BREVETOXIN ACCUMULATION AND PERSISTENCE DURING A *KARENIA BREVIS* (RED TIDE) BLOOM IN SOUTH TEXAS

A Thesis

by

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BA, University of Connecticut, 2012

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Red tide blooms composed of the harmful microalgae, *Karenia brevis,* are bi-annual events along the South Texas coast that cause marine and terrestrial animal mortalities. On September 13, 2015, a red tide bloom occurred from Port Aransas, TX to South Padre Island, TX and persisted in Corpus Christi Bay, TX until November 23, 2015. The objective of this study was to determine if brevetoxin (PbTx), produced by *K. brevis,* was passively or actively accumulated in macroalgae and if so, determine persistence time and depuration rates.

Sargassum was collected from Padre Island National Seashore on September 26, 2016 and co-cultured with seawater containing *K. brevis.* Accumulation of PbTx was measured for a 72-hr exposure period. The PbTx concentration was determined using ELISA (reported as PbTx-3 equivalents) and confirmed by HPLC-MS/MS (normalized to PbTx-1 standard). The *Sargassum* community was partitioned mechanically to include epiphytic algae (with concentrations as high as 26 ng/mL PbTx-3 equivalents) and toxin adsorbed on *Sargassum* (up to 51 ng/mL PbTx-3 equivalents). Concentrations as high as 100 ng/g PbTx-3 (by ELISA) and 934 ng/g PbTx-1 (by HPLC-MS/MS) were detected in *Sargassum* tissue.

As little information is available on PbTx in plant vectors; a second study assessed toxin depuration and persistence in macroalgae after the *K. brevis* bloom subsided. *Ulva intestinalis* was collected from Corpus Christi Bay on November 6, 2015 and incubated in f/10 media for a 30-day period to assess depuration. The epiphytic fraction contained over 17 ng/mL PbTx-3 equivalent and above 3 ng/g PbTx-3 equivalent in adsorbed PbTx over the 30-day period. *U.*

intestinalis tissue PbTx concentration remained above 110 ng/g PbTx-3 equivalent. These results confirm that PbTx can be present in the macroalgal community for at least one month after a *K. brevis* bloom subsides.

This study is the first to assess macroalgae as a vector for PbTx trophic transfer. Pelagic forms of macroalgae can accumulate and transfer PbTx into regions not experiencing red tides while toxin in attached forms can persist and be released to the environment after a bloom has disappeared. In future studies, examination of accumulation, depuration, and persistence of toxins in other macroalgal species should be considered. Studies on the transfer of marine algal toxins to different communities (i.e. beaches) could provide a better understanding of post-bloom effects.

DEDICATION

It has always been a dream of mine to help save the environment through science. To all the future scientists, I dedicate this research to you. I hope it inspires you, the way that I have been inspired to continue in this field.

ACKNOWLEDGEMENTS

I would first like to thank my advisor, Dr. Paul Zimba for his endless help and support throughout my time at Texas A&M – Corpus Christi. Without him, my dreams of becoming a scientist would not have been possible. Thank you for taking a chance on me and pushing me towards success. I would like to thank my committee members, Drs. Jeffrey Turner and Brandi Reese for being readily available when I needed their guidance. I would also like to give a big thank you to the Center for Coastal Studies for the funding they provided to me along with being my second home over the last two years.

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Without all of you my degree would not have been possible. Thank you all for the wonderful opportunity. I have grown so much over these two years and I am so excited to see what the future holds.

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INTRODUCTION

1.1 General Background on Algae

Algae are a diverse group of polyphyletic organisms that are classified by physical and chemical characteristics such as cell wall composition, photosynthetic pigments, and mode of reproduction (Bold and Wynne 1978). The 13 divisions recognized today (Table 1) are non-vascular, non-rooted, photosynthetic organisms that contain chlorophyll *a* (Richardson et al. 1983; Dawes 1998; Lobban et al. 2014). Two major size classifications are used: macroscopic and microscopic forms.

Coastal waters are home to a diverse community of perennial and ephemeral macroalgae and a diverse range of planktonic, epiphytic, and benthic species of microalgae (Pedersen and Borum 1996). Macroalgae are classified into "red" (Rhodophyta), "green" (Chlorophyta), and "brown" (Phaeophyta) plant lines based on their accessory pigments, reproductive patterns, and chemical composition (Bold and Wynne 1978). Macroalgae can have coenocytic or parenchymatous bodies that are visible with the naked eye and occur almost exclusively in marine environments (Lobban and Harrison 1994). These organisms can be found in the intertidal and littoral zones as attached or free-floating species, or in the pelagic zone where they are exclusively free floating.

The microalgae are comprised of organisms such as cyanobacteria (Cyanophyta), diatoms (Bacillariophyta), dinoflagellates (Dinophyta), cryptomonads (Cryptophyta), yellow-brown algae (Chrysophyta), and eustigmatophytes (Eustigmatophyta) (Richardson et al. 1983; Bold and Wynne 1985; Dawes 1998; Roy et al. 2011). A microscope is needed to see the various forms of microalgae which can occur as colonial, filamentous, and single cells. Microalgae can be found

in a variety of freshwater, marine, and terrestrial environments and have been found in extreme settings such as deserts and polar regions (Bold and Wynne 1985). These organisms are much more speciose in comparison to macroalgae.

An estimated 45% of oceanic primary productivity is attributed to algae (Bascom 1979). A variety of factors influence algal production such as turbulent wave action, currents, light availability, nutrients, grazing, and species interactions (Sand-Jensen and Borum 1991; Pedersen and Borum 1996). The algae are critical to nutrient cycling in open-ocean and coastal regions by providing essential nutrients to higher organisms (Bold and Wynne 1985; Yool and Tyrrell 2003).

Harmful Microalgae and Health Effects

Harmful algal blooms (HABs) occur when accumulated phytoplankton and macroalgae cause negative effects that threaten human or environmental health (Ahn et al. 2006). Organisms can be exposed to toxins through drinking water, bioaccumulation/biomagnification through diet, as well as dermal and aerial exposure (Landsberg et al. 2009). HABs can affect the marine environment by both non-chemical and chemical impacts leading to physiological impairment or mortality (Smayda 1997).

Non-chemical effects can lead to mechanical or physical damage or starvation (Smayda 1997). For example, non-chemical, physical damage (piercing) of fish and invertebrate gill filaments has occurred from the setae of chaetocerid diatoms (Horner et al. 1990; Yang and Albright 1992; Rensel 1993; Tester and Mahoney 1995).

Mechanical damage to larval invertebrates has been documented during a bloom of the dinoflagellate *Cochlodinium heterolobatum* (Ho and Zubkoff 1979). Starvation-impaired growth and lowered fecundity in bivalves have occurred during blooms of the brown tide species *Aureococcus phagefferens* (Tracey 1988).

Of the known phytoplankton taxa, only 2% (60-80 spp.) are considered to be harmful or toxic (Van Dolah 2000). These taxa include dinoflagellates that produce toxins such as saxitoxin, neosaxitoxin, brevetoxin (PbTx), okadaic acid, ciguatoxin, and *Pfiesteria* toxins, as well as the diatom *Pseudonitzschia* which can produce domoic acid (Glibert et al. 2005). Toxins are hypothesized to have arisen for chemical signaling as well as to deter grazers (Van Dolah 2000).

The ingestion of bio-accumulated toxin in marine animals can cause paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), amnesic shellfish poisoning (ASP), and diarrhetic shellfish poisoning (DSP) in humans (Van Dolah et al. 2001; Deeds et al. 2010). The ingestion and subsequent accumulation of toxin in marine animals, such as filter feeding oysters, has led to fish, seabird, and mammal mortalities (Anderson 1997; Carmichael 2001; Pierce and Kirkpatrick 2001; Landsberg 2002; Plakas et al. 2002). Hypoxia and anoxia resulting from biological/ chemical oxygen demand to degrade decomposing algal blooms can cause die-offs in marine systems (Graneli et al. 1989; Mahoney and Steimle 1979).

Karenia brevis in the Gulf of Mexico

With the increase of anthropogenic nutrient loading leading to eutrophic conditions andrising seawater temperatures from the increase in atmospheric CO_2, the occurrence of toxic bloom-forming algae are expected to become more common (Hallegraeff 1993; Paerl and Huisman

Table 1. A summary of the 13 divisions/classes of algae and their distinguishing characteristics. Adapted from Richardson et al. 1983, Dawes 1998, and Roy et al. 2011. * = Divisions present in this study.

Division/Class	Common Name	Chl(s)	Major Pigment(s)	% Marine	Species #
Cyanophyta	Blue-green algae	*a*	Myxoxanthin, Zeaxanthin, β-carotene	8	>2,698
Prochlorophyta		*a, b*	Zeaxanthin	75	4
***Rhodophyta**	Red algae	*a*	Zeaxanthin	98	7,097
***Chlorophyta**	Green algae	*a, b*	Lutein, Violaxanthin, *cis*-Neoxanthin, *β*-carotene	13	6,298
Prasinophyceae	Prasinophytes		Neoxanthin, MgDVP, Prasinoxanthin, Violaxanthin	99	240
Euglenophyta	Euglenoids	*a, b*	Diadinoxanthin	3	~1,000
***Phaeophyta**	Brown algae	*a, c*	Fucoxanthin	99	~1,500
Chrysophyta		*a, c*			
Prymnesiophyceae	Golden-brown algae		Fucoxanthin, Diadinoxanthin	20	1,000
Xanthophyceae	Yellow-brown algae		Diadinoxanthin	15	600
Bacillariophyceae	Diatoms		Fucoxanthin, Diadinoxanthin	50	~10,000
Raphidophyceae	Chloromonads		Diadinoxanthin, Fucoxanthin	50	15
***Dinophyta**	Dinoflagellates	*a, c*	Peridinin, Violaxanthin, Zeaxanthin, *cis*-Neoxanthin, *β*-carotene	90	~4500
Cryptophyta	Cryptomonads	*a, c*	Alloxanthin, Crocoxanthin, Monadoxanthin	50	200
Eustigmatophyta	Eustigmatophytes	*a, c*	Antheraxanthin, Vaucheriaxanthin	8	12

2009). Red tide in the Gulf of Mexico is caused by *K. brevis*, a bloom-forming toxic dinoflagellate that occurs in subtropical to south temperate coastal regions (Magaña et al. 2003). The Gulf of Mexico is a semi-enclosed basin dominated by two currents: the Yucatan Current and Loop Current (Tester and Steidinger 1997). The anticyclonic circulation pattern and associated eddies are the main driver of *K. brevis* transport throughout the Gulf of Mexico (Tester and Steidinger 1997). The western Gulf of Mexico is the only exception to this pattern with cyclonic circulation driving bloom initiation. Background concentrations of >100 cells L^{-1} have been documented while concentrations of 1000 cells L^{-1} are considered to be bloom concentrations (Geesey and Tester 1993).

Fish kills potentially caused by red tides were noted as early as 1528 by Spanish conquistadors (cited in Magaña et al. 2003). The first published report of red tide in South Texas described respiratory distress by the local population (Lund 1936). *K. brevis* blooms are best known from the west coast of Florida; however, there has been an increase in these blooms along the Texas coast during the past 20 years (Walsh et al. 2006).

In the past decade, South Texas has experienced increased HAB events with extensive red tide blooms now occurring biannually. Other toxin producers have been found with increased frequency in South Texas waters such as the dinoflagellate, *Gambierdiscus toxicus* (Villareal et al. 2007). In 2003, ciguatera toxin was found at six petrochemical platforms surveyed off Texas (Villareal et al. 2007). The presence of okadaic acid produced by the dinoflagellate, *Dinophysis ovum*, prompted the closure of oyster beds from March to April 2008 in Port Aransas, TX (Swanson et al. 2010). Along the Texas coast, *K. brevis* blooms have increased in frequency resulting in large estuarine fish kills (Campbell et al. 2013).

Walsh et al. (2006) developed a hypothesis to account for the sequence of physical and ecological events required for initiation of *K. brevis* blooms along the Florida coast (Figure 1). The sequence includes (1) low dissolved inorganic nitrogen (DIN) and phosphate (PO_4) ratios that eliminate phytoplankton competition; (2) transport of iron-rich Saharan dust aerosols that supports blooms of the diazotrophic cyanobacteria *Trichodesmium;* (3) nitrogen (N_2) fixation by *Trichodesmium* and conversion to organic nitrogen, which is leaked to the environment and used by *K. brevis* as a nutrient source; (4) upwelling to transport toxic dinoflagellates and diazotrophs to dissolved organic matter (DOM) rich coastal regions; (5) small *K. brevis* blooms grown from dissolved organic nutrients (DON) released from the termination of the co-occurring *Trichodesmium* bloom; (6) a supplementary nutrient source from fish mortalities caused by *K. brevis*; (7) large red tide blooms. For blooms in Texas waters, windrowing of algae on the continental shelf occurs in addition to upwelling (4).

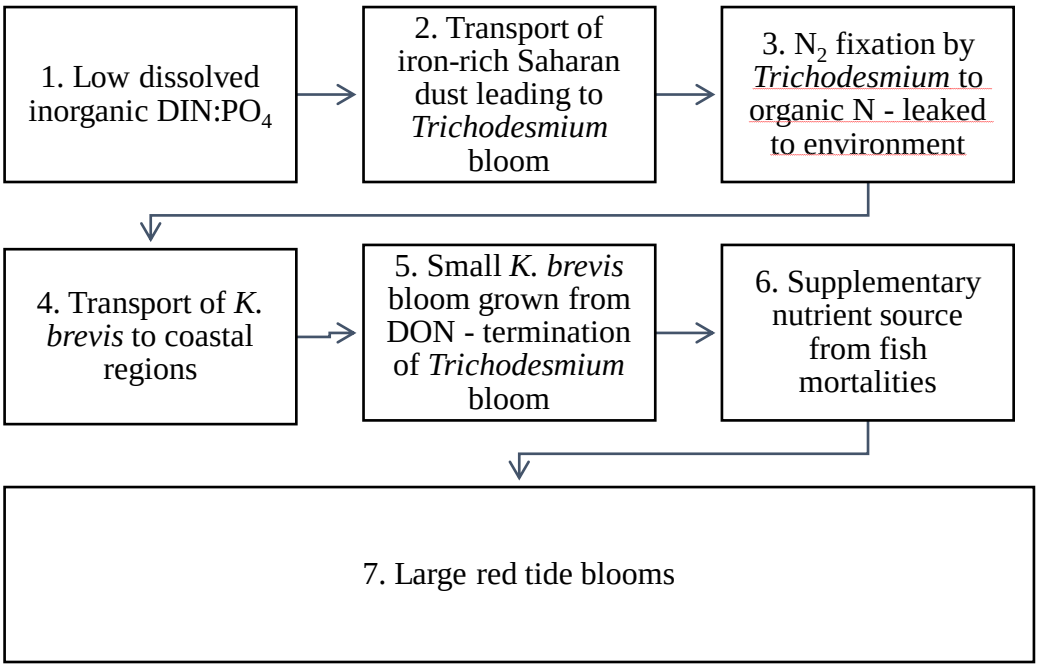

Figure 1. Red tide initiation in the Gulf of Mexico (Walsh et al. 2006).

Brevetoxin

K. brevis can produce brevetoxin (PbTx), a lipid soluble polyether neurotoxin that can cause animal mortalities, as well as human illness. In cell membranes, the binding of the toxin to site 5 of the voltage-gated sodium (Na^+) channels leads to longer mean opening times (Rein et al. 1994; Baden et al. 2005). The longer open period of voltage gated sodium channels leads to repetitive nerve firing and uncontrolled Na^+ influx by the toxin resulting in cell death (Kirkpatrick et al. 2004). There are two classes of PbTx – PbTx-1 (A-type) and PbTx-2 (B-type) with at least 11 known congeners (Figure 2). These PbTxs are classified by their trans-fused polyether ring structure; PbTx-1 has a 10-ring backbone and is considered to be a more potent toxin, while PbTx-2 has an 11-ring backbone and is more commonly produced by *K. brevis* (Poli et al. 1986; Baden 1989; Flewelling 2008). PbTx structures feature a lactone function on the A-ring, a spacer region between the lactone and the tail region, and a rigid tail region composed of

four rings (Baden 2005; Flewelling 2008). The side chain on the terminal ether ring of the PbTx is the region that differentiates between congeners.

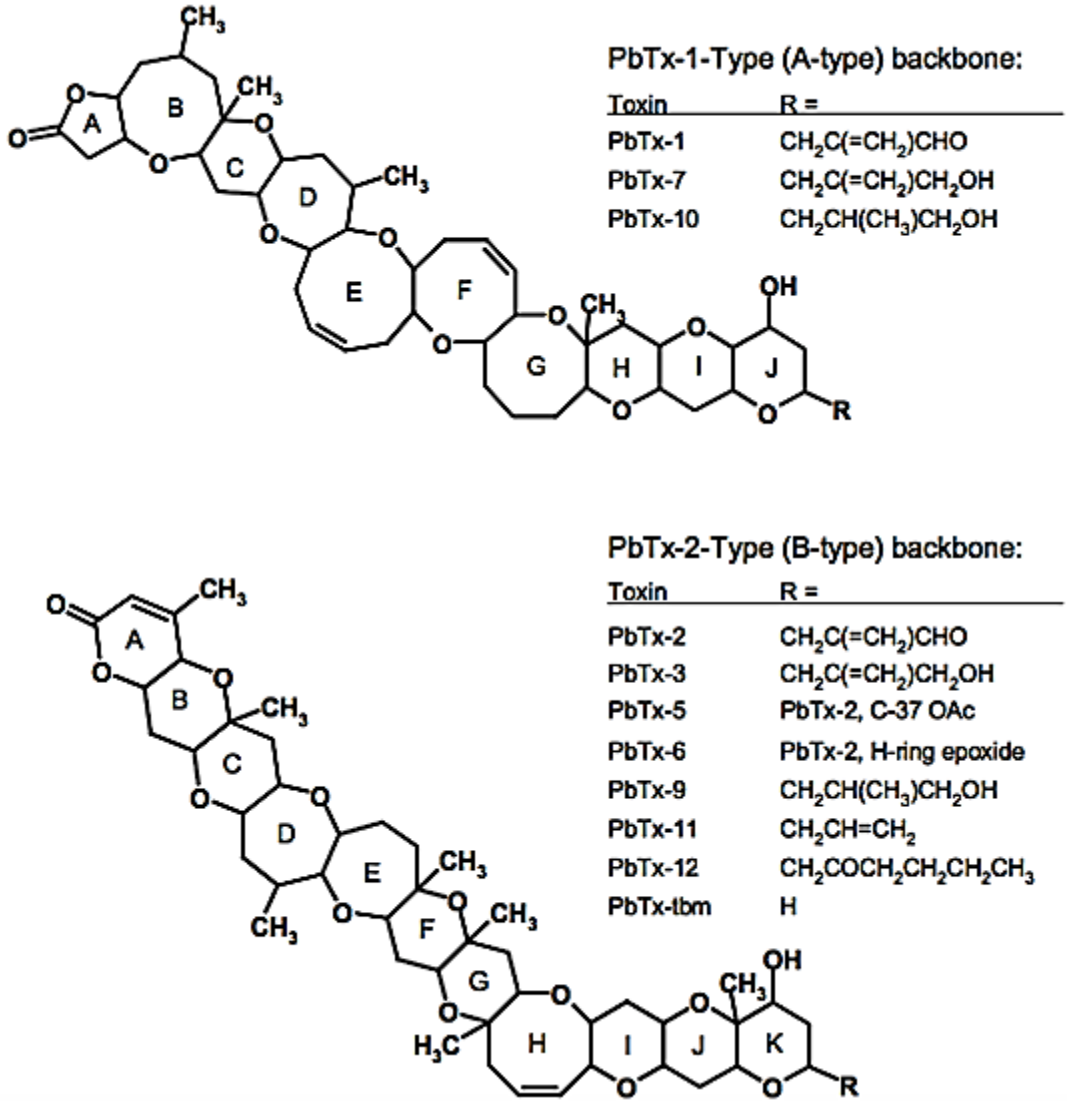

Figure 2. PbTxs produced by *Karenia brevis* and their corresponding chemical structure. Adapted from Flewelling 2008.

The production of PbTxs by *K. brevis* and subsequent uptake into organisms and the surrounding environment transforms the parent molecule into metabolites (Flewelling 2008). Research on the metabolism of PbTx has focused mainly on shellfish (Plakas et al. 2004). Little to no mortality

events of invertebrates have been documented from exposure to PbTx (Plakas et al. 2004, Sotka et al. 2012). The PbTx metabolites present in shellfish are the result of reduction, oxidation, and conjugation to form adducts (Plakas et al. 2004; Wang et al. 2004). PbTx metabolite formation is hypothesized to involve modifications to the side chain of the parent type (Flewelling 2008). The structural modifications to the side chain are believed to be part of the detoxification strategy by filter feeding shellfish (Flewelling 2008).

Neurotoxic shellfish poisoning from the consumption of shellfish and finfish can cause gastroenteritis and neurological effects, while the aerosolized toxin can cause severe respiratory irritation in humans and other mammals (Rafalski, 2012; Kirkpatrick et al., 2004). In addition to toxin production, mortality of fish has occurred from the clogging of gills after exposure to high densities of *K. brevis* (Rensel 1993). PbTx bioaccumulates in filter feeding bivalves by ingestion of *K. brevis* cells or by filtering toxic water (Plakas and Dickey 2010). The U.S. Food and Drug Administration (FDA) action level mandates shellfish bed closures when *K. brevis* densities exceed 5,000 cells/L^{-1} (Plakas and Dickey 2010).

I. Brevetoxin uptake and accumulation in *Sargassum* spp. from Padre Island National Seashore, Texas

1. Introduction

Sargassum spp. ecology

Sargassum is the most diverse genus of marine macroalgae, with more than 130 tropical-temperate species (Xie et al. 2013). *S. natans* (Linnaeus) Gaillon and *S. fluitans* (Børgesen) Børgesen form floating mats throughout the Gulf of Mexico and Sargasso Sea (Bold and Wynne 1985; Gower et al. 2011). They are unique holo-pelagic species with a single life history stage, reproducing exclusively by vegetative fragmentation (Lüning 1990). *Sargassum* originates in the western Gulf of Mexico and travels along the Loop Current in March annually. It has been estimated by Medium Resolution Imaging Spectrometry (MERIS) that the annual wet weight of *Sargassum* in the Gulf of Mexico reaches one million tons (Gower et al., 2011).

Extensive mats of *Sargassum* provide a floating ecosystem offering habitat and nutrition to over one hundred species including larval fish, invertebrates, and juvenile sea turtles (Morris and Mogelberg, 1973). Some organisms, such as the *Sargassum* fish (*Histrio histrio*), live in drifting mats of *Sargassum* throughout their lifetime. There are fifty species of commercially important fish such as tuna, billfish, jacks, and mahi mahi that utilize *Sargassum* as shelter and as a nursery area (Schoener and Rowe 1970). *Sargassum* is also essential to providing carbon to deep-sea environments as it degrades (Schoener and Rowe 1970; Hurd et al. 2014,). *Sargassum* has been found in the gut contents of the deep-sea brittlestar, *Amphiophiura bullata*, which suggests that *Sargassum* is important in deep-sea food webs (Schoener and Rowe 1970). *S. natans* and *S. fluitans* are the main components of South Texas marine wrack, providing habitat to

invertebrates and detritivores (Williams and Feigen, 2009). Beaches are often lacking essential nutrients and depend on marine wrack to provide carbon and nutrient inputs (Williams and Feigen, 2009).). The ingestion and breakdown of macrophyte wrack by intertidal invertebrates and catabolic decomposition by fungi and bacteria release nutrients to beach plant communities (Dugan et al. 2010; Koop et al. 1982). The spatial and temporal variability of macroalgae on beaches can be high in response to environmental and anthropogenic factors (Dugan et al., 2010). Beaches account for 70% of the coastline; marine wrack helps to control erosion of beaches by protecting sediment and absorbing wave energy (Bascom 1979; Williams and Feigen 2009). The wrack may enhance biomass accumulation by benthic microalgae and bacteria by supplying nutrients such as nitrogen and phosphorous (Olabarria et al., 2010).

Objective

The objective of this study was to determine if PbTx was passively or actively accumulated in *Sargassum* spp. This is the first study to examine the accumulation of PbTx in macroalgae. Toxin concentration was determined using enzyme-linked immunosorbent assay (ELISA) and high performance liquid chromatography equipped with tandem mass spectroscopy (HPLC-MS/MS). *Sargassum* is transported throughout the Gulf of Mexico by currents; resulting in exposure risk to organisms in non-bloom areas. Since *Sargassum* is the main component of South Texas marine wrack, organisms utilizing it as a refuge or as a food resource may be exposed to PbTx on beaches. Previous studies have reported seagrass and sediment as vectors for PbTx transport in the marine ecosystem (Flewelling 2008). Bird and terrestrial animal mortalities have been documented in Florida and Texas from contact with toxic sediment/through the ingestion of toxic fish and seagrass (Flewelling 2008; Rafalski 2012).

2. Methods

2.1. Experimental Design

On September 13, 2015, a red tide bloom was confirmed by Texas Parks and Wildlife (TPWD) with high *K. brevis* concentrations (> 10^6 cells/L^{-1}) at Packery Channel (Corpus Christi, TX) and Bob Hall Pier (Corpus Christi, TX). Horace Caldwell Pier (Port Aransas, TX), and mile markers 0, 5, 10 on Padre Island National Seashore (PINS) were reported to have moderate *K. brevis* concentrations (>10^5 and <10^6 cells/L^{-1}).

Reports from TPWD on September 23, 2015 showed an increase from moderate (9/15/15) to high concentrations (9/21/15) of *K. brevis* on PINS from the northern border to mile marker 20 (Figure 3). On September 26, 2015, seawater and *Sargassum* were collected from the water column at mile marker 10, Padre Island National Seashore, TX (Figure 3). As *K. brevis* is 20 to 40 μm in length, seawater was first filtered through 78 μm Nitex mesh (Sefar Inc. Buffalo, NY) to remove larger organisms. The filtrate was then concentrated using 7 μm Nitex mesh to capture high densities of *K. brevis.* Cell counts were performed at the initial sampling time using the Utermöhl (1958) method. A Carl Zeiss IM 35 inverted microscope (Carl Zeiss AB, Germany) and settling chambers were used to quantify *K. brevis* cells.

Figure 3. Sampling location at mile marker 10 on Padre Island National Seashore, TX where seawater and *Sargassum* were collected on September 26, 2015.

2.2. Sargassum spp. and K. brevis incubation

Four 5-gallon aquarium tanks containing 11 liters of the seawater (containing concentrated *K. brevis*) from PINS were equipped with air bubblers and controlled lighting (12:12 L/D; 350 photons m^{-2} s^{-1} photosynthetically active radiation (PAR)) and lids to contain aerosolized PbTx. *Sargassum* was added to each of the four replicate tanks (9 plants – wet weight between 5 -10 g) and the uptake of PbTx monitored by time course sampling. Sampling of *Sargassum* and seawater occurred at 12 hr intervals for 72 hrs. The baseline PbTx concentration in samples was

measured at Time 0. The sampling period was chosen because *Sargassum* and *K. brevis* are difficult to maintain in culture together for extended periods. *Sargassum* requires a highly turbulent environment while *K. brevis* will lyse if exposed to high turbulence.

2.2.1. Removal of surface bound PbTx and epiphytes from Sargassum

As PbTx can be found in epiphytes and attached to surfaces (Flewelling 2008), the *Sargassum* community was partitioned using a combined mechanical and chemical approach. One *Sargassum* plant was removed from each replicate tank and added to a 1 L plastic bottle containing 100 mL of 2-(N-morpholino) ethanesulfonic acid (MES) buffer and shaken for 60 seconds (1 revolution per second) to remove epiphytes. The resulting epiphyte slurry was subsampled (10 mL) and frozen at -80ºC in a 15 mL centrifuge tube. *Sargassum* was then rinsed with 20% methanol (MeOH) to remove adsorbed PbTx on the surface tissue. The resulting adsorbed slurry was subsampled (10 mL) and frozen at -80ºC in a 15 mL tube. The individual *Sargassum* plant was frozen at 80ºC until extracted for toxin analyses.

2.2.2. Toxin extraction from Sargassum tissue

Sargassum plants were homogenized individually using a combination of mortar and pestle and blender. A 1 g subsample of the plant was extracted using 9 mL of MeOH in a 50 mL centrifuge tube. The tube was vortexed for 30 seconds, sonicated for 20 seconds, then centrifugation at 3000 x for 10 minutes. The supernatant was removed and frozen at -80ºC until ELISA and HPLC-MS/MS analyses.

2.2.3. Toxin evaluation by ELISA

The Abraxis PbTx competitive ELISA assay (Abraxis Corporation, Warminster, PA) was used to quantify the total PbTx concentration (as PbTx-3 equivalents) in the samples. ELISA measures the biological activity of PbTx as the standards and samples compete for binding sites within the wells of the microtiter plate that are coated with sheep anti-PbTx antibodies. The colorimetric change, determined by a Biotek Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT), is inversely proportional to PbTx concentration. A PbTx-3 standard curve was run during each sample set to determine the concentration of PbTx present (Figure 15). Winooski, VT). Samples were diluted if the PbTx concentration exceeded the range of standard concentrations. The lower detection limit for PbTx in water samples was 0.05 ng/mL and 22.5 ng/mL in dried, diluted shellfish samples (Abraxis Corporation, Warminster, PA).

2.2.4. Toxin evaluation by HPLC-MS/MS

Each time point for tissue samples (0, 12, 24, 36, 48, 72 hrs) and water samples (0, 24, 72 hrs) were analyzed for PbTx presence and concentration by HPLC-MS/MS (n=36). Water samples collected at 12, 36, and 48 hours were lost. Water column and tissue extracts were analyzed by mass spectrometry on an Agilent 1200 HPLC (Agilent, Wilmington, DE) equipped with a 6410 triple quadrupole mass spectrometer. A Phenomenex C-18 Luna 2.5 μm 2 x 100 mm^2 analytical column (Phenomenex, Torrance, CA) was used along with a proprietary sodium adduct mixture for chromatographic separation of PbTx congeners. The mobile phase A consisted of water and the sodium adduct mixture while mobile phase B consisted of 95% acetonitrile (ACN) and the sodium adduct mixture. A blank of MeOH was performed before each run. The sample run time was 25 min with a gradient consisting of 0-20 min %B and 10-20 min %B. Flow rate was

maintained at 0.4 mL/min and the column temperature was maintained at 40ºC. Multiple reaction monitoring (MRM) method was used to monitor the following congeners: PbTx-1 (867 atomic mass units (AMU)), PbTx-2 (895 AMU), PbTx-3 (897 AMU), and PbTx-9 (899 AMU) (Roth et al. 2007). The PbTx congeners were quantified by their mass-to-charge ratio. The fragmentation pattern of the congeners was then compared to PbTx standards. Data was analyzed using Agilent MassHunter Qualitative Analysis software version B.07.00 (Agilent, Wilmington, DE). The toxin concentration was normalized using a PbTx-1 standard curve (Figure 16). The lower limit of detection of PbTx using HPLC-MS/MS is 0.01 ng/mL.

2.2.5 Pulse amplitude modulation

Chlorophyll- *a* fluorescence induction pulse amplitude modulation (PAM) techniques are used to assess the photophysiology of photosynthetic organisms non-invasively (Burdett et al. 2012). Pulses of actinic light at varied intensities and duration supply a saturating light source to the organism (Burdett et al. 2012). PAM provides information on the photosynthetic response to PAR to determine the current photosynthetic state of an organism (Falkowski and Laroche 1991). 100 mL of seawater from each replicate tank was concentrated onto a .2 μm filter at the initial sampling time and 24 hrs. To ensure chlorophyll was at ground state, samples were put in the dark for 30 minutes prior to PAM analysis. A Junior PAM (Heinz-Walz GmbH,Germany) was used to determine the quantum yield (1:Y (II)) at differing PAR levels.

2.2.6. Statistical Analyses

R Studio (version 0.98.1102) was used to perform statistical analyses. A one-way analysis of variance (ANOVA) assessed if there were any significant differences between PbTx

concentration in water, *Sargassum* tissue, adsorbed PbTx (MeOH) and epiphytes over time. Tukey's honest significant difference (HSD) post hoc test was used to determine if there were significant differences between individual sampling times. A Pearson's correlation was used to determine if a linear relationship was present between ELISA and HPLC-MS/MS analyses. For all tests, a significant difference required $p < 0.05$.

3. Results

Concentrated seawater analyses

TPWD reported *K. brevis* concentrations exceeding 10^6 cells/L^{-1} along the PINS shoreline on September 22, 2015. Samples collected on September 26, 2015 for this research contained *K. brevis* concentrations of 10^3 cells/L^{-1}.

Throughout the sampling period, PbTx was found in all seawater, *Sargassum* tissue, adsorbed on the surface of *Sargassum* (MeOH soluble), and epiphyte samples. PbTx was present in the concentrated seawater and remained between 11 ng ng/mL PbTx-3 equiv. and 13 ng/mL PbTx-3 equiv. over the 72-hr sampling period as determined by ELISA (Figure 4A) (see Appendix – Table 8 for values). The PAM results show a decrease in quantum yield of photosystem II (Y(II)) from 0 to 24 hrs (Figure 5). After 24 hrs, no algal fluorescence was detected in the seawater samples. The dissolved PbTx concentration remained between 11 and 13 ng/mL PbTx-3 equiv. over 72 hrs. No significant differences ($p = 0.0453$) in seawater PbTx concentrations were detected during the 72-hr sampling period (Table 2).

A similar pattern in dissolved PbTx concentration was found by HPLC-MS/MS analyses. PbTx remained between 80 ng ng/mL PbTx-1 equiv. and 93 ng/mL PbTx-1 equiv. over the 72-hr sampling period (Figure 4B) (see Appendix – Table 8 for values). No significant differences ($p = 0.9857$) were seen between PbTx concentration and sampling time (Table 2). Pearson's correlation showed 95% agreement between ELISA and HPLC-MS/MS analyses in seawater.

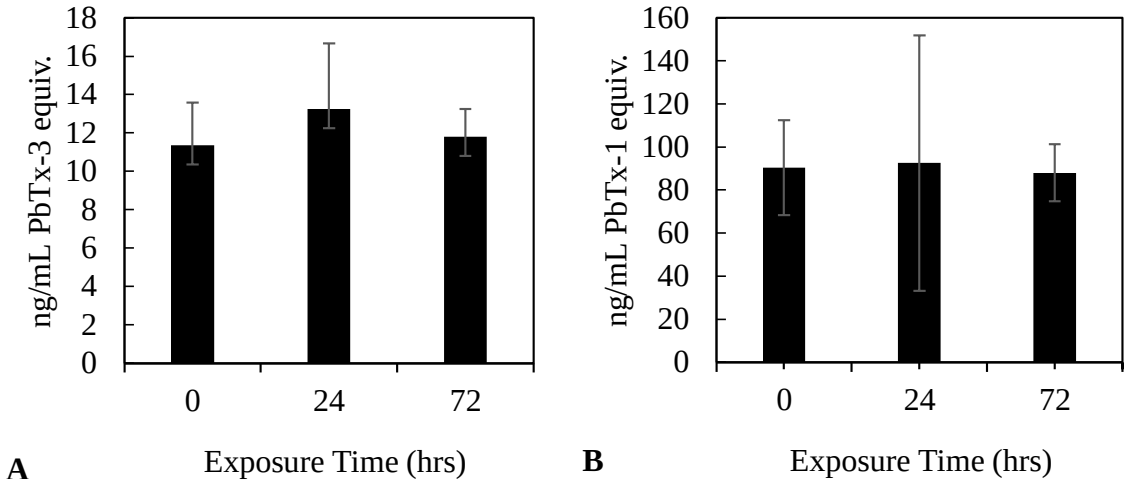

Figure 4. A. Mean PbTx concentration (+/- standard deviation) in seawater (n=4). PbTx concentration were expressed as PbTx-3 equiv. using ELISA analysis. B. Mean PbTx (PbTx-9 as PbTx-1 equiv.) concentration (+/- standard deviation) in seawater determined by HPLC-MS/MS.

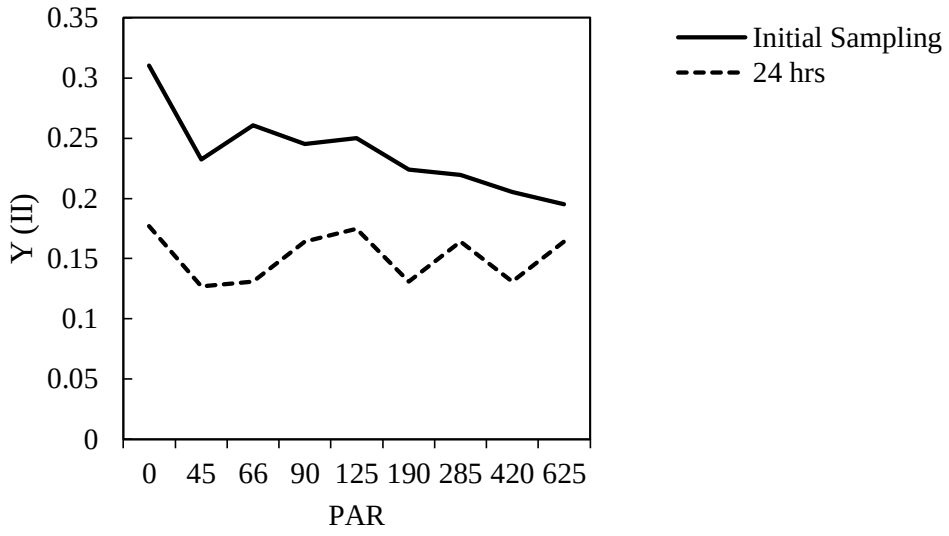

Figure 5. Mean quantum yield of Y(II) in concentrated seawater containing *K. brevis* at the initial sampling time and 24 hrs later (later time periods had no quantum yield response). Y(II) is the quantum yield of photosystem II in response to PAR.

Table 2. Summary of ANOVA results to determine statistical difference in PbTx concentration in seawater and the *Sargassum* community over the 72-hour sampling period. Analyses were performed using R Studio Version 0.98.1102. Significance is indicated at $p < 0.05$ by *, $p < 0.01$ by **, and $p < 0.0001$ by ***.

	ELISA		HPLC-MS/MS			
	PbTx-3 equiv.		PbTx-9 as PbTx-1 equiv.		PbTx-3 as PbTx-1 equiv.	
Sample Fraction	F-value	P-value	F-value	P-value	F-value	P-value
Seawater PbTx concentration	0.5464	0.6599	0.0144	0.9857	1.2997	0.3193
Adsorbed PbTx concentration	27.8170	1.094e-05***	-	-	-	-
Epiphyte PbTx concentration	14.2640	0.0003***	-	-	-	-
Sargassum tissue PbTx concentration	3.2260	0.0452*	7.3965	0.0006***	2.6206	0.0598

Sargassum epiphyte and adsorbed fraction analyses

PbTx was detected in the epiphyte fraction using ELISA analysis (Figure 6A). The PbTx concentration was highest at Time 0 (21 ng/mL PbTx-3 equiv.) and decreased to 3 ng/mL PbTx-

3 equiv. at 24 hrs (see Appendix – Table 8 for values). At 72 hrs, the PbTx concentration increased to 13 ng/mL PbTx-3 equiv. Significant differences ($p = 0.0003$) were identified between sampling time (Table 2) and epiphyte PbTx concentration. Tukey's HSD identified significant differences between Times 0 and 24 ($p = 0.0124$), 0 and 48 ($p = 0.0002$), and 0 and 72 hrs ($p = 0.0451$-see Table 3). Significant differences were also seen between Time 48 and 72 hrs ($p = 0.0210$).

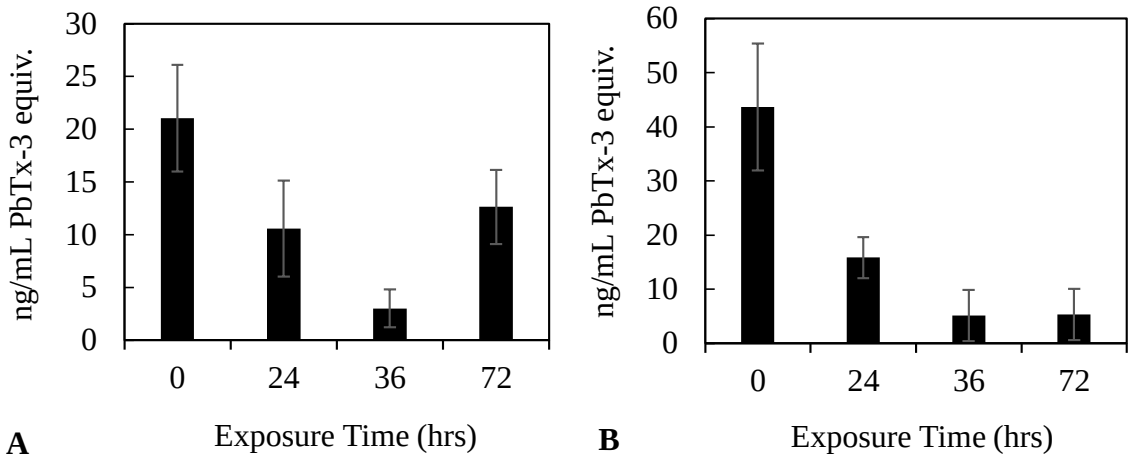

Figure 6. PbTx-3 equiv. determined by ELISA in two *Sargassum* fractions. A. Mean PbTx concentration (+/- standard deviation) in epiphytes removed by the MES rinse (n=4). PbTx-3 equiv. determined by ELISA. B. Mean PbTx concentration (+/- standard deviation) in the adsorbed (superficial) toxin on *Sargassum* spp. (n=4).

Table 3. Results of Tukey's HSD to compare differences between dates and PbTx concentrations from ELISA and HPLC-MS/MS. Significance is indicated at $p < 0.05$ by *, $p < 0.01$ by **, and $p < 0.0001$ by ***.

	ELISA	
	PbTx-3 equiv.	
Sample Fraction	Exposure Time (hrs)	Adj. P-value
Adsorbed PbTx concentration	24-0	0.0005***
	48-0	<0.0000***
	72-0	<0.0000***
Epiphyte PbTx concentration	24-0	0.0124**
	48-0	0.0002***
	72-0	0.0451*
	72-48	0.0210*
Sargassum tissue PbTx concentration	36-0	0.0507*
	HPLC-MS/MS	
	Exposure Time (hrs)	PbTx-9 as PbTx-1 equiv.
Sargassum tissue PbTx concentration	36-0	0.0007***
	36-24	0.0162*
	72-36	0.0008***

Toxin attached superficially to the surface of the *Sargassum* was removed by a MeOH rinse and analyzed by ELISA (Figure 6B). Levels of PbTx decreased from 44 ng/mL Pb-Tx-3 equiv. at Time 0 to 3 ng/mL Pb-Tx-3 equiv. at 36 hrs. From 36 to 72 hrs the PbTx concentration was unchanged (between 3-5 ng/mL Pb-Tx-3 equiv.). Significant differences ($p = 1.094e-05$) between PbTx concentration in the adsorbed PbTx and sampling time were identified (Table 2). Tukey's HSD identified significant differences between Time 0 and 24 ($p = 0.0005$), 48 ($p < 0.0000$), and 72 hrs ($p < 0.0000$) (Table 3).

Sargassum tissue analyses

The mean concentration of PbTx in *Sargassum* tissue was 24.3 ng/g PbTx-3 equiv. at Time 0 (see Appendix – Table 8 for values). The PbTx concentration increased during the study, nearly doubling in the first 24 hrs, then persisted at levels above 50 ng/g PbTx-3 equiv. for the remainder of the study (Figure 7A). Significant differences ($p = 0.0452$) in *Sargassum* tissue PbTx concentration were detected during the 72 hr sampling period (Table 2). Tukey's HSD identified significant differences between Time 0 and 36 hrs ($p = 0.0507$) (Table 3).

PbTx concentrations in *Sargassum* remained above 200 ng/g PbTx-1 equiv. during the study as measured by HPLC-MS/MS (See Appendix – Table 8 for values). An increase in PbTx concentration was seen from Time 0 to 36 hrs followed by decreasing concentrations from 36 to 72 hrs (Figure 7B). Significant differences ($p = 0.0006$) were seen between exposure time and PbTx concentration in *Sargassum* tissue (Table 2). Tukey's HSD identified significant differences between Time 0 and 36 ($p = 0.0007$), 24 and 36 ($p = 0.0161$), and 36 and 72 hrs ($p = 0.0008$) (Table 3).

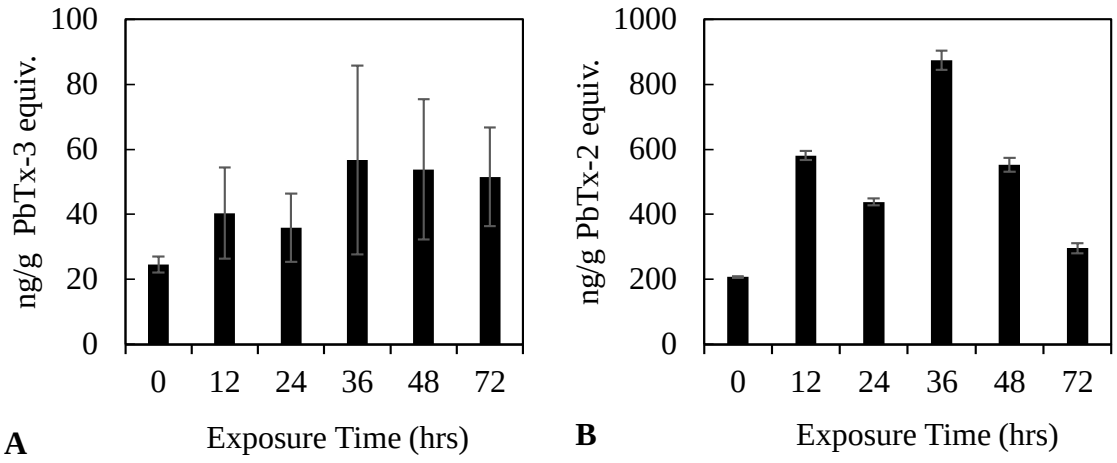

Figure 7. A. Mean PbTx concentration (+/- standard deviation) in *Sargassum* spp. (n=4) determined by ELISA as PbTx-3 equivalents. B. Mean PbTx (PbTx-9 as PbTx-1 equiv.) concentration (+/- standard deviation) in *Sargassum* determined by HPLC-MS/MS.

Total brevetoxin

The total PbTx concentration in *Sargassum* was pooled for *Sargassum* tissue, adsorbed PbTx (MeOH), and epiphytes (Figure 8A) and compared to the seawater PbTx concentration. The total PbTx in the *Sargassum* community was higher at 72 hrs than both Time 0 and the 24 hrs. Higher concentrations (78% PbTx-3 equiv.) of PbTx were found within the *Sargassum* tissue. PbTx was present at low concentrations (8% PbTx-3 equiv.) on the surface of the macroalgae and in epiphytes removed (18% PbTx-3 equiv.).

The results from HPLC-MS/MS identified that PbTX-9 was the most abundant congener present in the samples, accounting for 73% of the total PbTx identified (Figure 8B). PbTx-3 accounted for 27% of the total PbTx identified.

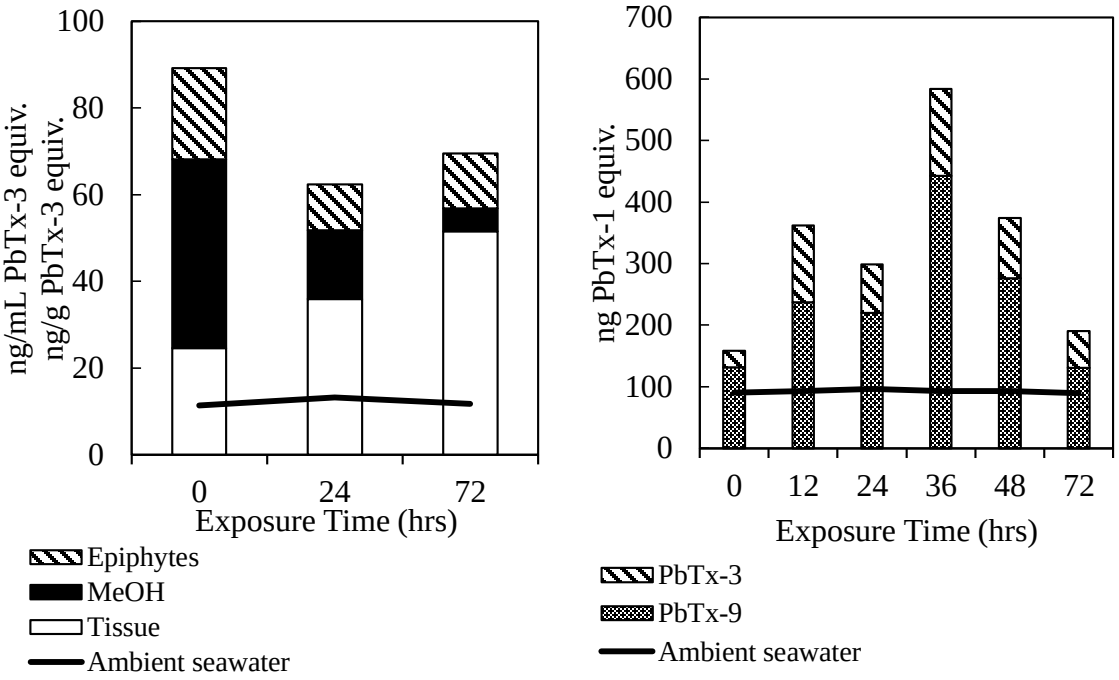

Figure 8. A. Total PbTx concentration associated with *Sargassum* during the 72 hr exposure period. PbTx-3 equiv. concentration determined by ELISA. B. Total PbTx concentration (PbTx-3, PbTx-9 as PbTx-1 equiv.) in *Sargassum* tissue determined using HPLC-MS/MS.

4. Discussion

This is the first study to examine the accumulation of PbTx in macroalgae. Both ELISA and HPLC-MS/MS analyses detected significant increases in PbTx concentration over time (Table 2), demonstrating that *Sargassum* can actively concentrate PbTx within its tissue (Figure 7A and B). A Pearson's correlation showed 95% agreement between ELISA and HPLC-MS/MS analyses in seawater and 65% agreement between tissue samples.

Sargassum tissue accumulated the highest concentration of PbTx followed by epiphytes and the adsorbed fraction. PbTx-9, a metabolite commonly found in shellfish (Plakas et al. 2004), was present at high concentrations in *Sargassum* tissue and seawater, while PbTx-3, found in fish and seagrass (Naar et al. 2007; Flewelling 2008), was present in low concentrations. PbTx persistence in *Sargassum* depends on how long the toxin is retained within tissue, attached to the surface, and retained in epiphytes. The release of toxin by depuration and *Sargassum* turnover time will determine how long PbTx persists in the environment after a bloom subsides. The accumulation and persistence of PbTx in macroalgae tissue could have serious implications for the organisms that utilize it as a resource and for the health of the marine ecosystem.

Previous studies examined PbTx accumulation in vascular marine plants and fish species (Naar et al. 2007; Flewelling 2008; Hitchcock et al. 2012). Flewelling (2008) reported accumulation in three seagrass species and found that PbTx in seagrass leaves and sheaths ranged from 0 to 5,327 ng/g PbTx-3 equiv. Epiphytes had higher PbTx concentrations (9536 ng/g PbTx-3 equiv.) compared to the seagrass blades (Table 4). In comparison, Hitchcock et al. (2012) found a maximum of 18 ng/g PbTx-3 equiv. in seagrass epiphytes. Stomach contents of bottlenose

dolphins (*Tursiops truncatus*), from a mass mortality event in Florida, contained fish species with 181 to 33,185 ng/g^{-1} PbTx-3 equiv. (Flewelling 2008). No *K. brevis* was present, yet the levels of PbTx in fish tissue suggest a bloom had recently occurred or fish consumed a food resource containing accumulated PbTx. The concentrations of PbTx in one gram of *Sargassum* determined by HPLC-MS/MS were similar to fish PbTx levels (see Appendix – Table 8 for values). This suggests that if *Sargassum* was ingested, mortalities could result in higher trophic levels.

Table 4. Comparison of results from studies looking at PbTx accumulation in seagrass, seagrass epiphytes, and sediments as determined by ELISA.

PbTx concentrations found per study		ELISA ng/g PbTx-3 equiv. Avg.	ELISA ng/g PbTx-3 equiv. Max.
This Study	*Sargassum* tissue	42	100
	Sargassum epiphytes	12	26
Flewelling 2008	Seagrass blades/sheaths	-	9536
	Seagrass epiphytes	-	5327
Hitchcock et al. 2012	Seagrass epiphytes	-	18

A sample of concentrated *K. brevis* cells from PINS was preserved for cells counts on September 26, 2015. *K. brevis* was present in very low (> 5,000 cells/L^{-1}) concentrations in the sample, yet higher concentrations (10^6 cells/L^{-1}) were found by TPWD during the same time period. The process of concentrating *K. brevis* cells in the field using netting may have caused cells to lyse, accounting for low cell counts in the sample. In the aquaria, the intensity of aeration and collision with *Sargassum* and the aquaria walls may have caused cells to lyse releasing toxin to the surrounding environment. After uptake from Time 0 to 36 hours, PbTx in tissue decreased from 36 to 72 hrs possibly in response to declining *K. brevis* health/cell lysis (Figure 4). The

decrease in photosynthetic yield to zero at Time 36 hrs suggests most *K. brevis* died during the first day of the experiment. A decrease in PbTx concentration occurred in epiphytes and adsorbed fractions during the 72 hr experiment. This could be due to lack of nutrients to sustain epiphytes, depuration by epiphytes, or increased absorption of PbTx in *Sargassum* tissue. Potential mechanisms for PbTx uptake are active absorption, passive adsorption through the surface, passive adsorption by epiphytic microalgae, and passive and active absorption by microfauna attached to the surface layer (Flewelling 2008). Understanding the mechanisms of PbTx bioaccumulation is crucial to understanding the fate of *Sargassum*-associated organisms.

A collection made on May 9, *Sargassum* from Port Aransas, TX, showed low concentrations (3 ng/g PbTx-3 equiv.) present in tissue. At the time, no *K. brevis* was present, so the *Sargassum* was transported from an area that had experienced a bloom. One million tons of *Sargassum* occurs in the Gulf of Mexico annually, therefore regions experiencing frequent *K. brevis* blooms (i.e. Florida) could advect PbTx as pelagic mats or beach wrack (Gower et al. 2012). In this study, PbTx concentrations exceeding 500 ng/g PbTx-3 equiv. were measured in one *Sargassum* plant. Theoretically, if all *Sargassum* accumulated similar levels of PbTx, the Gulf of Mexico would contain 22×10^{10} g PbTx-3 equiv. of toxin! Endemic organisms such as the *Sargassum* fish, *Histrio histrio,* could face direct impacts from PbTx exposure. *Sargassum* could contribute to mortalities on beaches through contact, ingestion, or transfer of toxin to sediments. Mortality events of migratory shorebirds, domestic dogs, tree frogs, coyotes, squirrels, and raccoons have been documented (Rafalski 2012).

Persistence of PbTx, associated with macroalgal communities, could adversely affect aquatic organisms. Flewelling (2008) found that PbTx concentrations in seagrass persisted at levels

exceeding 100 ng/g PbTx-3 equiv. for one to two months following a bloom event. This time period corresponds to the seagrass blade turnover time of 60 days for *T. testudinum* (Flewelling 2008). In comparison, *Sargassum* can live for up to one year in the pelagic marine environment and could serve as a toxin vector.

It is predicted that there will be an increase in the appearance and intensity of HABs resulting from anthropogenic nutrient loading and global climate change (Hallegraef 1993; Van Dolah 2000; Anderson 2002; Paerl and Huisman 2009). This study has demonstrated that *Sargassum* actively accumulated PbTx in tissue and was present as adsorbed and epiphytic fractions during a *K. brevis* bloom. *Sargassum* transport has the potential to impact communities in the Gulf of Mexico yielding the need for further research on vectors of biological toxins. In future studies on marine toxin accumulation, other macroalgae species (i.e. “red”, “green” plant lines) should be considered. Studies on the transfer of toxins to different communities (i.e. release of PbTx to beaches as *Sargassum* senesces) could provide understanding of post-bloom effects. As harmful algal blooms intensify, increased levels of toxin could be transferred to marine communities.

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II. Brevetoxin depuration in *Ulva intestinalis* (Linnaeus) Nees from Corpus Christi Bay, TX

1. Introduction

Brevetoxin metabolism and depuration

Metabolism and depuration of brevetoxin (PbTx) have typically been examined in filter-feeding bivalves such as the oyster, *Crassostrea virginica*, and the clam, *Mercenaria mercenaria* (Plakas et al. 2004). The ingestion of *K. brevis* cells or dissolved PbTx leads to accumulation of toxin within tissue until these animals can depurate in a clean water source. Species-specific metabolism of PbTx have been reported in bivalves (PbTx-9) (Plakas et al. 2004; Flewelling 2008). These filter feeding organisms have the ability to depurate toxin actively from their tissue, but depuration can take up to eight months (Plakas et al. 2004). Little to no mortality events of bivalves have occurred by exposure to PbTx (Plakas et al. 2004). Biomagnification of toxin could occur in higher trophic levels from the consumption of bivalves that have bio-accumulated PbTx (Tester et al. 2000). PbTx transfer from bivalves to fish has been documented with low levels persisting in fish livers for up to a year (Naar et al. 2007; Bricelj et al. 2012). Top predator mortalities, including the bottle nose dolphin (*Tursiops truncatus*), have occurred from the ingestion of fish with high concentrations of PbTx by trophic transfer of toxin (Flewelling 2008). The persistence of PbTx in tissue could pose a threat to consumers after a *K. brevis* bloom has senesced (Plakas et al. 2004).

Toxin accumulation and persistence in vascular plants

The accumulation and persistence of PbTx have only been studied in vascular marine plants. In Florida, seagrass communities were shown to retain high levels of PbTx for weeks and low levels for months (Flewelling 2008, Hitchcock et al. 2012). Post-bloom concentrations of PbTx

have been found at 10s of ng (g dry wt)$^{-1}$ in seagrass epiphytes and 1 to 10 ng (g dry wt)$^{-1}$ in seagrass bed sediments when *K. brevis* is not present (Flewelling 2008). Microcystin, a cyanobacterial toxin, has accumulated in freshwater vascular plants, such as *Ceratophyllum demersum* and *Elodea canadensis* (Pflugmacher et al. 1999; Pflugmacher et al. 2001). Studies on the persistence of toxins in marine macroalgae have not been published.

Ulva intestinalis (Linnaeus) Nees

Ulva intestinalis (Linnaeus) Nees is a cosmopolitan green tide species typically found in marine intertidal communities. Intertidal invertebrates such as amphipods, larval crabs and bivalves use *U. intestinalis* as a refuge and food source (Sotka et al. 2009). *U. intestinalis* can be found in colonies up to 20 cm in length (Bold and Wynne 1985) that attach to surfaces by a holdfast (Ruangchuay et al. 2012). The laminar structure and high surface area of this species allows for increased exposure to the environment. Previous studies have used this species as an indicator of environmental health. Some studies have examined the presence of heavy metals in *U. intestinalis* tissue while others have used this species as an indicator of eutrophication (Teichberg et al. 2010, Villares et al. 2001).

Objective

The objective of this study was to determine the persistence and subsequent depuration rate of PbTx in *U. intestinalis* over a 30-day period. This was the first study to examine PbTx persistence in macroalgae. If PbTx is stored in this macroalgae, *U. intestinalis* may serve as a vector for PbTx transport to higher trophic levels leading to widespread mortality events. As studies have examined PbTx in shellfish, seagrass, sediments, and vascular plants (Pflugmacher

et al. 1998; Plakas et al. 2004; Flewelling, 2008; Hitchcock et al. 2012), there is a need for studies assessing toxin accumulation in non-vascular marine plants.

2. Methods

2.1. Experimental Design

On September 18, 2015, background concentrations (<1000 cells/L^{-1}) of *K. brevis* were reported in Corpus Christi Bay, TX by Texas Parks and Wildlife (TPWD). By November 2, 2015, high concentrations (>10^6 cell/L^{-1}) along the southeast side of the bay were reported (Figure 9). A survey of Corpus Christi Bay macroalgae was performed on November 5, 2015 to identify sampling locations and taxa that had accumulated PbTx. *U. intestinalis* was selected because the species was abundant and contained PbTx based on preliminary analysis. The sampling site at Doddridge Park, TX (Figure 9) was chosen because expansive *U. intestinalis* mats were present and for the ease of access. Three rocks (no greater than 15 cm) covered in the algal mat were collected on November 6, 2015. Replicate five-gallon aquarium tanks (n=3) were filled with 11 liters of f/10 media prepared with aged (>6 months), filtered (0.2 µm) seawater. F/10 media was used to provide *U. intestinalis* with nutrients over the 30-day period. Every seven days the media was replaced with 11 liters of new f/10. Air bubblers, lighting (12:12 L/D; 350 µmol photons m^{-2} s^{-1} PAR), and lids were added to each of the tanks at setup.

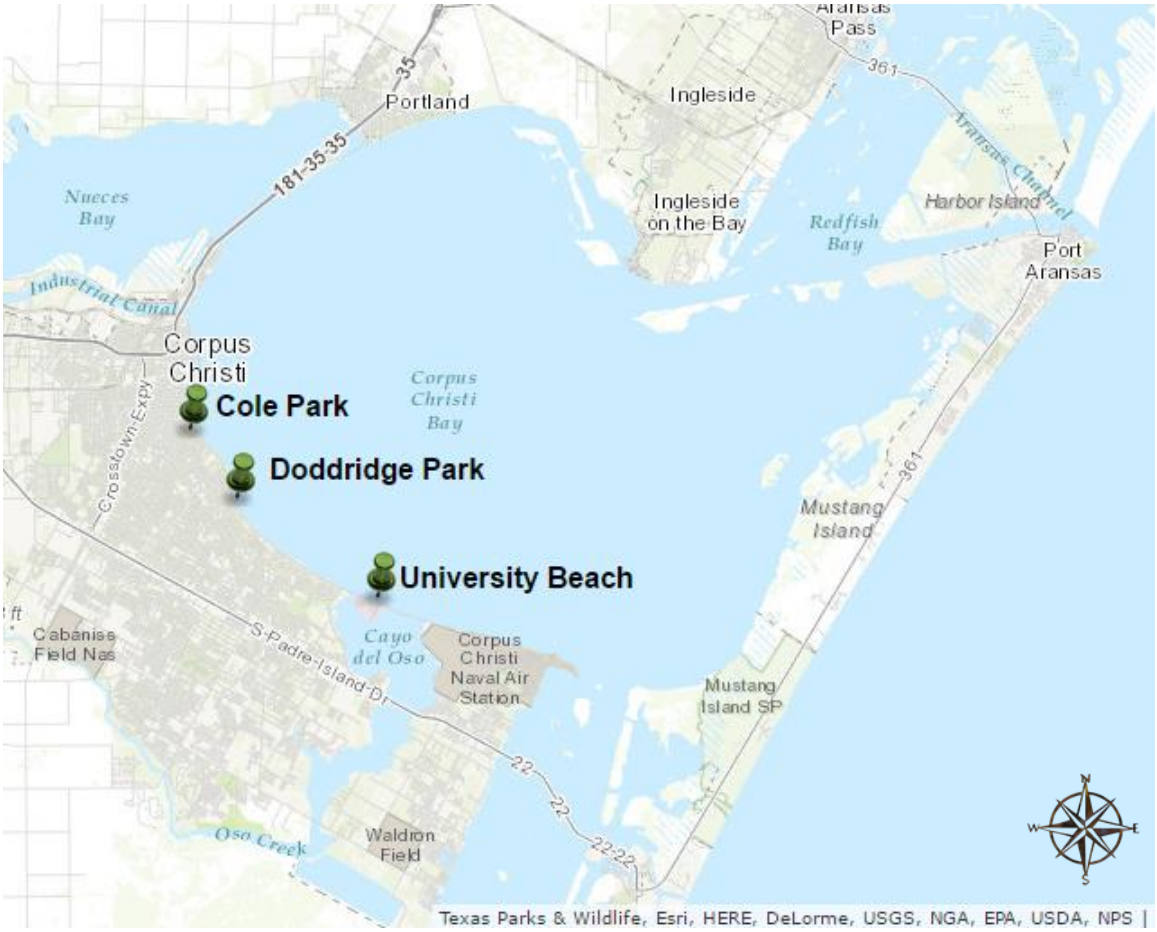

Figure 9. Sampling locations along Corpus Christi Bay, TX where macroalgae were collected for a preliminary survey at Cole Park, Doddridge Park, and University Beach on November 5, 2015. *U. intestinalis* for the experiment was collected at Doddridge Park on November 6, 2015.

2.1.1. Removal of surface bound PbTx and epiphytes from U. intestinalis

Brevetoxin can be found in epiphytes and attached to surfaces (Flewelling 2008). The *U. intestinalis* community was partitioned using a combined mechanical and chemical approach. One gram of *U. intestinalis* was collected from each replicate tank for ELISA analysis on days 0, 3, 6, 9, 12, 16, 20, and 30 to determine PbTx concentration in the fractionated *Ulva* community. *U. intestinalis* was processed by first removing epiphytes using 2-(N-morpholino)ethanesulfonic acid (MES) buffer. *U. intestinalis* was added to microfuge tubes with 1 mL of MES and shaken

for 60 seconds to release epiphytes. *U. intestinalis* was then added to microfuge tubes with 1 mL of methanol (MeOH) and shaken to remove adsorbed toxin. Each of the resulting slurries were stored in microfuge tubes at -80°C prior to analysis. *U. intestinalis* was frozen at -80°C until PbTx extraction from tissue.

2.1.2. Toxin extraction from U. intestinalis tissue

Each sample of *U. intestinalis* tissue was homogenized using a mortar and pestle. The 1 g sample was extracted using 1 mL of MeOH in a 2 mL microfuge tube. The tube was vortexed for 30 seconds, sonicated for 20 seconds, then centrifuged at 3000 x for 10 minutes. The supernatant was removed and frozen at -80ºC until ELISA analyses.

2.1.3. Toxin evaluation by ELISA

The Abraxis PbTx competitive ELISA assay (Abraxis Corporation, Warminster, PA) was used to quantify the total PbTx concentration (as PbTx-3 equivalents) in the three *U. intestinalis* fractions. ELISA measures the biological activity of PbTx, as the standards and samples compete for binding sites within the wells of the microtiter plate that are coated with sheep anti-PbTx antibodies. The colorimetric change, determined by a Biotek Synergy HT Multi-Mode Microplate Reader (BioTek Instruments, Inc., Winooski, VT), is inversely proportional to PbTx concentration. A PbTx-3 standard curve was run during each sample set to determine the concentration of PbTx present (Figure 17). Samples were diluted if the PbTx exceeded the range of standards. The lower detection limit for PbTx in water samples was 0.05 ng/mL and 22.5 ng/mL in dried, diluted shellfish samples (Abraxis Corporation, Warminster, PA).

2.1.4 Statistical Analyses

A one-way analysis of variance (ANOVA) was performed to determine if statistically different concentrations of PbTx were present in the *U. intestinalis* fractions over the thirty-day incubation period. R Studio (version 0.98.1102) was used to perform the ANOVA. A post hoc Tukey's honest significant difference (HSD) analysis was performed to assess the significant differences between sampling times if significant differences were identified in the ANOVA analyses.

3. Results

Preliminary survey of Corpus Christi Bay macroalgae on November 5, 2016

Four Rhodophyte species and two Chlorophyte species were collected on November 5, 2015 from Corpus Christi Bay, TX (Figure 9). The preliminary survey (Figure 10) found the highest PbTx concentration of 66.42 ng/g PbTx-3 equiv. present in the Rhodophyte *Gelidiella* sp. The two *Gracilaria* spp. had similar PbTx concentrations (11.00 ng/g PbTx-3 equiv.), while *Hypnea musciformes* had the lowest PbTx concentration present of the Rhodophytes. The Chlorophyte, *Cladophora vagabunda*, had the second highest PbTx concentration of 20.97 ng/g PbTx-3 equiv. While *U. intestinalis* had the second lowest concentration of PbTx present, it was in high abundance and widely distributed at each of the sampling sites and was used in the depuration experiment.

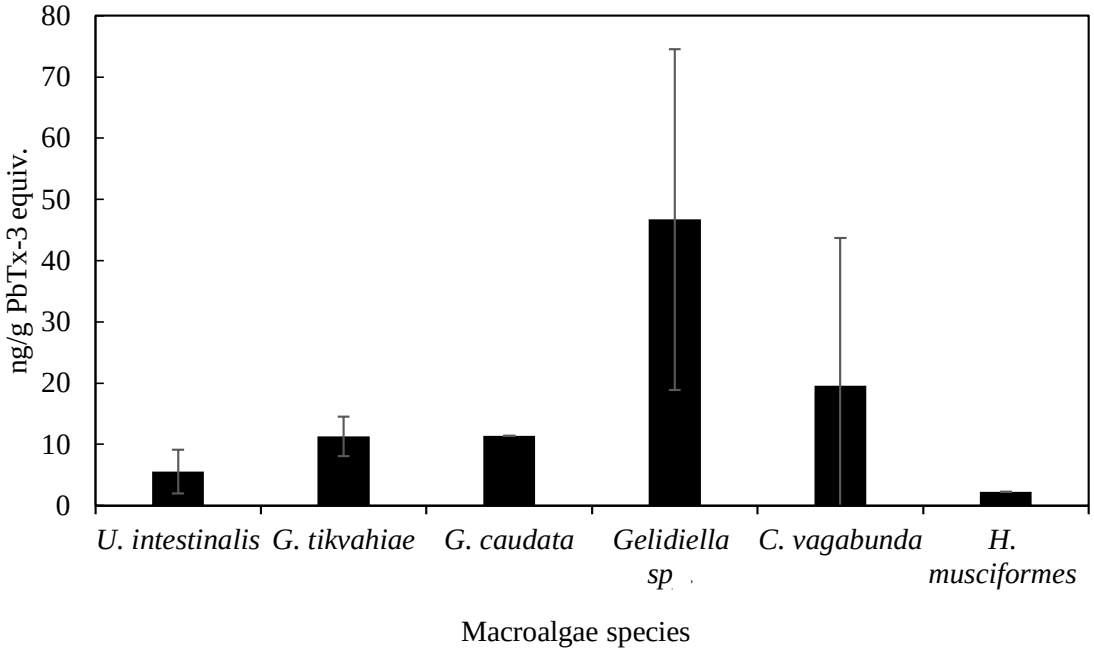

Figure 10. Results from the preliminary survey of Corpus Christi Bay macroalgae collected on November 5, 2015 from Doddridge Park, Cole Park, and University Beach. PbTx concentration was determined by ELISA and expressed as PbTx-3 equiv.

Brevetoxin concentration in U. intestinalis epiphytes, adsorbed fraction, and tissue

PbTx was found in all *U. intestinalis* epiphytes, adsorbed on the surface of *U. intestinalis* (MeOH soluble), and within algal tissue samples. In *U. intestinalis* epiphytes, PbTx remained between 16.66 ng/mL PbTx-3 equiv. and 38.61 ng/mL PbTx-3 equiv. for the thirty-day experiment (Figure 11). The maximum PbTx concentration found in all epiphyte samples was 53.13 ng/mL PbTx-3 equiv. on day six (See Appendix – Table 11 for values). No significant differences ($p = 0.2053$) in epiphyte PbTx concentrations were found over the sampling period (Table 5).

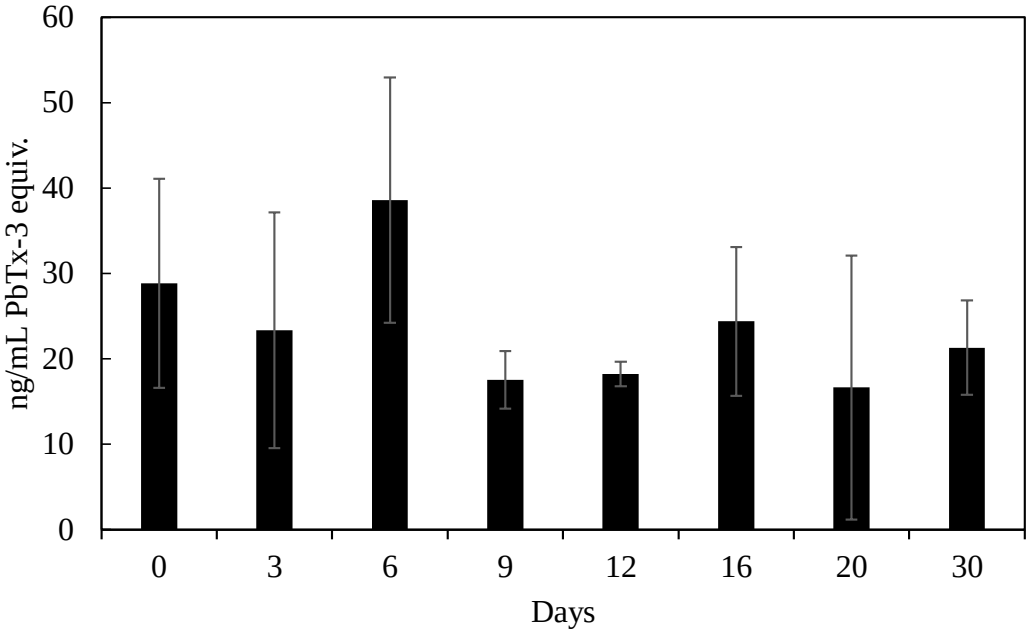

Figure 11. Mean PbTx concentration (+/- standard deviation) in *U. intestinalis* epiphytes over the thirty-day depuration period. PbTx concentration determined using ELISA.

Table 5. Summary of ANOVA to determine statistical differences in *U. intestinalis* PbTx concentration over the thirty-day sampling period. Analyses were performed using R Studio Version 0.98.1102. Significance is indicated at $p < 0.05$ by *.

	ELISA	
	PbTx-3 equiv.	
	F value	P value
Adsorbed PbTx concentration	0.5164	0.8093
Epiphyte PbTx concentration	1.7040	0.2053
U. intestinalis tissue PbTx concentration	7.3639	0.0130*

Lower concentrations of PbTx were found adsorbed to the surface of *U. intestinalis* compared to epiphytes. The PbTx concentration remained between 5.57 and 9.71 ng/mL PbTx-3 equiv. (Figure 12) over the thirty-day sampling period. The maximum PbTx concentration (17.31 ng/mL PbTx-3 equiv.) in the adsorbed fraction was measured on day zero (See Appendix – Table 11 for values). No significant differences ($p = 0.8093$) in the adsorbed PbTx concentration were

found over the experiment (Table 5).

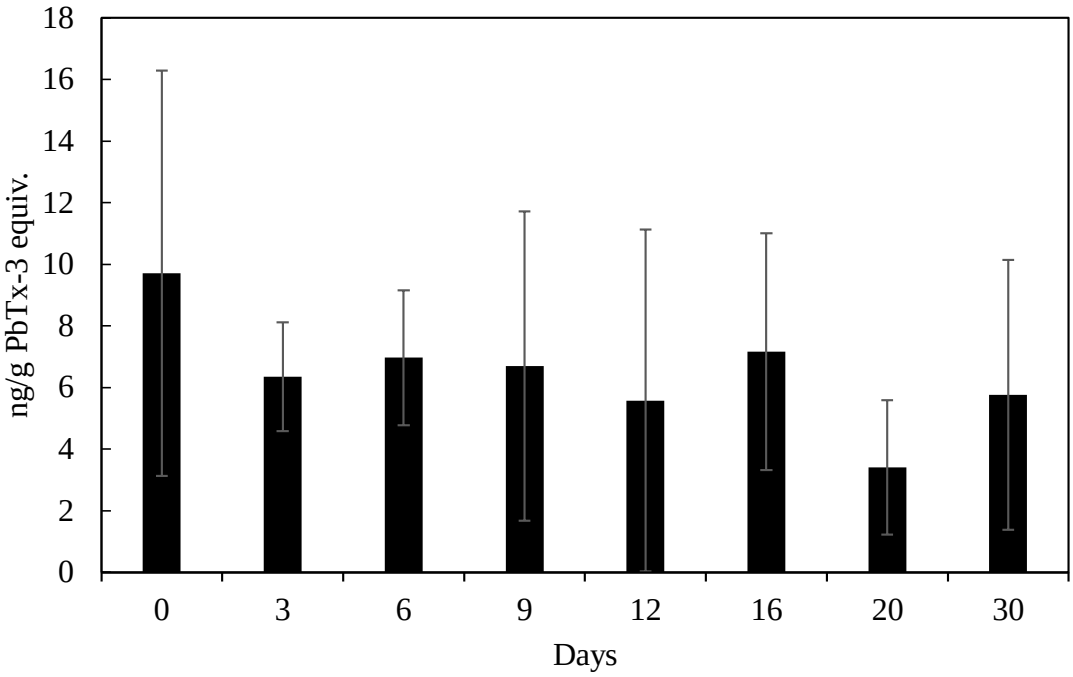

Figure 12. Mean PbTx concentration (+/- standard deviation) adsorbed to *U. intestinalis* during the thirty-day depuration period. PbTx concentration determined using ELISA.

Tissue concentrations of PbTx were higher than in the adsorbed fraction and epiphytes (Figure 13). Concentrations of PbTx increased on days three and six relative to initial concentrations. The maximum concentration of 419.11 ng/g PbTx-3 equiv. occurred on day six. After day six, the PbTx concentration decreased until day twelve where the PbTx concentration remained between 110.30 and 131.58 ng/g PbTx-3 equiv. for the remainder of the study (See Appendix – Table 11 for values). Significant differences ($p = 0.0074$) were seen between sampling time and PbTx concentration in *U. intestinalis* tissue (Table 5). Tukey's HSD identified significant differences between days six and zero ($p = 0.0076$), day six and day twelve (0.0372), day six and day sixteen ($p = 0.0390$), and day six and day thirty ($p = 0.0425$) (Table 6).

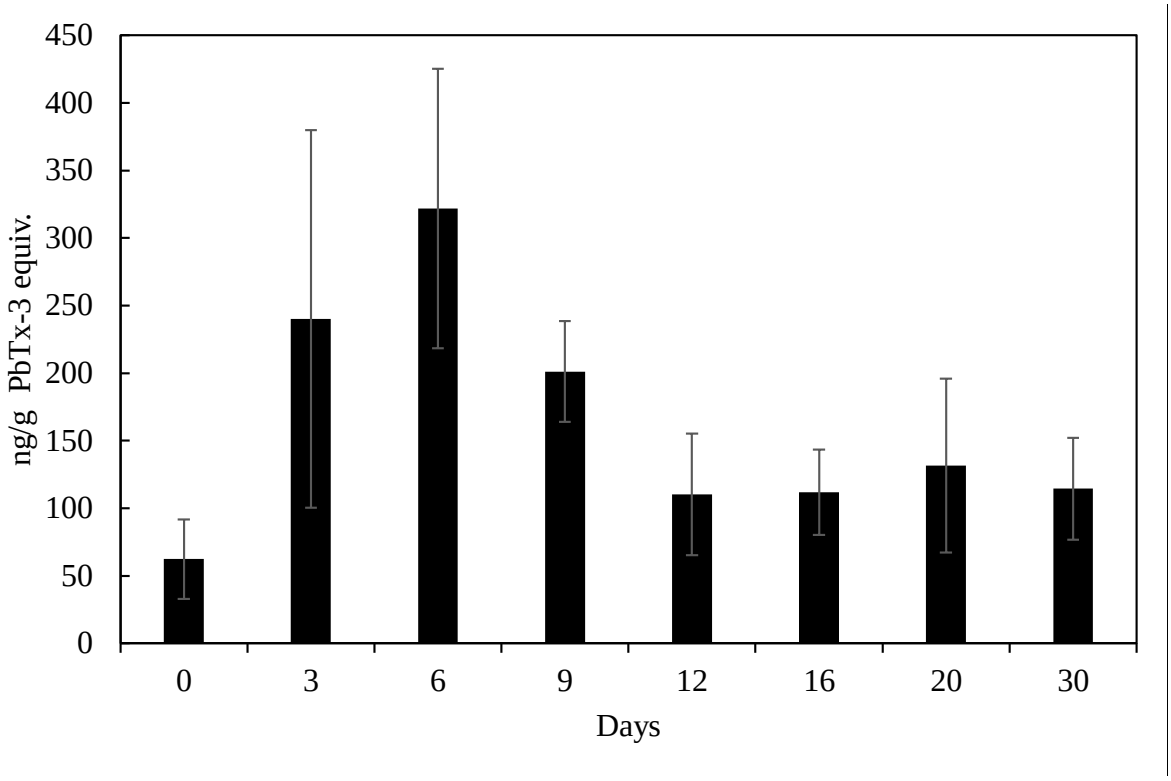

Figure 13. Mean PbTx concentration (+/- standard deviation) in *U. intestinalis* tissue during the thirty-day depuration period. PbTx concentration determined by ELISA.

Table 6. Results of Tukey's HSD analysis of ELISA data to compare differences between specific dates and PbTx concentrations in *U. intestinalis*. Significance is indicated at $p < 0.05$ by * and $p < 0.01$ by **.

	ELISA	
	PbTx-3 equiv.	
	Day	Adj. P-value
U. intestinalis tissue PbTx concentration	6 - 0	0.0076**
	6 - 12	0.0372*
	6 - 16	0.0390*
	6 - 30	0.0425*

The total PbTx consisting of epiphyte, adsorbed, and tissue PbTx was summed. The average concentration of PbTx for the 30-day experiment was 191.77 ng PbTx-3 equiv. Total PbTx increased from 100.85 ng PbTx-3 equiv. on day zero to 367.47 ng PbTx-3 equiv. on day six (Figure 14). After day six, a decrease in PbTx occurred until day twelve. The PbTx remained

between 134.12 and 151.64 ng/g PbTx-3 equiv. from day twelve until day thirty. The highest PbTx was present in *U. intestinalis* tissue (62%) while 28% was found in epiphytes, and 10% in the adsorbed fraction.

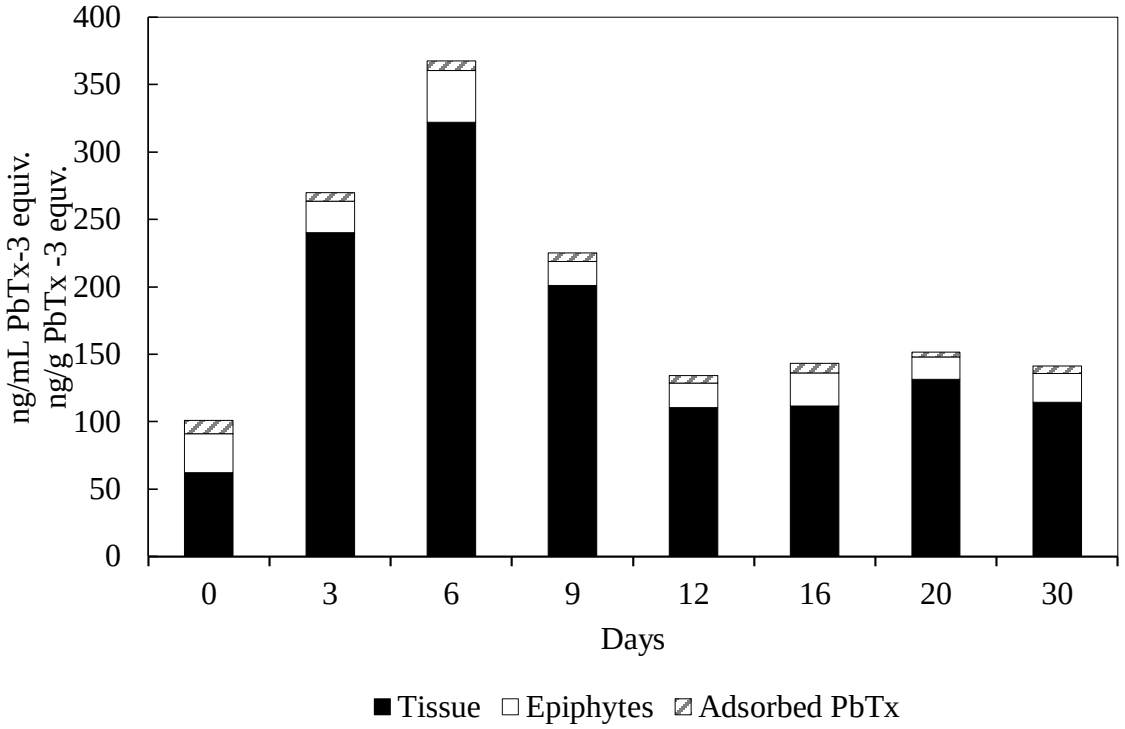

Figure 14. Total PbTx concentration in *U. intestinalis* tissue, epiphytes, and adsorbed fractions. PbTx-3 equiv. concentration determined by ELISA.

4. Discussion

This is the first study to examine persistence and depuration of PbTx in macroalgae. Four genera of red and green algae contained PbTx (Figure 10), demonstrating accumulation of PbTx in multiple plant lines. The *U. intestinalis* community of epiphytes, the adsorbed fraction, and tissue all contained PbTx with the highest concentration in tissue (Table 11). The toxin concentration in *U. intestinalis* tissue remained above 110.3 ng/g PbTx-3 equiv. and persisted for

at least thirty days without depuration.

Previous studies on toxin persistence have focused on seagrasses, sediments (Flewelling 2008; Hitchcock et al. 2012), freshwater vascular plants (Pflugmacher et al. 1999), and filter-feeding bivalves (Plakas et al. 2002; Plakas and Dickey 2010). Marine vascular plants (i.e. seagrasses) retained PbTx concentrations greater than 100 ng/g PbTx-3 equiv. in roots, rhizomes, and blades for weeks post-bloom, while lower levels persisted for one to two months (Flewelling 2008) (Table 7). Seagrass epiphytes retained PbTx concentrations greater than 100 ng/g PbTx-3 equiv. for one to two months (Flewelling 2008), while lower mean concentrations of 25 ng/g PbTx-3 equiv. were found to persist for up to eight months in both epiphytes and sediments (Hitchcock et al 2012). This study found similar epiphyte PbTx concentrations to Hitchcock et al. (2012), as *U. intestinalis* epiphytes retained a mean concentration of 24 ng/mL PbTx-3 equiv. over thirty days (Table 7). There is the potential for lower concentrations of PbTx to persist in *U. intestinalis* tissue and epiphytes for months post-bloom.

Algal toxins, such as the cyanobacterial toxin, microcystin, were found in freshwater vascular plants (i.e. *Ceratophyllum demersum*) (Pflugmacher et al. 1999). Uptake and subsequent metabolism of this toxin occurred in different regions of the plant (i.e. roots vs. leaves), affected translocation, and caused mortalities (Pflugmacher et al. 1999). In contrast, shellfish and amphipods were unaffected by PbTx -- the speed of detoxification depended on their ability to depurate toxin (Plakas et al. 1999; Sotka et al. 2009). Bivalves, such as the eastern oyster, *Crassostrea virginica*, and the northern quahog, *Mercenaria mercenaria*, that had actively accumulated PbTx, depurated large amounts of toxin for two weeks post exposure (Plakas et al.

2004; Griffith et al. 2013). These species retained lower concentrations in tissue for months post-bloom with little to no mortality events (Plakas, et al. 2004; Griffith et al. 2013).

Table 7. Comparison of *U. intestinalis* PbTx persistence compared to previous studies assessing PbTx in seagrass, sediments, and shellfish. All studies used ELISA kits for toxin assessment.

PbTx persistence period per study		ELISA	
		Avg. ng PbTx-3 equiv.	Time period
This Study	*U. intestinalis* tissue	162	>30 days
	U. intestinalis adsorbed PbTx	7	-
	U. intestinalis epiphytes	24	>30 days
Flewelling 2008	Seagrass blades/sheaths	> 100	1 -2 months
	Seagrass epiphytes	> 100	1 - months
Hitchcock et al. 2012	Seagrass epiphytes	11	≤ 8 months
	Sediment	25	≤ 8 months
Plakas and Dickey 2010	Eastern oyster - *Crassostrea virginica*	-	≤ 6 months

Sotka et al. (2009) used *U. intestinalis* coated with PbTx-2 and -3 extracts as a food source for the amphipod *Ampithoe longimana*. This amphipod was not deterred from eating the coated *U. intestinalis*, nor was survivorship or growth impacted. While many grazers prefer *Ulva* spp. as a food source (Hurd et al. 2014), some may prefer other macroalgal species such as the green species *Cladophora vagabunda* (Jimenez et al. 1996) or red algae *Gelidiella* over *Ulva*, resulting in higher PbTx exposure. There is the potential for biomagnification of toxin in organisms ingesting invertebrates if benthic herbivores readily feed on foods containing PbTx (Sotka et al. 2012).

In the intertidal zone, differences in *U. intestinalis* exposure to PbTx could be caused by wave action, tides, water column patchiness, or differing accumulation of toxin because of age/exposure time. Differing exposure could have led to non-uniformity of PbTx within the *U. intestinalis* mat. Future studies should collect one cm^3 of *U. intestinalis* at three different areas on the rock (n=3) to normalize the PbTx concentration versus single sample collections that were replicated by tank. *U. intestinalis* grows from an apical meristem with newest growth occurring closest to the substrate; the plant could be tested for differences in PbTx concentration in old versus new growth. Field sampling of attached macroalgal communities could also provide information on toxin persistence in the intertidal community after a bloom of *K. brevis*. Measuring the PbTx concentration (post-bloom) within a designated area of attached macroalgae (> thirty days) could elucidate how long PbTx persists in a natural environment.

Due to the hydrophobic nature of PbTx, these molecules tend to adsorb to surfaces, suspended particles, and sediment (Pierce et al. 2008). PbTx may have been adsorbed to the rock substrate where the *U. intestinalis* mats were attached. The increase in *U. intestinalis* tissue concentrations of PbTx from day zero to day six (Figure 13) could have resulted from the release of PbTx by the substrate and subsequent absorption by the macroalgae, as no *K. brevis* was present. Evaluation of toxin content of macroalgal substrates should be tested in future work.

Although the PbTx concentration did not decrease over thirty days in the *U. intestinalis* community, a longer experiment with improved methods could track depuration in future work. To increase depuration, volumes of seawater greater than 11 liters should be implemented. If frequent water changes are not considered, a PbTx removal method such as clay flocculation, or

a recirculating system equipped with a bio-filter, could be used to trap dissolved PbTx (Pierce et al. 2004). As toxin is being released from the macroalgae to the seawater, concentrating the seawater and measuring the dissolved PbTx could confirm how much toxin is being released into the aquaria.

In summary, PbTx accumulated in both Rhodophyte and Chlorophyte macroalgae species. Persistence of PbTx was confirmed in *U. intestinalis* epiphytes, the adsorbed fraction, and tissue for at least thirty days after a *K. brevis* bloom. Depuration did not occur in this species and should be evaluated in future studies.

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APPENDIX

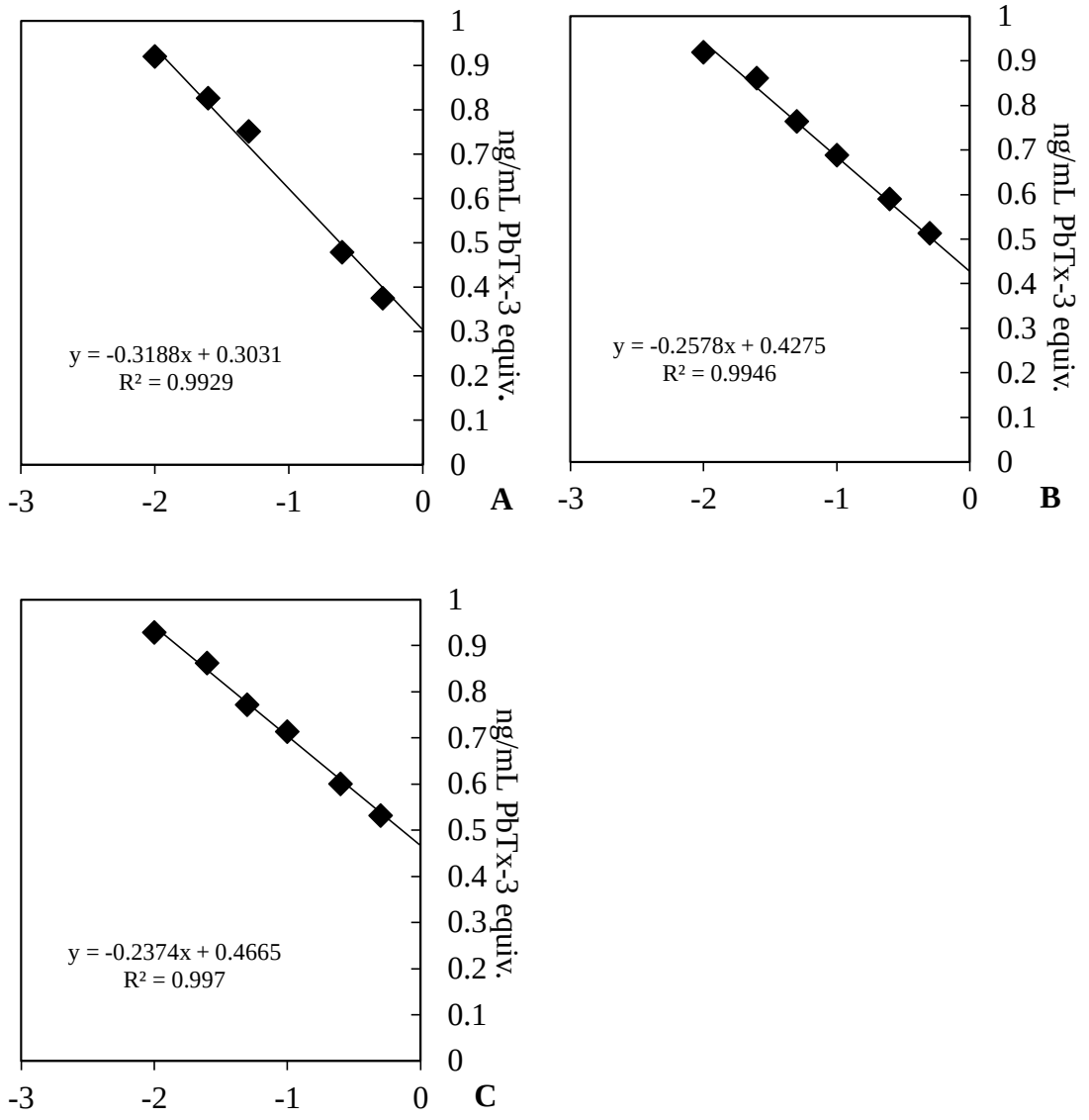

Figure 15. Standard curves used to quantify ELISA analysis results in. A. Seawater samples B. MeOH and MES samples. C. *Sargassum* tissue samples.

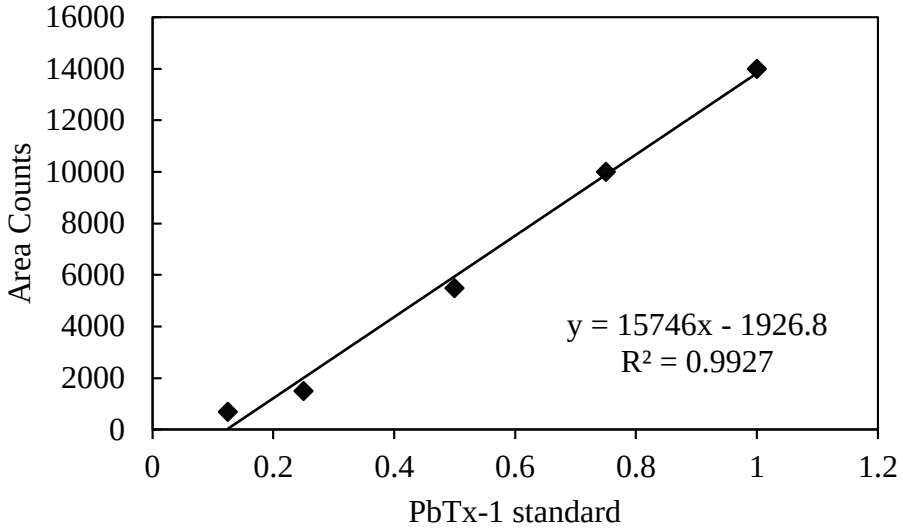

Figure 16. HPLC-MS/MS standard curve determined using a PbTx-1 standard.

Table 8. Summary of results from ELISA and HPLC-MS/MS analyses of *Sargassum*. The *Sargassum* community was fractionated to include adsorbed PbTx, epiphyte PbTx, and tissue PbTx. Average, standard deviation, maximum and minimum concentrations of PbTx are shown.

		ELISA				HPLC-MS/MS							
		PbTx-3 equiv.				PbTx-9 as PbTx-1 equiv.				PbTx-3 as PbTx-1 equiv.			
		Avg.	Stdev.	Max.	Min.	Avg.	Stdev.	Max.	Min.	Avg.	Stdev.	Max.	Min.
	hr	ng/mL				ng/mL				ng/mL			
PbTx seawater concentration	0	11.35	2.23	13.37	8.63	90.42	21.96	119.44	67.14	0.00	0.00	0.00	0.00
	24	13.24	3.42	17.74	9.41	92.51	59.42	153.52	15.21	3.54	4.11	7.61	0.00
	72	11.81	1.43	12.94	9.95	88.03	13.30	101.13	69.48	1.74	3.47	6.95	0.00
Sargassum tissue PbTx concentration	0	24.57	2.47	27.21	21.25	207.62	105.64	339.88	81.48	0.00	0.00	0.00	0.00
	12	40.37	14.03	59.17	28.19	403.78	181.09	612.06	223.35	177.09	147.39	392.80	61.92
	24	35.93	10.54	43.42	20.31	360.93	204.59	474.96	149.68	77.44	48.66	125.01	56.48
	36	56.72	28.96	99.85	37.54	737.84	217.80	934.22	530.00	136.11	44.67	195.23	93.17
	48	53.80	21.56	70.08	22.27	455.18	90.94	535.94	325.40	97.88	73.68	176.22	0.00
	72	51.55	15.20	68.41	31.70	218.44	39.98	273.29	177.77	77.41	54.22	115.25	0.00
Sargassum epiphyte PbTx concentration	0	21.05	5.04	26.00	15.92								
	24	10.57	4.57	16.72	5.69								
	36	5.11	4.73	9.65	0.66								
	72	12.63	3.50	16.15	8.51								
PbTx adsorbed on *Sargassum* surface	0	43.61	11.73	50.87	26.13								
	24	15.85	3.77	20.93	11.81								
	36	3.00	1.81	5.16	0.79								
	72	5.30	4.73	10.16	0.55								

Table 9. Results of Tukey's HSD to compare differences between dates and PbTx concentrations in *Sargassum* analyzed using ELISA. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

		ELISA			
		PbTx-3 equiv.			
	Exposure Time (hr)	Difference	Lower	Upper	Adj. P-value
Ambient seawater PbTx conc.	24-0	1.90	-3.04	6.83	0.55
	72-0	0.47	-4.47	5.40	0.96
	72-24	-1.43	-6.37	3.50	0.70
Adsorbed PbTx conc.	24-0	-27.77	-42.21	-13.33	0.0005***
	48-0	-38.50	-52.94	-24.06	0.0000***
	72-0	-38.32	-52.75	-23.88	0.0000***
	48-24	-10.73	-25.17	3.71	0.17
	72-24	-10.55	-24.98	3.89	0.18
	72-48	0.19	-14.25	14.63	1.00
Epiphyte PbTx conc.	24-0	-10.47	-18.72	-2.22	0.0124**
	48-0	-18.05	-26.29	-9.80	0.0002***
	72-0	-8.41	-16.66	-0.17	0.0451*
	48-24	-7.58	-15.82	0.67	0.07
	72-24	2.06	-6.19	10.30	0.87
	72-48	9.63	1.39	17.88	0.0210*
Sargassum tissue PbTx conc.	24-0	11.36	-17.94	40.66	0.74
	36-0	29.23	-0.07	0.75	0.0507*
	48-0	17.77	-13.87	49.42	0.43
	72-0	26.98	-2.32	56.27	0.07
	36-24	17.87	-11.43	47.17	0.36
	48-24	6.41	-25.23	38.06	0.96
	72-24	15.62	-13.68	44.91	0.48
	48-36	-11.46	-43.10	20.19	0.78
	72-36	-2.26	-31.55	27.04	0.99
	72-48	9.20	-22.44	40.85	0.88

Table 10. Results of Tukey's HSD to compare differences between specific dates and PbTx concentrations in *Sargassum* analyzed using HPLC-MS/MS. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

		HPLC-MS/MS							
		PbTx-9, PbTx-1 equiv.				PbTx-3, PbTx-1 equiv.			
	Exposure Time (hr)	Difference	Lower	Upper	Adj. P-value	Difference	Lower	Upper	Adj. P-value
Ambient seawater PbTx concentration	24-0	2.09	-71.70	75.88	0.99	3.55	-2.59	9.68	0.28
	72-0	-2.40	-76.18	71.39	0.9	1.74	-4.40	7.88	0.71
	72-24	-4.49	-78.27	69.30	0.98	-1.81	-7.95	4.33	0.69
Sargassum tissue PbTx concentration	12-0	196.15	517.15	40.66	0.41	177.09	10.16	344.02	0.0340*
	24-0	153.30	-167.70	474.30	0.65	77.44	-89.49	244.37	0.68
	36-0	530.22	209.22	851.21	0.0007*	136.11	-30.83	303.04	0.15
	48-0	247.56	-73.44	568.56	1.00	97.88	-69.05	264.81	0.45
	72-0	10.82	-310.18	331.82	0.36	77.41	-89.52	244.35	0.68
	24-12	-42.85	-363.85	278.15	0.99	-99.65	-266.58	67.28	0.43
	36-12	334.07	13.07	655.06	0.03	-40.99	-207.92	125.95	0.96
	48-12	51.41	-269.59	372.41	0.99	-79.21	-246.14	87.72	0.66
	72-12	-185.33	-506.33	135.67	0.46	-99.68	-266.61	67.26	0.43
	36-24	376.91	55.91	697.91	0.0162*	58.67	-108.27	225.60	0.86
	48-24	94.26	-226.74	415.26	0.93	20.44	-146.49	187.37	0.99
	72-24	-142.48	-463.48	178.52	0.72	-0.03	-166.96	166.91	1.00
	48-36	-282.66	-603.65	38.34	0.10	-38.23	-205.16	128.71	0.97
	72-36	-519.40	-840.39	-198.40	0.0008*	-58.69	-225.63	108.24	0.86
	72-48	-236.7400	-557.74	84.26	0.22	-20.47	-187.40	146.47	0.99

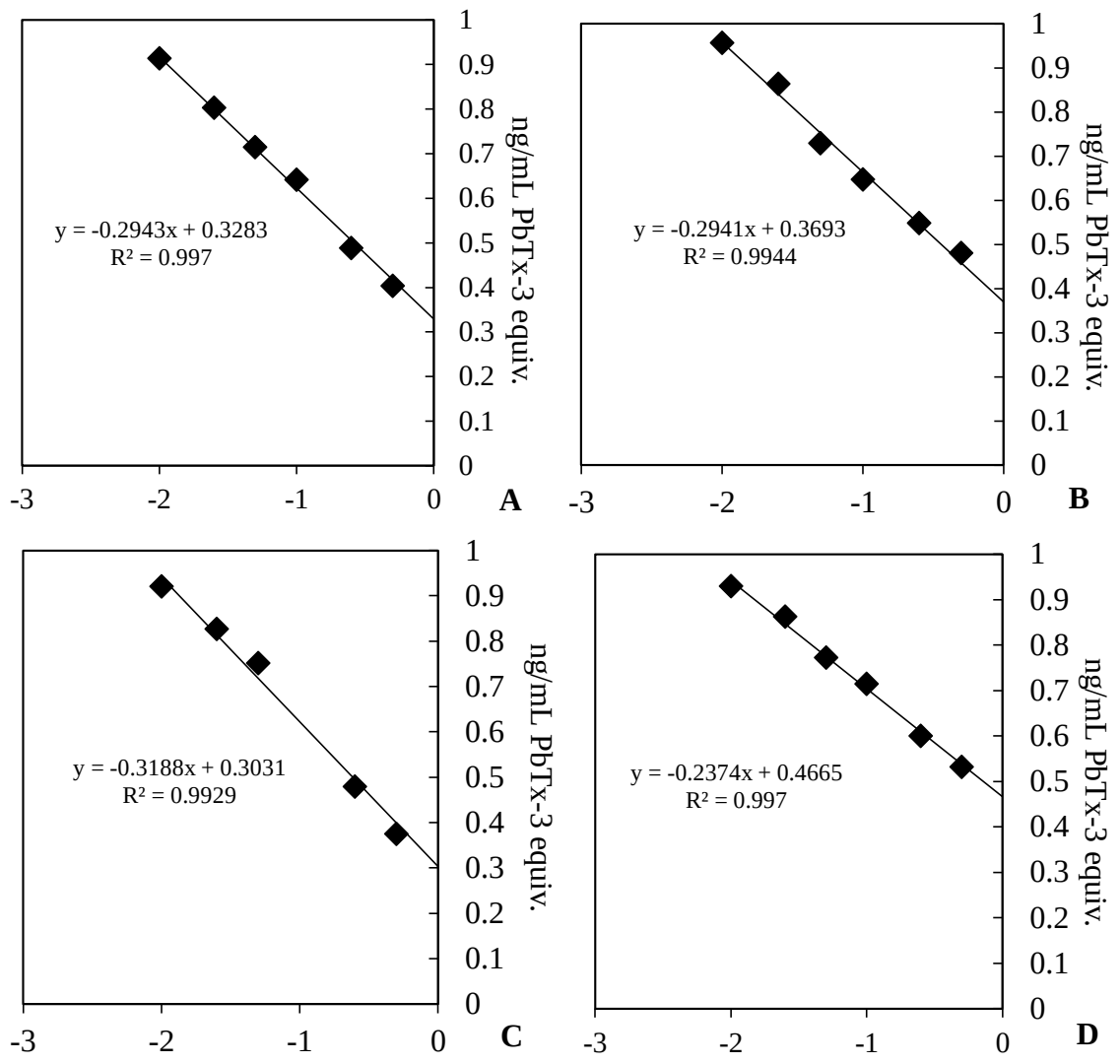

Figure 17. Standard curves determined by ELISA analysis. A. Preliminary survey of macroalgae B. MES samples. C. MeOH samples. D. *U. intestinalis* tissue samples.

Table 11. Summary of results from ELISA analyses of *U. intestinalis* as PbTx-3 equiv. The *U. intestinalis* community was fractionated to include adsorbed PbTx, epiphyte PbTx, and tissue PbTx. Average, standard deviation, maximum and minimum concentrations of PbTx are shown.

		ELISA			
		ng/g PbTx-3 equiv.			
	Day	Avg.	Std. Dev.	Max.	Min.
Adsorbed PbTx concentration	0	9.71	6.58	17.31	5.85
	3	6.35	1.76	8.26	4.79
	6	6.97	2.19	9.47	5.40
	9	6.70	5.02	12.46	3.28
	12	5.57	5.55	11.94	3.04
	16	7.16	3.84	10.85	3.18
	20	3.40	2.18	5.79	1.51
	30	5.76	4.39	8.88	0.74

		ELISA			
		ng/g PbTx-3 equiv.			
	Day	Avg.	Std. Dev.	Max.	Min.
Epiphyte PbTx concentration	0	28.82	12.25	41.40	16.94
	3	23.35	13.78	39.25	15.25
	6	38.61	14.37	53.13	24.39
	9	17.53	3.37	24.39	15.44
	12	18.24	1.43	21.42	16.94
	16	24.4	8.72	25.47	10.21
	20	16.66	15.96	37.82	9.3
	30	21.31	5.51	27.43	16.73

		ELISA			
		ng/g PbTx-3 equiv.			
	Day	Avg.	Std. Dev.	Max.	Min.
U. intestinalis tissue PbTx concentration	0	62.32	29.35	95.40	39.38
	3	240.26	139.68	397.85	131.7
	6	321.89	103.30	419.11	213.42
	9	201.14	37.32	244.22	178.71
	12	110.30	45.06	142.87	58.88
	16	111.75	31.48	137.52	76.66
	20	131.58	64.35	195.32	66.64
	30	114.42	37.77	155.58	81.37