero (Dunnett's multiple comparison test, $p < 0.05$)

DISCUSSION

We demonstrate that striped mullet and Atlantic menhaden exposed to brevetoxin-producing *Karenia brevis* cultures accumulate toxin over a 10 h period in visceral and somatic tissues. Previously, this exposure design was used to monitor toxin in the blood of mullet and showed that uptake is proportional to the toxin content of the culture water, and reaches a blood to water ratio of approximately 0.5 after 24 h (Woofter et al. 2005). The present

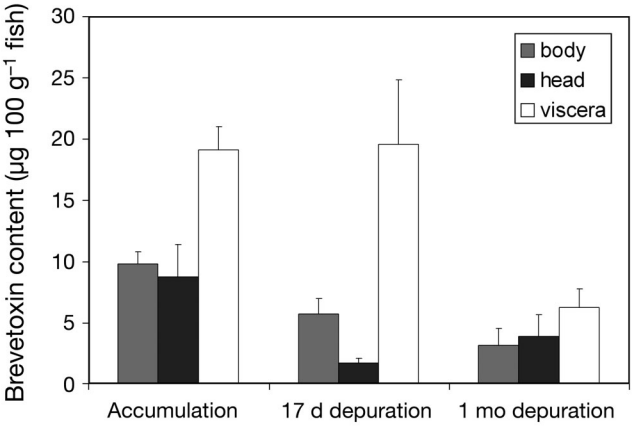

Fig. 5. *Brevoortia tyrannus*. Brevetoxin content (normalized to µg 100 g^{-1} fish) in body, head and viscera of Atlantic menhaden after 10 h exposure to *Karenia brevis* and depuration in *K. brevis*-free water. Values are means ± SE (n = 4) after 10 h accumulation and 17 d and 1 mo (31 d) depuration

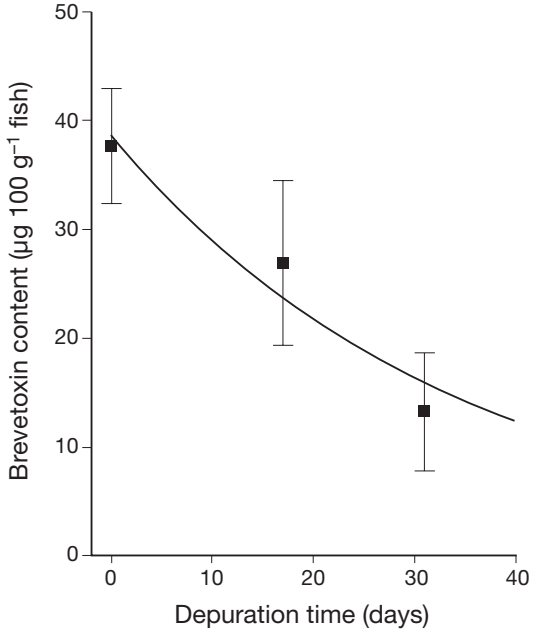

Fig. 6. *Brevoortia tyrannus*. Time of elimination of brevetoxin from Atlantic menhaden. Values of summed body concentrations (body + head + viscera, normalized to µg 100 g^{-1} fish) are given as means ± SE (n = 4). The elimination curve was generated as a 1-phase exponential decay

study indicates that the uptake of toxin is greater in fish tissues than blood; however, because the whole mullet was not analyzed, a whole body to water ratio could not be determined for this species. On the other hand, collection of the entire menhaden in 3 sections (head, body, viscera) permitted determination of a whole body to water ratio of 57. This value reflects certain limitations of experimental design such as the length of exposure, as well as feeding and absorption rates. A repeat exposure of menhaden to *K. brevis* after 48 h of depuration resulted in only a 55% increase in whole body toxin content, indicating that these fish are less likely to proportionally accumulate toxin upon repeated exposure. It is possible that the less than proportional accumulation of toxin can result in part from a feeding deterrence after recent toxin exposure.

Our studies of aqueous exposure of planktivorous fish to *Karenia brevis* indicate uptake of toxin by intestinal absorption. Previously it was shown that the uptake of toxin into blood is slow, with a maximal accumulation at 8 h (Woofter et al. 2005), which is consistent with intestinal absorption rates for mullet (Perers & De Silva 1978). The initial high content of brevetoxin in the viscera of menhaden also supports intestinal absorption of toxin. Menhaden are planktivores, whereas mullet are often observed as detritivores with adaptability to plankton feeding. Indeed, a field study of mullet grazing on a dinoflagellate bloom of *Kryptoperidinium* indicated that this fish can quickly change its feeding pattern to planktivory under heavy bloom conditions (Odum 1968). Grazing fish such as mullet and menhaden have the capacity to filter large volumes through specialized gill rakers that sort particles to the esophagus for digestion (Sanderson et al. 2001). Particle sizes that can be sorted range from a whole dinoflagellate to as small as a bacterium. Given the fragile nature of the *K. brevis*, it is possible that the fish may also ingest toxin associated with lysed cell fragments as well as whole cells.

After a 10 h exposure, toxin accumulated to different concentrations in the various tissues of the mullet. Analysis of our data on a concentration basis (ng g^{-1} tissue) as presented in Fig. 1, found that the mean levels of brevetoxin were highest in the intestine and liver, with the intestinal values showing high fish to fish variability, likely due to different luminal contents of *Karenia brevis*. However, when adjusted for the weight of each organ (ng 100 g^{-1} fish) as presented in Fig. 2, the muscle was the major accumulating tissue for brevetoxin during the 10 h feeding period. Analysis of menhaden data yielded a VSR of 2.2 ± 0.3 (SE). The second exposure of menhaden after 48 h did not lead to a difference in VSR (2.5 ± 0.4). These results indicate that the viscera represent double the somatic body stores for brevetoxin in menhaden after 10 h of exposure and that a second *K. brevis* exposure within 48 h leads to increased toxin burden without a change in distribution between viscera and somatic stores. Previous work demonstrated that mullet retain detectable toxin in their blood for up to 5 d, suggesting that they may carry a toxin burden long after being exposed to a bloom of *K. brevis* (Woofter et al. 2005). We found that toxin is measurable in all tissues 8 wk post-exposure in mullet.

Interestingly, the amount of toxin in mullet decreased quickly in intestine and muscle, whereas liver showed an increase of toxin that peaked at 4 wk. By contrast, the viscera in menhaden remained unchanged for 17 d of depuration, during which time the somatic compartment was eliminating toxin. Based upon the organ elimination data from the mullet study, the delayed loss of toxin from viscera of menhaden may result from redistribution from intestine and muscle to the liver, where toxin is metabolized and recycled through enterohepatic pathways prior to elimination.

Comparison of the accumulation of brevetoxin in menhaden in this study correlates well with the values obtained from menhaden found in stomach contents of bottlenose dolphins examined in the 2004 Florida Panhandle mortality event. Menhaden collected from the dolphin stomach contents had muscle concentrations of 900 ng g^{-1} and visceral concentrations of 20 000 ng g^{-1} (Anonymous 2004, Flewelling et al. 2005). These values are 5- to 10-fold higher than toxin concentration values we measured in the body and the viscera of the menhaden exposed in the laboratory (169 ± 22 and 1858 ± 247 ng g^{-1}). Comparison of viscera brevetoxin concentration in menhaden from bottlenose dolphin stomach contents and menhaden fed measured levels of toxin indicates that the bloom would have had 10 times more toxin (approximately 65 µg l^{-1}) than present in the algae in our treatment tanks. Given that blooms regularly reach several million cells per liter and that our cell concentrations for the menhaden exposure were set at 275 000 cells l^{-1}, this suggests that (to achieve the toxin burden) the fish found in the stomach contents of the dolphins likely grazed on an estimated bloom of 2.75×10^6 cells l^{-1}. Mullet have been reported to graze on *Karenia brevis* cells in holding tanks at densities in excess of this range (4×10^6 cells l^{-1}) containing 62 µg l^{-1} of brevetoxin, and concentrated toxin (600 ng g^{-1} at 24 h) in their viscera (Naar et al. 2007). Although this value for mullet is lower than the concentration of brevetoxin viscera of menhaden, it is consistent with a lower accumulation potential of mullet. Taken together, these results indicate that menhaden grazing on a bloom of several million cells per liter for ≥10 h can achieve the toxin levels similar to those recovered from the stomachs of dolphins from the 2004 Florida Panhandle mortality event.

The unusual aspect of the 2004 Florida Panhandle bottlenose dolphin mortality event was that no bloom of *Karenia brevis* was detected; yet the dolphins were found to have full stomach contents, indicating that they had gorged on highly toxic fish (dominated by menhaden). Our findings with menhaden fed *K. brevis* indicate that these fish retain a toxin load with a half-elimination time of 24 d and opens the possibility that the menhaden recovered from the bottlenose dolphin stomachs may have encountered the toxic bloom much earlier than they were consumed. Given that the Gulf of Mexico menhaden were also completing their inshore migration at this time (Lassuy 1983), it is also possible that they encountered the suspected *K. brevis* bloom some distance from where the fish were consumed by the dolphins. Unfortunately, the elimination pattern we describe from the visceral organs of mullet, with rapid clearance from the intestinal tract and delayed redistribution to the liver, precludes using high visceral versus low muscle content of planktivorous fish as an indicator of recent exposure of planktivorous fish to *K. brevis*.

In summary, Atlantic menhaden accumulated brevetoxin at a whole body to water ratio of 57 when exposed to cultures of *Karenia brevis* for 10 h. Toxin accumulated in menhaden with a whole body VSR of 2.2 over this period and was eliminated with a half-life of approximately 24 d. Comparison of these results with the 2004 Florida Panhandle dolphin mortality event indicates that the toxin concentration of menhaden recovered from stomach contents of dead dolphins is consistent with menhaden grazing over a period of 10 h on a bloom of approximately 3×10^6 cells l^{-1} containing approximately 60 µg brevetoxin l^{-1} of seawater. Our elimination data provide evidence that menhaden have the potential to remain a lethal vector to dolphins for several weeks after they encounter a *K. brevis* bloom. The slow elimination and the migration of menhaden at the time of the event offer both temporal and spatial explanations as to how an event such as the 2004 Florida Panhandle dolphin mortalities may occur in the absence of an observable *K. brevis* bloom.

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