EVALUATION OF BREVETOXIN ACCUMULATION AND DEGRADATION IN COASTAL MAMMALS, BIRDS, AND FISH FOUND MORIBUND ON TEXAS BEACHES DURING RED TIDE BLOOMS

by

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ABSTRACT

Karenia brevis red tides in the Gulf of Mexico can have profound negative effects on human and animal health, local economies, and the coastal ecosystem (Landsberg et al., 2002; Landsberg et al., 2009). It is presumed that beached fish, either deposited on the upper surf line or buried in the sand, that have accumulated brevetoxin may pose a health risk to scavenging terrestrial animals. Because brevetoxicosis has been implicated in recent mortality events of birds and canids along the Texas coast, it is important to understand the availability of brevetoxin through these fish to the terrestrial ecosystem. Freshly dead striped mullet (*Mugil cephalus*) and pinfish (*Lagodon rhomboides*) were collected during the 2009 and 2011 red tide blooms, respectively. Fish were placed above and below sand in mesh enclosures and exposed to natural conditions; fish were removed for up to 77 days to quantify the brevetoxin concentration in the muscle tissue. ELISA was used to measure the concentration of PbTx-3 equivalents in the muscle samples to generate brevetoxin degradation models. The concentration of brevetoxin decreased 75% from the initial levels within 8 days, with concentrations of less than 90% within 15-23 days. No difference was found between the rates of brevetoxin degradation among species, but nonlinear regression methods were used to determine that brevetoxin degrades faster in samples located above the sand. Such information can be utilized in beach closure recommendations concerning pet safety and also to assess the risk of brevetoxicosis in coastal ecosystems.

Brevetoxin was quantified in a number species of dead fish, birds, and mammals collected along the beach in fall 2009 and 2011. Brevetoxin was measured in tissues of 16 fish, 5 bird, and 1 mammal species found dead during the red tides whose toxin levels

or toxin accumulation had not previously been reported. Concentrations in the livers of fish averaged 2647 ± 508 ng PbTx-3 eq. g^{-1}, whereas an average concentration of 878 ± 749 ng PbTx-3 eq. g^{-1} was detected in fish found dead along the Texas Coastal Bend, USA. The concentration of brevetoxin detected in fish was not correlated to *K. brevis* cell density, which suggests a differential susceptibility to brevetoxin toxicity among species, different routes of toxin exposure, or variation in toxin production in *K. brevis* strains in different areas. The majority of bird species analyzed included double-crested cormorants (*Phalacrocorax auritus*); red knots (*Calidris canutus*), American coots (*Fulica americana*), a mourning dove (*Zenaida macroura*), a brown pelican (*Pelecanus occidentalis*), an eared grebe (*Podiceps nigricollis*), and a masked booby (*Sula dactylatra*) were also sampled. Brevetoxin was detected in the liver, muscle, and/or gastro-intestinal tract samples of all birds tested. Dead coyotes that were collected in Padre Island National Seashore during the 2011 bloom had a brevetoxin concentration of 165 ± 94 ng PbTx-3 eq. g^{-1} in their muscle. These results demonstrate that the extent of red tide impact and brevetoxin exposure can affect the entire coastal ecosystem.

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1. Introduction

Phytoplankton are an important food resource for higher trophic levels and produce nearly 50% of the oxygen used by heterotrophic organisms in aquatic systems. Typically, numerous species or guilds proliferate; however, single species can occasionally develop blooms (Smayda and Reynolds, 2001). Harmful algal blooms (HABs) include both autotrophic and heterotrophic species that disrupt aquatic and/or terrestrial life or the services and products they provide (GEOHAB, 2001). Harmful impacts can result from the production of toxic metabolites or by reaching dense concentrations that may affect physical habitat and water quality (GEOHAB, 2001). Recent research and observations suggest that the frequency, distribution, and severity of HABs have increased in the past two decades (Hallegraeff, 1993; Landsberg, 2002). While the exact reason for the increasing number of HABs is unknown, anthropogenic forcing resulting in climate change and eutrophication may be a causative factor (Walsh et al., 2006). Bioactive metabolites from microalgae have been implicated in mortality and sickness of many organisms, including invertebrates and humans (reviewed in Landsberg, 2002; Smith et al., 2008). Toxicity can occur directly through contact with the source organism or water exposure, or indirectly through food web transfer and biomagnification (Landsberg et al., 2009). Toxin production has been recognized in many divisions of microalgae including: dinoflagellates, diatoms, euglenoids, cyanobacteria, raphidophytes, and prymnesiophytes (reviewed in Landsberg, 2002; Zimba et al., 2010). Theories regarding the reason for production of microalgal toxins include competitor inhibition, grazer deterrence, chemical signaling, and evolutionary artifacts (Van Dolah, 2000). Algal blooms can also be considered harmful to the

environment when the decomposition of higher than normal cell densities cause hypoxic or anoxic conditions resulting in fish, wildlife, invertebrate, or plant stress or mortality (Hallegraeff, 1993). Light attenuation caused by dense algal blooms can significantly reduce the amount of solar energy available to aquatic vegetation, and subsequent degradation of these plant materials can further deplete oxygen available to aquatic organisms (Buskey et al., 1996; Gastrich and Wazniak, 2002). In addition, dense blooms of some silicoflagellates and diatoms such as *Chaetoceros* spp. can cause gill irritation in fish and build-up of mucus that can result in mortalities from asphyxiation (Erard-Le Denn and Ryckaert, 1990; Harrison et al., 1993).

Karenia brevis (Davis) G. Hansen et Moestrup, previously known as *Gymnodinium breve* and *Ptychodiscus brevis* (Daugbjerg et al., 2000), is most often linked to dinoflagellate bloom events or red tides along the Gulf of Mexico coastline that result in massive fish kills and toxicity episodes (Walsh et al., 2006). The first documented report of fish kills associated with *K. brevis* occurred in 1844 (Ingersoll, 1882). Ficunane (1964) determined that concentrations of *K. brevis* exceeding 1,000 cells L^{-1} constitutes a bloom in the Gulf of Mexico. Red tides are usually reported along the western coast of Florida and historically occurred every four to five years; more recently, however, they seem to occur almost annually (CBBNEP, 1997; Kusek et al., 1999; Walsh et al., 2006). Historically, *K. brevis* blooms occurred less frequently along the Texas coast, occurring on average every 17 years. In the last decade, however, the prevalence of blooms along Texas has also increased (Villareal et al., 2001; Maganan et al., 2003).

The mechanisms responsible for bloom formation in the Gulf of Mexico are complex, and the lack of understanding as to why they occur more frequently leaves resource managers with few options to predict or control red tides. Walsh and Steidinger (2001) suggest that red tide blooms are able to sustain themselves on the nitrogenous compounds leaked from the nitrogen-fixing cyanobacteria *Trichodesmium* spp. Iron in Saharan dust stimulates the growth of the *Trichodesmium* in offshore waters; once fixed, nitrogen is available to stimulate increased growth of *K. brevis* in the late summer and fall (Walsh et al., 2001; Walsh et al., 2006). In coastal waters, sediment resuspension may provide the nutrients needed for bloom maintenance. In addition, studies have shown that blooms may correspond with increased rainfall and shelf break fronts that provide nutrients and the potential for transportation of cells to more favorable growth conditions (reviewed in Vargo et al., 2009). Cells concentrated by currents into shallow nearshore waters results in exposure to elevated nutrients derived from coastal runoff that promotes further cell growth (Vargo et al., 2004; Walsh et al., 2006; Vargo et al., 2008). When *K. brevis* cell density is high in coastal waters, nutrients can be remineralized from organisms killed during the bloom and become available to sustain *K. brevis* growth (Walsh et al., 2006). Lastly, it has been suggested that anthropogenic nutrient loading from the Mississippi River may contribute to the maintenance of blooms and more frequent occurrence of the red tides along the Texas coast (Walsh et al., 2006; Vargo et al., 2009).

Van Dolah et al. (2009) detected a high genetic diversity in *K. brevis* clones that may affect cellular physiology and the subsequent formation and toxicity of strains within bloom assemblages. One study demonstrated that concentration and the types of toxins

can vary up to 3-fold among clones of *K. brevis* isolated from Texas and Florida waters (Baden and Thomas., 1988). Errera et al. (2010) determined that brevetoxin concentration can vary up to 10-fold among *K. brevis* clones, and that 7 out of 8 clones produced more PbTx-1 compared to PbTx-2 when the salinity of the growth media was decreased from 35 to 27. In addition, Brown et al. (2006) found variation in the toxicity of *K. brevis* with salinity and growth phase, with increased toxin production at salinities less than seawater. Tester et al. (2008) determined that cell abundance and chlorophyll-*a* concentration were strongly correlated during red tides and are a good indication of brevetoxin concentration in the water at cell densities greater than 50,000 cells L^{-1}.

K. brevis produces a suite of neurotoxins, called brevetoxins (PbTxs), which are harmful to many organisms. Brevetoxins are a class of cyclic polyether toxins that bind with high affinity to site five of the voltage-gated sodium nerve channels and produce membrane depolarization (Catterall and Risk, 1981; Baden et al., 2005). Repetitive firing in nerves is caused when binding results in a longer average open time for sodium channels and inhibition of sodium channel inactivation (Baden et al., 2005). Brevetoxins are grouped into two classes based on their structure: A-type, Type 2, or PbTx-1-type brevetoxins include those with a 10-ring backbone, whereas those containing an 11-ring backbone are referred to as B-type, Type 1, or PbTx-2-type toxins (Risk et al., 1979; Baden, 1989). Whereas A-type brevetoxins are considered more toxic, B-type toxins are more prevalent (Risk et al., 1979; Baden, 1989). There are 12 readily recognized polyether compounds produced by *K. brevis* (Prasad and Shimizu, 1989; Baden et al., 2005; Bourdelais et al., 2005; Fire et al., 2011). PbTx-1 and PbTx-2 are considered the parent congeners found in cells, whereas PbTx-3, -6, -7, -9, -10, PbTx-3 42-carboxylic

acid (CBA), cysteine-PbTx-B (A), its sulfoxide, and others are considered to be extracellular metabolites formed by oxidation or reduction in the environment and animal tissues (Pierce et al., 2001; Bourdelais et al., 2005; Pierce et al., 2008).

Neurotoxic Shellfish Poisoning (NSP) is caused by ingestion of brevetoxin-contaminated shellfish by humans (McFarren et al., 1965; Music et al., 1973; Hemmert, 1975). Human exposure to NSP results in nausea, diarrhea, paresthesias, tingling of lips or tongue, muscle ache, lack of coordination, temperature reversal (cold feels hot), vertigo, and constriction of the throat (Morris et al., 1991). Filter-feeding bivalves bioaccumulate brevetoxins when they ingest *K. brevis* cells or by absorbing dissolved toxins from the water (Plakas et al., 2002). The current regulatory limit for brevetoxins in seafood is 800 ng g^{-1}, and shellfish harvesting areas are closed when *K. brevis* densities exceed 5,000 cells L^{-1} (USFDA, 2005; Naar et al., 2007). Increased monitoring since 1970 has resulted in no cases of NSP from legally harvested shellfish; however, threats to human health still exist from aerosolized brevetoxins and potentially through the consumption of other contaminated materials (Watkins et al., 2008). Because *K. brevis* is an unarmored dinoflagellate that is easily lysed by ocean turbulence, brevetoxin can be released and transported by wind (Pierce, 1986; Cheng et al., 2005). Respiratory irritation and water discoloration occurs at *K. brevis* concentrations greater than 1,000 cells L^{-1}, while shellfish poisoning occurs at 5,000 cells L^{-1} and fish kills at 1×10^5 cells L^{-1} (Steidinger et al., 1998). Intermittent respiratory irritation can occur with brief exposure, but more severe symptoms can occur in individuals with compromised lung function due to disease (Fleming et al., 2005).

Brevetoxin is a potent ichthyotoxin that has caused massive fish kills along the Gulf of Mexico coast (Baden et al., 1979; Risk et al., 1979). The link between aquatic animal mortalities and *K. brevis* blooms was formally identified after a 1948 red tide (Gunter, 1951), and many more aquatic animal mortality events have been attributed to brevetoxin exposure (reviewed in Landsberg, 2002; Landsberg et al., 2009). While most bivalve molluscs are unaffected by the toxin, exposure to brevetoxin may greatly impact recruitment of bay scallops (Landsberg, 2002). In addition, brevetoxin exposure has been demonstrated to reduce survival of bivalve larvae and increase mortality of shrimp, sponges, sea urchins, coral, and crabs (Steidinger et al., 1972; Landsberg, 2002; Leverone, 2006; Ross et al., 2010). Brevetoxins have also been implicated in the deaths of birds, turtles, marine mammals, and sharks (Landsberg, 2002; Fire et al., 2008; Nam et al., 2010; Walsh et al., 2010; Wetzel et al., 2010).

Despite the extensive historical accounts of animal mortality and the increasing co-occurrence of *K. brevis* blooms and fish kills, the impact of red tides on the aquatic and coastal ecosystems is poorly characterized (Landsberg, 2002). Only recently have studies demonstrated that prey fish are potential vectors for transfer of brevetoxin to higher trophic levels which can cause mortality of organisms such as dolphins, manatees, sharks, and seabirds (Kreuder et al., 2002; Landsberg, 2002; Shumway et al., 2003; Flewelling et al., 2005; Naar et al., 2007; Fire et al., 2008; Landsberg et al., 2009; Nam et al., 2010). Despite the ichthyotoxicity of brevetoxin, bioaccumulation can occur in live fish, thereby serving as vectors for brevetoxin transfer to higher trophic levels (Flewelling et al., 2005; Naar et al., 2007). Thus, it is important to understand the susceptibility of different fish species to brevetoxin and the potential transfer to higher

trophic levels. The research described in subsequent chapters includes a study to assess how brevetoxin degrades over time in beached fish, resulting in a degradation model that can be used to manage beach closures to pets following a red tide fish kill event. In addition, brevetoxin concentrations were measured in fish, bird, and mammal specimens associated with the 2009 and 2011 Texas red tide blooms. This is the first attempt to measure brevetoxin in animal tissues from Texas, and findings support observations of respiratory distress, symptoms of neurotoxicity, and death of organisms (TPWD, personal communication). This work will also aid in the effort to understand the extent to which red tides affect coastal ecosystems.

2. Brevetoxin Degradation in Beached Fish along the Texas Coast

Introduction

Brevetoxins associated with red tide blooms can cause sickness or mortality in a number of organisms; even humans are at risk of intoxication via the consumption of contaminated shellfish or inhalation of aerosols (Steidinger et al., 1973; Blanchard, 1975; Baden, 1983; Pierce et al., 1986; Poli et al., 2000). Brevetoxins accumulate in fish through absorption of toxins across gill membranes, consumption of contaminated prey, or ingestion of *K. brevis* cells (Catterell and Risk, 1981; Tester et al., 2000). Brevetoxins can accumulate in live fish and have the potential to biomagnify through the food web (Flewelling et al., 2005; Naar et al., 2007). Brevetoxins have been detected in the tissues of both planktivorous and piscivorous fishes and in the marine predators of these fishes including dolphins and sharks (Naar et al., 2007; Fire et al., 2008; Flewelling et al., 2010). Terrestrial consumers of these fishes, such as birds and mammals, may also be at risk of brevetoxin accumulation and subsequent sickness or mortality (van Deventer et al., 2012, and references therein).

Recent anecdotal observations of neurotoxicity and mortality in dogs, coyotes, and bobcats during Florida red tides suggests that brevetoxin can be transferred to terrestrial mammals in the coastal ecosystem (van Deventer et al., 2012, and references therein). Bird fatalities including double-crested cormorants (*Phalacrocorax auritus*), red-breasted mergansers (*Mergus merganser*), and lesser scaup (*Aythya affinis*) resulting from brevetoxin exposure have been previously reported from severe red tide blooms such as the 1973 Florida bloom (Landsberg, 2002). Van Deventer et al. (2012) analyzed

tissues of birds found dead or sick during the 2005 Florida red tide and determined that birds scavenging on dead beached fish may be exposed to harmful levels of brevetoxin. Although fish may die from oxygen deprivation resulting from hypoxic conditions created by a phytoplankton bloom and not directly from toxin exposure and accumulation, a high proportion of these fish will have detectable levels of brevetoxin in their livers, gastrointestinal tract, and muscle that can be transferred to the terrestrial food web (Flewelling et al., 2005; Naar et al., 2007; Fire et al, 2008).

Non-lethal levels of brevetoxin can accumulate in fish and may be transferred to higher trophic levels even when there is no bloom (Flewelling et al., 2005; Naar et al., 2007; Zimba et al., in prep.). Thus, more brevetoxin may be available to the coastal ecosystem through food web transfer than previously thought. Linares et al. (2009) demonstrated that brevetoxin was detectable in shrimp 15 days after exposure to *K. brevis* cells was terminated. Similarly, brevetoxin and its metabolites were detected in oysters 60 days after dosing stopped (Plakas et al., 2004). Khan et al. (2010) determined that both solar and UV radiation destroy brevetoxin, but that the ether linkage prohibits efficient biological breakdown. Kieber et al. (2010) suggests that degradation of PbTx-2 in seawater is a photosensitive process, accelerated by organic matter, trace metals, and oxygen concentration.

Only recently has the availability of brevetoxin to terrestrial animals when red tide fish kills cause massive amounts of dead fish to wash up on shore been elucidated (van Deventer et al., 2012). While not thoroughly documented in the scientific literature during other red tides, dead pelicans and other scavenging birds, coyotes, and dogs were found in close proximity to fish kills during the 2009 and 2011 *K. brevis* blooms along

Padre Island National Seashore in Texas (TPWD, personal communication). It is presumed that beached fish that accumulate brevetoxin and are deposited on the upper surf line or buried in the sand may pose a health risk to animals ingesting these materials.

The goal of this study was to measure degradation of brevetoxin in beached fish killed by *K. brevis* red tides. Many organisms scavenge on fish carcasses that are beached during a red tide and the presence of elevated brevetoxin concentrations in their tissues will corroborate observations of respiratory distress and neurological symptoms prior to death (TPWD, personal communication; Rafalski et al., in prep.). It is predicted that brevetoxin in aerobic conditions (fish on beach) will degrade faster than in those buried in the sand and subjected to more anaerobic conditions, and that the rate of degradation will decrease over time (Khan et al., 2010).

Materials and methods

Fish

Freshly dead (eyes not glazed and firm to the touch) striped mullet (*Mugil cephalus)* and pinfish (*Lagodon rhomboides)* were collected during the 2009 red tide at Corpus Christi Bay and during the 2011 bloom at Packery Channel, respectively. Whole fish were stored at -80˚C until processed. *Karenia brevis* cell densities in Corpus Christi Bay exceeded 150,000 cells mL^{-1} at the time of the 2009 collection, whereas cell densities in Packery Channel where the 2011 samples were collected were 22,000 cells mL^{-1}. The average size of the striped mullet used in this study was 24.01 ± 2.82 cm, whereas the average size of the pinfish was 17.62 ± 1.54 cm.

To adequately estimate brevetoxin in fish muscle, a preliminary study was completed to assess the variability in the distribution of brevetoxin throughout the muscle

of an individual fish. Five striped mullet were thoroughly rinsed with deionized water and then wiped with acetone to remove any excessive external brevetoxin or *K. brevis* cells; seven plugs (~1 gram each) of the lateral muscle were collected along the lateral axis from behind the head to the rear caudal peduncle.

A similar procedure was used to extract tissue from the fish in the exposure study. Two tissue samples were removed from each fish and pooled for analysis prior to placement in the enclosures; this allowed for the assessment of initial brevetoxin concentration in every fish.

Enlosure and Sampling Procedure

Two enclosures (one per fish species) were constructed to expose the collected fish to natural conditions while preventing disturbance (Fig. 1). Enclosures were constructed using 3 cm diameter PVC pipe with the dimensions 61x61x91 cm. Wire mesh panels (1 cm squares) were attached to each side of the enclosure with zip ties. Replicate fish (n=3) were placed in biobags (0.5mm mesh size) to deter grazing by macroinvertebrates and to organize and expedite removal for sampling. Ten bags of each fish species were placed on top of the sand, and 10 additional bags were buried 15-30 cm below the surface under sieved beach sand (1cm square mesh). An additional bag of 5 fish was placed above and below-ground to normalize toxin concentration on a weight basis to account for water loss in the muscle due to dessication.

Fig. 1. Enclosures constructed to expose fish to natural conditions.

One bag of fish (n=3) was removed from each enclosure and location on day 1, 2, 3, 4, 8, 12, 15, 23, 46, and 77, beginning on October 29, 2011. Due to the prediction that the brevetoxin degradation would follow a logarithmic trend, sampling was concentrated at the beginning of the experiment. Muscle samples were removed from each fish in close proximity to the initial tissue cores. Each time muscle samples were collected, fish from the weight normalization bag were weighed to model water loss. As fish dried, the volume of tissue taken for a one gram sample would be greater than the volume sampled at time zero. It is assumed that the greater volume would contain more brevetoxin; as toxin was to be normalized per gram of tissue, a correction factor was generated for the weight of the fish based on the percent change in the weight of an additional set of fish in each location over time. The percent change in the brevetoxin concentration measured in

fish tissue during the time course compared to its initial value was applied to the mean value of brevetoxin concentration in each species to construct a model of toxin concentration over time

Toxin Extraction and Evaluation

Brevetoxin was extracted by homogenization and sonication of the tissue in acetone (9 mL g^{-1} tissue), followed by centrifugation (2885xg for 5 minutes). The acetone extract was stored at -80˚C for ELISA and HPLC-MS/MS analyses. Total brevetoxin (as PbTx-3-type equivalents) was quantified using the Abraxis brevetoxin (NSP) competitive ELISA (enzyme-linked immunosorbent assay) kit (Abraxis LLC, Warminster, PA). Brevetoxin that is present in the standards and samples compete for the binding site of sheep anti-brevetoxin antibodies that coat the wells of the microtiter plate; the concentration of PbTx-3 equivalents (PbTx-3 eq.) in the samples is based on a colorimetric change and is interpolated from a standard curve of PbTx-3 standards. The lower limit of detection of the kit is 22.5 ng PbTx-3 eq. g^{-1} for shellfish samples (Abraxis LLC).

While the ELISA quantification method has high sensitivity and efficiency, accurate determination of brevetoxin congeners requires high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS). In HPLC-MS/MS, brevetoxin congeners are chromatographically separated and individually quantified by a mass-to-charge ratio and fragmentation pattern that is compared to toxin standards. For each sampling date, one fish of each species and from each location (above versus below) was analyzed using HPLC-MS/MS (n=30). Its initial tissue sample was also analyzed to determine how the particular congeners degraded. Samples were

processed from the fish tissues as described in Plakas et al. (2002), Wang et al. (2004), and Naar et al. (2007). The acetone extract was evaporated under nitrogen (Peak Scientific, Billerica, MA) and resolubilized in 80% aqueous methanol. After a hexane partition, the methanol layer was dried again under nitrogen. The extract was resolubilized in 25% aqueous methanol and applied to a pre-conditioned C-18 solid-phase extraction column under vacuum (Phenomenex, Torrance, CA). After a water rinse, the C-18 SPE columns were eluted with methanol and concentrated under nitrogen. Fragmentation voltages and collision-induced dissociation energy values were optimized for PbTx-1 (867 AMU), PbTx-2 (895 AMU), PbTx-3 (897 AMU), and PbTx-9 (899 AMU) using Agilent's MassHunter Optimization program (version B.02.01) and authentic standards (MARBIONIC, Wilmington NC). Because brevetoxin is often present in the form of metabolites, transitions from the literature were also used for several congeners: PbTx-6:911-893 AMU, PbTx-10:871-853 AMU, Cyst-PbTx-2:1018-1000 AMU, and Ox-Cyst-PbTx-2:1034-1016AMU. An Agilent 1200 HPLC equipped with a 6410B triple quadrapole mass spectrometer and a Phenomenex Luna C-18 3µm 150x3 mm^2 analytical column was used for chromatographic separation. The solvent gradient consisted of acidified (0.1% formic acid) acetonitrile (ACN) and water with initial conditions of 50:50 ACN/H_2O to 95:5 ACN/H_2O over 45 min in positive ion mode with the probe at 4kV and 350˚C.

Data Analysis

R (version 2.11.1) was used to perform statistical analyses. Linear regression analysis and one-way ANOVA were used to determine if there was any significant variation in the distribution of toxin within an individual fish. Welch's two sample t-test

was used to compare the water-loss in fish between locations. One-way ANOVA with Tukey's HSD test was used to compare brevetoxin degradation among groups. The segmented package was installed to perform break-point regression analyses of brevetoxin concentration over time to generate a model of degradation.

Results

Individual Toxin Variation in Striped Mullet

One-way ANOVA confirmed that there was no significant difference in brevetoxin concentration throughout the lateral muscle of the five fish ($F= 0.507$, p-value=0.798, Fig. 2). In addition, no significant linear trend was detected in the brevetoxin concentration within individual striped mullet (average slope = -22 ± 39, $R^2 = 0.0383$, p-value=0.26).

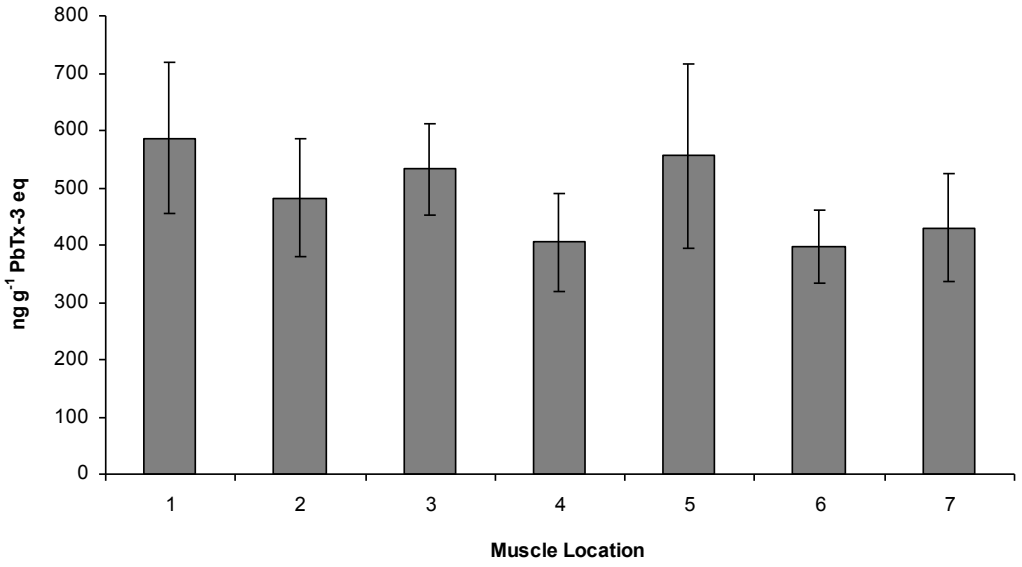

Fig. 2. Average brevetoxin concentration in striped mullet (*Mugil cephalus*) muscle locations (n=5); muscle 1 corresponds to a core of lateral muscle taken from behind the head of the fish, while muscle 7 corresponds to a location on the rear caudal peduncle.

Weight Normalization

Weight normalization curves were generated for striped mullet and pinfish from a sample of 5 fish positioned above and below-ground (Fig. 3). From these curves, a correction factor for the weight of tissue taken on each day over the time course was ascertained from these curves. For instance, on day 23, the striped mullet located below-ground weighed 60% of what they weighed on time zero. Thus, only 0.6 g of tissue was taken to make up the 1 g sample for brevetoxin analysis. The fish located on top of the sand lost more weight over time than the fish located below the sand. On average, fish lost 54% of their weight in above-ground locations whereas below-ground fish lost 27%. The weight loss of the pinfish located above and below-ground was not significantly different ($t=0.709$, p-value=0.487), while a more rapid weight loss occurred in above-ground mullet when compared to fish located below-ground ($t=-5.691$, p-value<0.001).

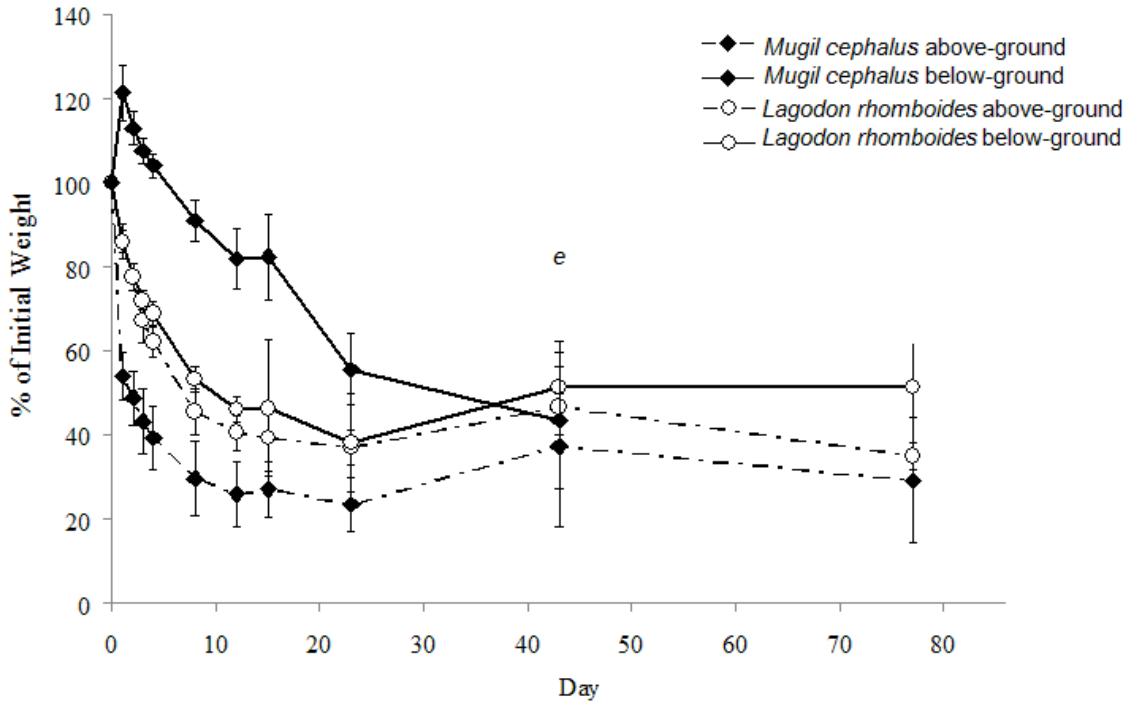

Fig. 3. Percent change in weight of fish over time due to dessication.

Brevetoxin Degradation

The initial brevetoxin concentration in the muscle of the 53 striped mullet was 402 ± 214 ng PbTx-3 eq. g^{-1}, whereas the concentration in pinfish muscle averaged 455 ± 265 ng PbTx-3 eq. g^{-1}. The percent change in the brevetoxin concentration measured in fish tissue during the time course (final concentration subtracted from the initial concentration, divided by the initial concentration) compared to its initial value was applied to these mean values to construct a model of toxin concentration over time (Fig. 4, A-D). Less than 25% of the initial brevetoxin concentration was detected after 8 days of exposure. Greater than 90% degradation occurred after 15-23 days in each species and location, but brevetoxin was still detected in all fish after 77 days. There was no

significant difference in the rate of brevetoxin degradation in the dead fish among species per one-way ANOVA ($F=0.222$, p-value=0.878; $n=12$).

Segmented linear (nonlinear regression) methods were used to estimate breakpoints in degradation rates for each fish species and location. This model resulted in a break point that separated rapid degradation in brevetoxin concentration from where the rate of degradation slowed significantly (Fig. 4, A-D). The times where the slope values changed (break points) corresponded to 3.4 and 5.1 days for the striped mullet above-ground and those buried below the sand, respectively. The rate in brevetoxin degradation changed after 4.0 and 4.5 days for the pinfish located above-ground and below-ground, respectively. The average rate of degradation was faster above-ground (91.6 ± 0.44 ng PbTx-3 eq. g^{-1} day^{-1}) for both striped mullet and pinfish when compared

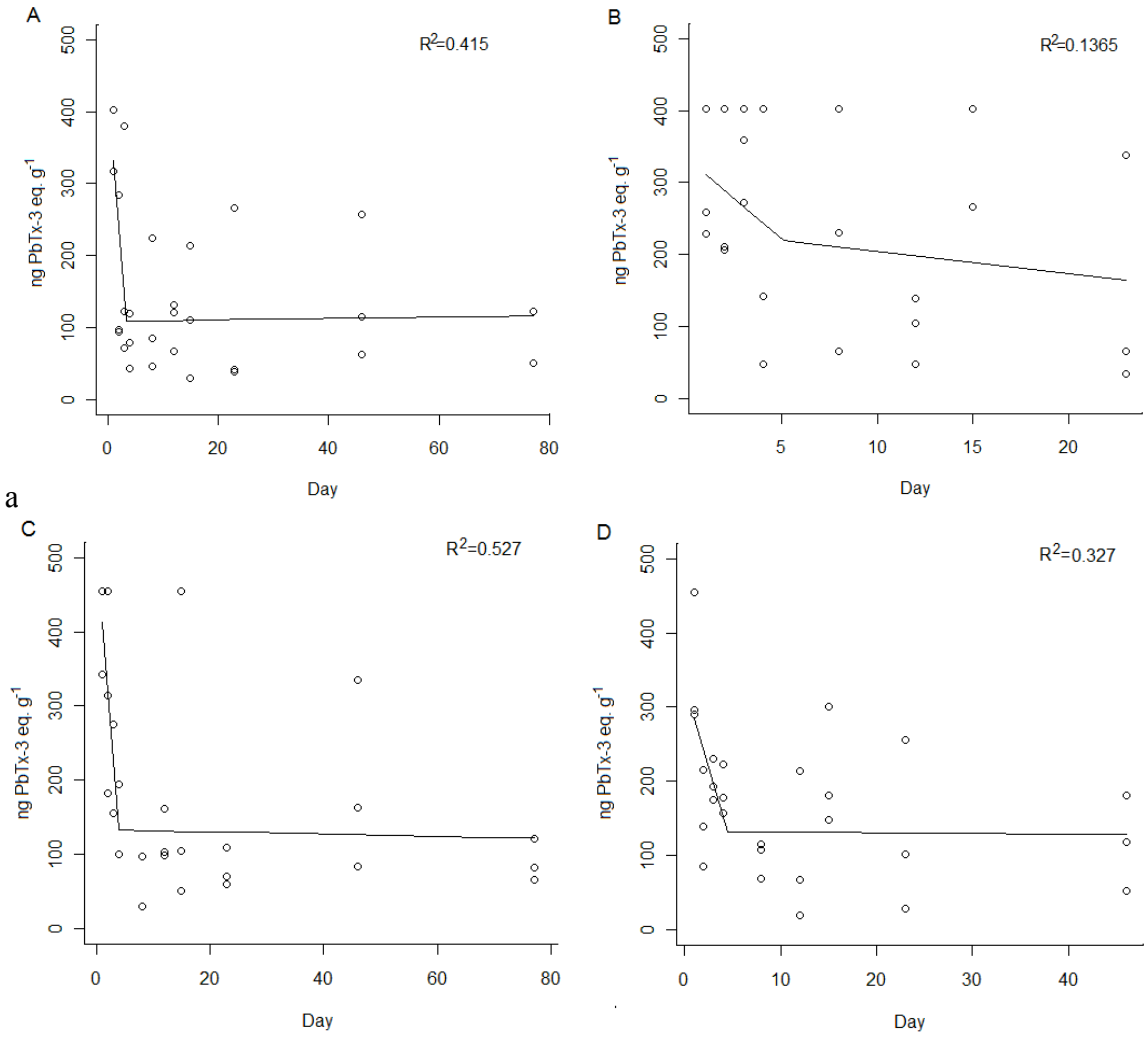

Fig. 4, A-D. Nonlinear regression analysis of brevetoxin degradation in individual fish. (A) Striped mullet (*Mugil cephalus*) located above-ground (B) *M. cephalus* located below-ground (C) Pinfish (*Lagodon rhomboids*) located above-ground (D) *L. rhomboides* located below-ground.

to the rate of those buried in the sand (32.8 ± 14.6 ng g^{-1} day $^{-1}$) before these changes in slope per the nonlinear regression analysis.

LC-MS/MS Analysis

A breakdown product of PbTx-1 and PbTx-9 (m/z 867 and 899, respectively) were the predominant brevetoxin congeners detected in all 60 samples analyzed using HPLC-MS/MS. PbTx-1 transitions 867.5-221.1 AMU and 867.5-93.1 AMU were

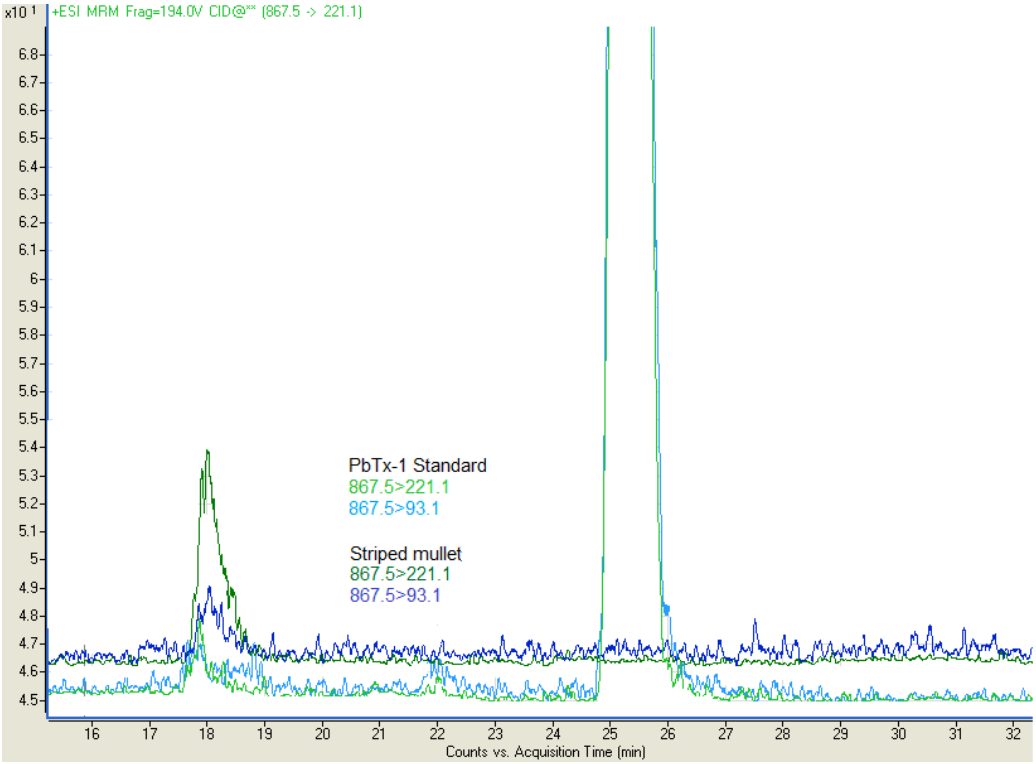

Fig. 5. PbTx-1 transitions 867.5-221.1 and 867.5-93.1 were monitored in multiple reaction monitoring mode (MRM). The authentic PbTx-1 standard had one peak located at 18 minutes and one at 25.2 minutes. When monitored for the same transitions, a peak was detected in the striped mullet samples that coincided with the 18 minute peak. monitored in multiple reaction monitoring mode (MRM).

The authentic PbTx-1 standard had one peak located at 18 minutes and one at 25.2 minutes. When monitored for the same transitions, the striped mullet sample coincided with the 18 minute peak (Fig. 5). In both species and locations, there was a smaller decrease in the concentration of PbTx-1 than PbTx-9 between an individual's initial measurement and its measurement through the time course (Fig. 6). The concentration of PbTx-1 decreased an average of 7 ± 4%, whereas PbTx-9 decreased an average of 50 ± 5% during the study period (n=30). Cyst-PbTx-2 was detected in all 9 of the fish analyzed for additional brevetoxin congeners and metabolites via selected ion monitoring (SIM) mode, while trace amounts of PbTx-2 was found in 2 of the samples.

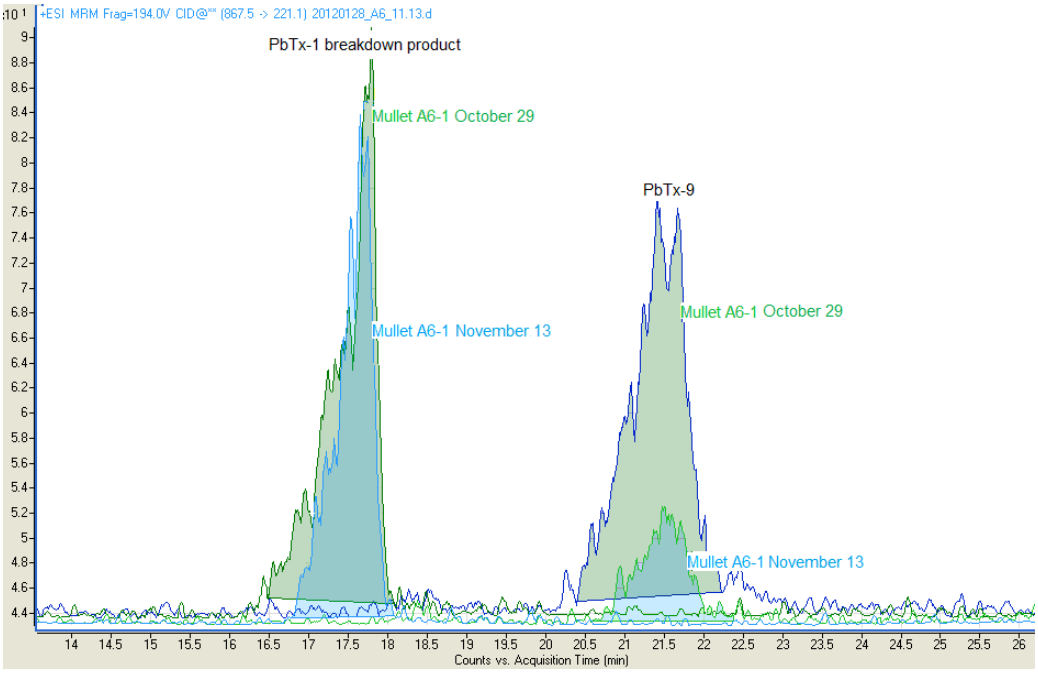

Fig. 6. Brevetoxin profile detected in a striped mullet via HPLC-MS/MS from November 13 compared to its initial profile on October 29.

Discussion

There was no significant variation in the brevetoxin concentration within an individual fish (Fig. 1). Thus the reduction in brevetoxin concentration over time can be attributed to degradation of the toxin and not due to variation in toxin throughout the muscle of the fish. In some instances, particularly at day 1 or 2, fish had a higher concentration of brevetoxin when compared to initial concentrations. The small amount of variation detected within a fish (± 64 ng PbTx-3 eq. g^{-1}) might account for this discrepancy. The weight loss in the fish was attributed to evaporative water loss and was subsequently used to normalize the weight of the tissue taken for toxin extraction. Fish

appeared to gain weight twice over the course of the exposure; this followed precipitation events and further corroborates that the weight loss was due to desiccation.

Nonlinear regression analyses of toxin concentration in this study suggests that brevetoxin initially degrades faster in fish that are exposed to sunlight and aerobic conditions, which corroborates finding by Khan et al. (2010) regarding brevetoxin degradation in seawater. Brevetoxin degradation decreased after 3.4-4 days in fish positioned above-ground, whereas below-ground fish required 4.5-5.1 days to exhibit a similar decrease. From a management perspective, it is recommended that resource managers may want to keep dead fish from being buried so that toxin degradation can be accelerated.

When examining the availability of brevetoxin to the terrestrial system, it is important to consider both the amount of toxin and its isoform (Naar et al., 2007). In this study, a breakdown product of PbTx-1 was found in tissues of all the fish, which corroborates previous work that the parent brevetoxin is unstable, reactive, and metabolized in tissue (Plakas et al., 2002). However, such a breakdown product has not previously been reported in animal tissues. From HPLC-MS/MS analyses, PbTx-9, a B-type congener, was the most abundant degradation product detected by ELISA. PbTx-2 and cyst-PbTx-2 were also detected via selected-ion monitoring (SIM) mode. These results are similar to those of Naar et al. (2007), who detected the brevetoxin congeners cys-PbTx-2, PbTx-3, ox-cys-PbTx-2 in pinfish livers in decreasing concentration. PbTx-2, PbTx-3, PbTx-6, and PbTx-9 were detected in striped mullet viscera in decreasing concentration by Naar et al. (2007). Ionization and fragmentation optimization of standards would increase the sensitivity of detection of these congeners and metabolites

(Grebe and Singh, 2011). Effects of freezing and storage of these congeners and metabolites is also unknown (Fire et al., 2007). It is likely that animals that scavenge on dead fish would likely ingest all parts of the fish, including the viscera, skin, and any associated phytoplankton cells. Higher concentrations of brevetoxin are detected in fish viscera (Naar et al., 2007), and that the parent congeners are associated with *K. brevis* cells. Since PbTx-1 and -2 are considered to be the more potent forms of brevetoxin (reviewed in Landsberg et al., 2002), the findings of this study suggest that fish carcasses are more toxic than once thought (Naar et al., 2007). While it has been suggested that the brevetoxin metabolites are less toxic than the other congeners, they can still cause sickness or mortality (Plakas et al., 2004).

Between November 4 and December 31. 2009, 11 coyotes (*Canis latrans*) and 3 domestic dogs were found dead or sick within Padre Island National Seashore, Texas during a red tide bloom that started in October. An additional 6 coyotes and 5 dogs were reported as sick, exhibiting neurological symptoms such as tremors, paralysis, and seizures; it is believed that a number of deaths or incidences of sickness went unreported. Additional dead and sick coyotes that exhibited similar signs of brevetoxicosis were found during the 2011 Texas red tide bloom; 492 ± 22 ng PbTx-3 eq. g^{-1} were detected in coyote muscle samples (n=2, Rafalski et al., in prep.). The Padre Island National Seashore superintendant suggested in late November that pet owners refrain from bringing their dogs to the beach and enacted a 20 day dog ban in the park on 9 December. Results from this study suggest that the availability of brevetoxin to these animals through the ingestion of dead fish is highest up to 6 days after a red tide fish kill; after this point, the rate of degradation of brevetoxin slowed significantly, and brevetoxin was

still present in fish after 77 days. Mortality events in upper trophic levels often lag behind a bloom event; this is presumably due to the time it takes for lethal levels of toxin to accumulate in consumer organisms (Kreuder et al., 2002; Flewelling et al., 2005). These findings along both the Texas and Florida coast suggests that one route of toxin exposure for coyotes and other scavengers is through the ingestion of brevetoxin-laden dead fish (van Deventer et al., 2012).

Mortality events of shorebirds are associated with red tides but have not always been thoroughly documented (reviewed in Landsberg, 2002; Shumway et al., 2003; van Deventer et al., 2012). Brevetoxin was detected in the livers and muscles of shorebirds including sanderlings (*Calidris alba*) and ruddy turnstones (*Arenaria interpres*) found dead during the 2005 Florida red tide (van Deventer et al., 2012) and in red knots (*Calidris canutus*) during the 2011 Texas red tide (Rafalski et al., in prep.). It is suggested that shorebirds may opportunistically scavenge on dead fish when they cannot find their preferred forage items due to high mortalities of invertebrates from the red tide bloom; shorebirds likely feed on flies, maggots, and other decomposers located with fish carcasses (Gunter et al., 1951; Simon and Dauer, 1972; Landsberg, 2002; Landsberg et al., 2009; van Deventer et al., 2012). Based on a white Leghorn feeding experiment where a total dose of 198,000 ng PbTx per chick was required to kill a chick, van Deventer et al. (2012) suggested that lethal levels of brevetoxin could be accumulated in shorebirds through the consumption of dead fish during red tides. Findings of this study could further be applied to this model to assess the availability and toxicity of brevetoxin to the terrestrial food web. For instance, as it has been shown that brevetoxin degrades

rapidly in the first 4-6 days in dead fish in this study; using toxin levels from freshly dead fish tissues may overestimate the availability of brevetoxin to consumers.

Observations by Texas Parks and Wildlife Department officials corroborate scavenging on dead fish by birds and mammals with brevetoxicosis and subsequent mortality in the terrestrial component of the coastal ecosystem (TPWD, personal communication; van Deventer et al., 2012 and references therein); the models from this experiment could be combined with information on toxicity of brevetoxin to terrestrial animals to assess the potential availability of toxin, recognizing that the levels decrease in dead fish over time. Since the climatology of Texas differs from that of Florida (significantly fewer rainfall events in Texas), this work may only provide a model for Texas beaches. However, this research contributes to the work done on degradation of brevetoxin in aquatic systems, and gives wildlife managers a potential timeframe for when beaches may be safe for humans and their pets following a severe *K. brevis* bloom.

3. Brevetoxin Concentrations in Texas Fish, Birds, and Mammals

Introduction

Red tide events have caused massive mortalities of fish, birds, and mammals in the Gulf of Mexico (reviewed in Landsberg et al., 2009; Van Deventer et al., 2012). Most red tide field studies have been conducted along the Florida coast since bloom occurrence is nearly an annual event (Kusek et al., 1999; Walsh et al., 2006). Red tides blooms have also occurred more frequently on the Texas coast in recent years (Villareal et al., 2001; Magaña et al., 2003). According to the Texas Parks and Wildlife Department (TPWD) beach census, over 5.5 million fish were killed during the 2009-2010 red tide and over 4.4 million died during the 2011-2012 *K. brevis* bloom along the Texas coast. It is estimated that more than 2 million striped mullet were found dead on the beaches along the coast (TPWD, personal communication). These estimates are extremely conservative as many fish are eaten prior to beaching, sink, float out to sea, or land in remote areas not routinely surveyed. High mortalities of scaled sardine (*Harengula jaguana*), Gulf kingfish (*Menticirrhus littoralis*), Atlantic bumper (*Chloroscombrus chrysurus*), pinfish (*Lagodon rhomboides*), ladyfish (*Elops saturus*), spot (*Leiostomus xanthurus*), hardhead catfish (*Ariopsis felis*), Gulf menhaden (*Brevoortia patronus*), and pigfish (*Orthopristis chrysoptera*) occurred along Texas beaches during these red tides. Important recreational fish species such as red (*Sciaenops ocellatus*) and black drum (*Pogonias cromis*), southern flounder (*Parlichthus lethostigma*), and spotted seatrout (*Cynoscion nebulosus*) were also killed (TPWD, unpublished data).

In addition, red tides and brevetoxins have been linked to bird mortality events along the Florida coast (Quick and Henderson, 1974; Forrester et al., 1977; Kreuder et al., 2002; Shumway et al., 2003; Landsberg et al., 2007). Large numbers of double-crested cormorants (*Phalacrocorax auritus*), red-breasted mergansers (*Mergus merganser*), and lesser scaup (*Aythya affinis*) were found dead during a Florida red tide event from October 1973 to May 1974 (Forrester et al., 1977) and morbidity and mortality of double-crested cormorants during Florida red tides have occurred sporadically for 30 years (Kreuder et al., 2002; Landsberg et al., 2009). Few studies have been completed to link brevetoxin exposure to bird morbidity and mortality events during Texas red tides; however, a number of sick or dying double-crested cormorants and other birds have been found along the Coastal Bend Gulf beaches and barrier islands during Texas red tides (TPWD, personal communication; Tony Amos, personal communication). A large number of sick double-crested cormorants were received by the Animal Rehabilitation Keep (ARK) at the University of Texas at Austin's Marine Science Institute, Port Aransas, Texas in fall 2011. The birds exhibited symptoms consistent with brevetoxicosis including erratic behavior, inability to hold up the head and neck, limited flying ability, sudden violent spasms, and being in unusual places for an aquatic bird (Tony Amos, personal communication). These symptoms are consistent with those associated with brevetoxin toxicity in double-crested cormorants in Florida (Kreuder et al., 2002). Other species of birds found dead on beaches in close proximity to these sick or dead double-crested cormorants included red knots (*Calidris canutus*), American coots (*Fulica americana*), a mourning dove (*Zenaida macroura*), a brown pelican (*Pelecanus*

occidentalis), an eared grebe (*Podiceps nigricollis*), and a masked booby (*Sula dactylatra*).

The objective of this research was to quantify the concentration of brevetoxin in fish, birds, and mammals to further assess the extent of the impact of red tides on coastal ecosystems. Previous studies have focused on quantifying the brevetoxin found in live prey fish of dolphins (Naar et al., 2007; Fire et al., 2008). Van Deventer et al. (2012) reported brevetoxin concentrations found in dead thread herring (*Opisthonema oglinum* Lesueur), scaled sardines (*Harengula jaguana* Poey), and mullet (*Mugil* spp.). By measuring the brevetoxin found in dead fish on the beach, this study will contribute to the understanding of the differential body burden of brevetoxin in fish species and will also help to quantify how much toxin may be available to terrestrial consumers (reviewed in Landsberg et al., 2002; Naar et al., 2007; van Deventer et al., 2012). This study also aims to begin to elucidate the risk of brevetoxin accumulation in migratory and resident birds found in Texas. The Laguna Madre system is one of the most important habitats along the Gulf Coast for wintering and migratory shorebirds, and many species that use the Central Flyway winter in large numbers in Texas (Ballard et al., 2010). Many migratory and resident species feed on plants and animals in the Laguna Madre while making frequent flights to freshwater to reduce the salt loads ingested while foraging (Ballard et al., 2010). This study will help to characterize the risk of red tides and brevetoxin exposure to these birds and others that primarily winter near waters that can contain *K. brevis* cells through the fall and into the winter months. The last objective of this study was to assess the amount of brevetoxin found in dead mammals collected during the red tides that exhibited signs of brevetoxicosis. These findings will support the anecdotal

evidence suggesting that coastal mammals are killed by brevetoxin (Van Deventer et al., 2012, and references therein).

Materials and methods

Fish samples were obtained from four locations along the Coastal Bend of Texas (Fig. 1). Freshly dead fish (eyes not glazed, firm to the touch, and visually intact) were collected either from Gulf or bay beaches during the red tide bloom. Many carcasses exhibited signs of scavenging by birds and other animals including missing eyes, bore holes, and further predation. Fish were stored at -80˚C, necropsied, and tissue samples of liver and muscle were taken. During November 2012, sick or dead birds found on Mustang Island or north Padre Island were collected by the ARK, the Texas Parks and Wildlife Department, or the CBBCP; carcasses found along South Padre Island were collected by the National Park Service (Fig. 3.1). Birds and coyotes were necropsied at Texas A&M University-Corpus Christi and liver, muscle, and/or gastrointestinal tract (GIT) content samples were frozen at -80˚C until brevetoxin could be extracted and analyzed. Dog muscle and liver samples, received from a Houston veterinarian, had been preserved in formalin and arrived desiccated.

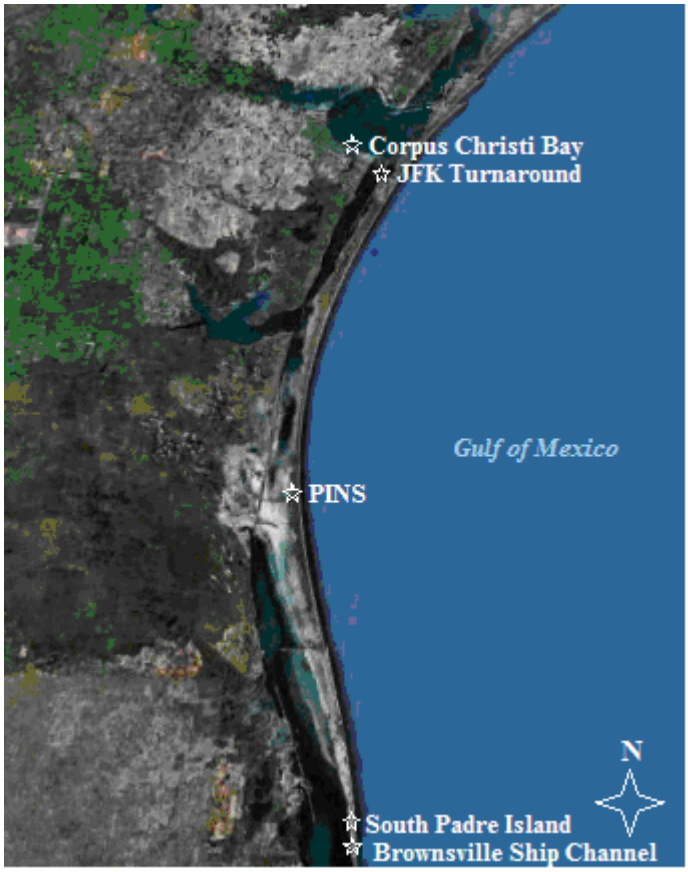

Fig. 7. Sampling locations along the Texas Coastal Bend, U.S.A.

Brevetoxin was extracted by homogenization and sonication of the tissue in acetone (9 mL g^{-1} tissue), followed by centrifugation (2885xg for 5 minutes). The acetone extract was stored at -80˚C for ELISA and HPLC-MS/MS analysis. Total brevetoxin (as PbTx-3-type equivalents) was quantified in tissue samples using the Abraxis brevetoxin (NSP) competitive ELISA (enzyme-linked immunosorbent assay) kit (Abraxis LLC, Warminster, PA). Brevetoxin that is present in the standards and samples compete for the binding site of sheep anti-brevetoxin antibodies that coat the wells of the microtiter plate; the concentration of PbTx-3 equivalents (PbTx-3 eq.) in the samples is based on a colorimetric change and is interpolated from a standard curve of PbTx-3

standards. The lower limit of detection of the kit is 22.5 ng PbTx-3 eq. g^{-1} for shellfish samples (Abraxis LLC).

HPLC-MS/MS was used to identify the specific brevetoxin congeners in water samples and a subset of fish, bird, and mammal samples. Water was sampled from Padre Island National Seashore when fish were collected. Whole water was applied to a pre-conditioned C-18 solid-phase extraction column under vacuum (Phenomenex, Torrance, CA). After a water rinse, the C-18 SPE columns were eluted with methanol and concentrated under nitrogen for PbTx confirmation. For the animal samples, the acetone extract was evaporated under nitrogen (Peak Scientific, Billerica, MA) and resolubilized in 80% aqueous methanol. After a hexane partition, the methanol layer was dried again under nitrogen. The extract was resolubilized in 25% aqueous methanol and applied to a pre-conditioned C-18 solid-phase extraction column under vacuum (Phenomenex, Torrance, CA). After a water rinse, the C-18 SPE columns were eluted with methanol and concentrated under nitrogen. Fragmentation voltages and collision-induced dissociation energy values were optimized for PbTx-1 (867 AMU), PbTx-2 (895 AMU), PbTx-3 (897 AMU), and PbTx-9 (899 AMU) using Agilent's MassHunter Optimization program (version B.02.01) and authentic standards (MARBIONIC, Wilmington NC). As brevetoxin is often present in the form of metabolites, transitions from the literature were used for the identification of several congeners: PbTx-6:911-893 AMU, PbTx-10:871-853 AMU, Cyst-PbTx-2:1018-1000 AMU, and Ox-Cyst-PbTx-2:1034-1016AMU. An Agilent 1200 HPLC equipped with a 6410B triple quadrapole mass spectrometer and a Phenomenex Luna C-18 3µm 150x3 mm^2 analytical column was used for chromatographic separation. The solvent gradient consisted of acidified (0.1% formic

acid) acetonitrile (ACN) and water with initial conditions of 50:50 ACN/H_2O to 95:5 ACN/H_2O over 45 min in positive ion mode with the probe at 4kV and 350˚C.

Data was analyzed using version 2.11.1 of the statistical platform R. Welch's two sample t-test was used to compare brevetoxin accumulation among juvenile and adult pinfish. One-way ANOVA with Tukey's HSD test were used to compare brevetoxin concentration among groups of fish with different dietary preferences.

Results

Brevetoxin was detected using ELISA in the muscle and/or liver of all 150 fish collected from fall 2009 and 2011 (Table 1). The highest concentration of brevetoxin in liver was found in a pinfish while the highest concentration detected in a muscle sample was found in a shrimp eel (3664 and 2223 ng PbTx-3 eq. g^{-1}, respectively). Brevetoxins found in the muscle of fish ranged from 47 to 2223 ng PbTx-3 eq. g^{-1}, while liver concentrations ranged from 1924 to 3664 ng PbTx-3 eq. g^{-1}. The average concentration found in small pinfish was 2.4 times larger than that found in larger pinfish; however no significant difference was detected in the means when analyzed with a Welch's two sample t-test (p-value=0.07; average sizes of 5.64 and 17.62 ± 1.54 cm, respectively). Overall, the average concentration in livers of fish (2647 ± 508 ng PbTx-3 eq. g^{-1}, n=15) was significantly higher than the concentration found in fish muscle (mean of 878 ± 749 ng PbTx-3 eq. g^{-1}, n=29, p-value<0.05). Congeners confirmed by LC-MS/MS include a PbTx-1 breakdown product, which was found in greater concentration than cyst-PbTx-2 and PbTx-9 (n=18), while ox-cyst-PbTx-2 was detected in 3 fish, and PbTx-2 was detected in 5 fish. Fish species collected were categorized as herbivore, planktivore,

benthic omnivore, or piscivore (Table 1); analysis by one-way ANOVA detected no significant difference in the concentration of brevetoxin in muscle samples of these groups ($F = 1.855$, p-value = 0.159).