Development of Accelerated Solvent Extraction - Solid Phase Microextraction Method for the Analysis of Brevetoxins in Sediment Samples

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Abstract

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Amanda Joanne Gabriel

The inability of modern harmful algal bloom detection techniques to identify past *K. brevis* events has left the field without access to almost 300 years of historical data essential to the assessment of their apparent increase in frequency and intensity. As such, the marine sediments underlying *K. brevis* affected areas, and the final sink of the brevetoxins they produce when in bloom, represent the only means of establishing the spatial and temporal history of past occurrences.

In this work, a novel accelerated solvent extraction - solid phase microextraction method has been developed and optimized for the extraction and quantification of brevetoxins from marine sediments. Although the extraction method requires further optimization to improve its extraction efficiency and robustness, the method provides an effective means of recovering brevetoxins from most normal marine sediments, with total recoveries of spiked sediments as high as 62.0 ± 9.5% for brevetoxin-2 and 61.6 ± 6.5% for brevetoxin-3. The development of the method has allowed for several procedural pitfalls to be identified, including the potential presence of several contaminants and interferences detrimental to the precise and accurate quantification of brevetoxin using mass spectrometry. The most important among these limiting factors is the presence of high concentrations of co-extracted hydrophobic organic carbon, which affects the extraction efficiency of brevetoxins through the entrainment and sedimentation of extracted brevetoxins from the sediment extract by the formation of organic matter-rich flocculates. A strong relationship exists between organic carbon concentration and brevetoxin recoveries, which allows correcting for the effect of flocculation.

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Contribution of Authors

All analyses were carried out by A. Gabriel under the supervision of Y. Gélinas. A. Gabriel wrote the first draft of the article reporting this work (Chapter 2), with inputs from Y. Gélinas. The analyses (elemental analysis and triple quadrupole mass spectrometry measurements) were completed at the Concordia University Centre for Biological Applications of Mass Spectrometry (CBAMS) and PERFORM Centre - Clinical Analysis Suite facilities. A. Tessier provided guidance throughout the HPLC-MS method development as well as maintained the instrumentation at both facilities. A. Leri performed preliminary extraction method assessments and undergraduate student J. Shapiro occasionally helped with the weighing and extraction of samples.

Table of Contents

List of Figures

List of Tables

List of Acronyms

ASE	Accelerated Solvent Extractor
ESI	Electrospray Ionization
GOM	Gulf of Mexico
HAB	Harmful Algal Bloom
HPLC	High Performance Liquid Chromatography
MIP	Molecularly Imprinted Polymers
MI-SPME	Molecularly Imprinted Solid-Phase Microextraction
MRM	Multiple Reaction Monitoring
MS	Mass Spectrometry
MSPD	Matrix Solid Phase Dispersion
NOAA	National Oceanic and Atmospheric Administration
NSP	Neurotoxic Shellfish Poisoning
PbTx-2	Brevetoxin-2
PbTx-3	Brevetoxin-3
PEG	Polyethylene Glycol
PP	Polypropylene
SON	Sonication
SOX	Soxhlet
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
VGSC	Voltage Gated-Sodium Channel
WFS	West Florida Shelf
OC	Organic Carbon

1. General Introduction

1.1. Harmful Algal Blooms

Harmful algal blooms (HAB), commonly referred to as red tides, are caused by the accumulation of one or more rapidly proliferating species of phytoplankton or algae in surface waters. Their occurrence is a worldwide phenomenon and affects the coastal waters of all continents as well as freshwater lakes and rivers.[1] This domination of an ecosystem by aggregations of these organisms often results in visible water discoloration, turning waterways shades of red, brown, green, or yellow depending on the pigmentation of the prevalent algae species.[2]

Although stunning in appearance, these colorful displays result in a myriad of adverse effects to the surrounding environment and ecosystem that are long lived and far reaching. The high concentrations of algae associated with HABs result in increased competition for available nutrients, the impedance of sunlight to photosynthetic and marine plant life, the displacement indigenous wildlife and contribute to the formation of hypoxic or anoxic dead zones in affected waterways.[2,3] One example is the blue-green algae HAB that took place in Lake Erie in the summer of 2015 where the bloom manifested itself as a dense, bright green scum that at its peak covered over 775 km^2 of the lake's surface.[4] This HAB resulted in a severely hypoxic "dead zone" within the lake's central basin where dissolved oxygen remained undetectable for a month-long period.[5,6]

In addition to their negative environmental impacts, HABs result in the annual expenditure of millions of dollars in an effort to mitigate harm to affected human populations and to fund growth within this field of research. The occurrence of HABs have resulted in economic losses estimated at 82 million dollars per year in the United States due to the implementation of environmental monitoring programs, public advisory services, and losses in revenue to recreation, tourism, and commercial fishery industries.[7] While many of the harmful effects of HABs are due to non-toxic ecological and economical effects, certain HABs owe their destructiveness to the synthesis and dispersal of toxins that alter cellular processes in affected organisms and are responsible for a series of debilitating shellfish poisoning syndromes in humans.[1]

1.1.1. *Karenia Brevis* Red Tides

One type of HAB is caused by the brevetoxin producing, photosynthetic marine dinoflagellate *Karenia Brevis*.[8] *K. brevis* is an unarmored, non-cyst producing, positively phototactic vegetative plankton that measures between 18 and 45 μm in diameter and is differentiated from other Karenia species by its bulbous apical protrusion and spherical nucleus, as seen in Figure 1-1.[8,9] These coastal, salt-water blooms are characterized by above-background cellular concentrations of 10^3 cells L^{-1} or more and commonly reach levels considered "high" in status, according to the National Oceanic and Atmospheric Administration (NOAA), when these levels exceed 10^6 cells L^{-1}.[8,10]

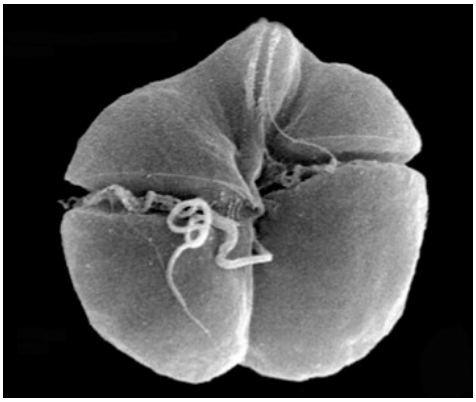

Figure 1-1. *K. Brevis* marine dinoflagellate responsible for toxic red tide HABs. Modified from ref. [9].

Although *K. brevis* born red tides are known to occur in the coastal waters of New Zealand, Japan, and South Africa,[11] nowhere else in the world are blooms as frequent and long lasting as those that occur annually within the Gulf of Mexico (GOM) and along the West Florida Shelf (WFS).[8] Here, *K. brevis* HABs are an almost yearly event which occur most frequently in the late summer to early fall and have been known to last anywhere from a few weeks to over a year in duration, covering areas of over to 25000 km^2.[8,12] The developmental stages of a *K. brevis* HAB are heavily dependent upon the seafloor topography specific to the continental shelves within the GOM.[8] For this reason, HABs of this type have the ability to develop fully offshore, without ever impacting the coastline and its inhabitants, as well as migrate inshore and cause great economic and health concerns for affected populations.[8] The

developmental stages of a of *K. brevis* HAB most commonly involve its offshore, mid-shelf, *initiation* from a live seed stock of *K. brevis* cells present at background concentrations; inner shelf *growth* in population and increase in biomass as cells adapt to their environment; advancement and *maintenance* along the coast by winds and currents; followed by the least understood, and least studied, *termination* of the bloom.[8]

There are several monitoring programs in place for the detection of *K. brevis* red tides which range from visual detection by pilots used to search for water discoloration or patches of dead fish, to satellite imagery and spectral or pigment sensor technology.[8] Sampling efforts are also being made in the form of water samples at fixed and offshore stations collected on a monthly basis, as well as samples obtained whenever possible by volunteer fishermen, ships of opportunity and others with an interest in contributing.[8] While these monitoring efforts make it possible for the public to remain advised of current HAB status, early detection or predictive systems are still largely unavailable. Bloom confirmation often comes too late as satellite imagery can only detect surface blooms classified as medium by the NOAA, at levels of 10^5 cells L^{-1},[8] which are reached 2 to 8 weeks after cell growth begins.[13] Even non-scheduled water sampling occurs only as a response triggered by water discoloration, fish kills, and negative health effects in humans once high cellular concentrations of 10^6 cells L^{-1} are reached.[8]

1.1.1.1. *Karenia Brevis* Red Tide Initiation and Apparent Long-Term Increase

A significant portion of the research performed on *K. brevis* red tides is focused on determining the chemical, biological, and physical environments that allow for their initiation, though much remains unknown and several hypotheses are still under debate.[14] Commonly attributed to a dynamic balance of natural growth prompters, wind and ocean circulation patterns, as well as anthropogenic influences leading to eutrophication, the triggers of *K. brevis* HABs are diverse and in constant evolution as local, and global, climate as well as industrial and agricultural practices change.[1,2] Although occurrences of *K.brevis* HABs have been recorded all over the world, the high incidence and apparent increase of deadly and long lasting blooms within the GOM has made it the focal point of the search for an understanding of these initiation mechanisms.

The initiation of *K. brevis* blooms is known to be a naturally occurring process where a combination of data collected using cultured cells in artificially defined growth media and in the field have been used in its assessment. This has allowed for the evaluation of the necessary climate, basic nutritive requirements and growth prompters that contribute to the increase from background cellular concentrations to hazardous bloom levels.[2,8] These studies revealed that both temperature and salinity play significant roles in the geographic distribution and occurrences of *K. brevis* red tides. Both cultured and field data demonstrated optimal growth conditions are seasonally characteristic of commonly affected waters throughout the GOM.[8] In addition to these sea-surface parameter findings, the effect of nutrient availability has been widely assessed and studies have revealed that terrestrial sources of both organic and inorganic phosphorous and nitrogen as well as trace metals, chelators, and vitamins promote *K. brevis* growth and increases in cell biomass. [8,13]

While the knowledge of what environmental conditions and nutrient supplies allow for *K. brevis* bloom initiation are important, even more critical are the physical processes that control their supply. The GOM is characterized by a series of interconnected surface ocean currents that cumulatively compose a large-scale circulation feature known as the Loop Current, seen in Figure 1-2.[8,13]

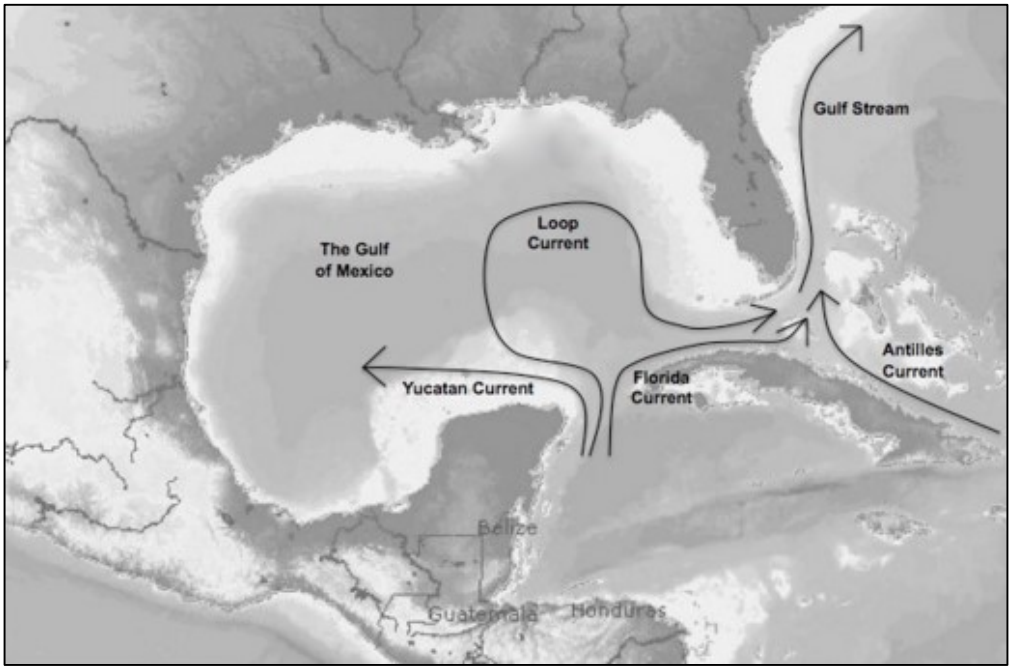

Figure 1-2. The Loop Current enters the GOM as the Yucatán Current, passing between the Yucatán peninsula and Cuba, and exits as the Florida Current, passing between the Dry Tortugas and Cuba. It then meets with the Gulf Stream along the U.S. South Atlantic Bight.[15,13]

Bloom initiation zones, occurring 18 to 74 km offshore,[8,13] are associated with the fronts caused by upwelling and seasonally variant spin-off eddy formation along the boundary of this system.[1,8,13,14] These fronts, along with the dynamic mixing regions that result from the Loop Current's interaction with the Texas, West Florida, and Mexico mid-shelves, become the optimal light and nutrient environment for *K. brevis* cell and biomass growth as limiting nutrients from bottom waters are delivered into the euphotic zone.[8,13,14] In addition to supplying an environment suitable for the growth and accumulation of *K. brevis* cells, the Loop Current is also responsible for the transportation of these harmful cells out of the GOM and into the Gulf Stream where they spread to the U.S. South Atlantic Bight and as far as North Carolina, 1500 km away.[3,13] Furthermore, the spin-off eddies resulting from the Loop Current also allow for the introduction of growing *K. brevis* populations into the near shore waters of the gulf where they have access to anthropogenic nutrient inputs.

Although climate controls many of the fundamental parameters responsible for *K. brevis* bloom initiation and though these blooms have been occurring long before the settlement of man in affected regions, the effect of anthropogenic influences on these blooms in near-shore waters cannot be ignored. These anthropogenic inputs have resulted in the widespread eutrophication of coastal waters and changed the supply and composition of the nutrient pool available for algae proliferation, particularly in the case of nitrates and phosphates.[1,2,3,8,12,14] The resulting favourable nutrient conditions not only increase the intensity and maintenance of *K. brevis* blooms but also have the ability to artificially initiate blooms in these near-shore waters.[14] Industrial and agricultural effects in urbanized coastal regions include pollutants like mariculture born waste, agricultural and phosphate mining runoff, sewage effluents, atmospheric inputs of NO_x emissions, and changes to local food chains through the aquaculture of primary *K. brevis* grazers.[1,2,3,8,12]

Due to increases in initiation, growth, and maintenance parameters, the occurrence of *K. brevis* red tides within the GOM has increased in frequency, geographic distribution, and duration, with an increase of 13- to 18-fold in cell concentration over a 50-year period.[12,16] Since both climate driven processes and artificial nutrient loading have the ability to promote *K. brevis* cell growth and increases in biomass, there is an ongoing debate as to whether the apparent increase in these HABs is due to natural or anthropogenic causes.[17] While certain expansions in *K. brevis* affected areas can be attributed to phenomena such as climate change and its effect on seasonal sea-surface temperature, storms, and currents, they can also result from changing human activities and increases in anthropogenic eutrophication of coastal waters.[2,3] An investigation of the long-term increase of *K. brevis* HAB events in the GOM by Brand and Compton compared occurrences from the years 1954-1963 to those in the 1994-2002 period.[12] The study demonstrated increases in the occurrence, duration, seasonality, maximum intensity, cellular abundance, as well as on and offshore geographic distribution of blooms within the 1994-2002 period.[12] These changes were attributed to increased nutrient abundance due to greater inputs born of human activity though this hypothesis is not agreed upon by the entire scientific community. Some attribute the apparent increase in *K. brevis* red tides to a raised awareness of the scope of the issue, sampling biases, and the improvement of the tools and techniques used for bloom detection and tracking.[2,12] In order to assess the long-term changes in

the occurrence, and the conditions prompting them, the research field requires a proxy to identify and determine the intensity of historical *K. brevis* events.

1.1.1.2. Brevetoxins

The most dangerous and damaging attribute of *K. brevis* red tides is its production of a suite of neurotoxins known as brevetoxins (PbTx).[12] These lipid soluble, polycyclic, methylated polyether compounds, with molecular weights that range from 867 to 953 g/mol, exist as a group of derivatives of two structural backbones, A (PbTx-1) and B (PbTx-2) as seen in Figure 2-1.[18,19] The most prevalent brevetoxin species observed during a *K. brevis* red tide event are PbTx-1, PbTx-2, and PbTx-3, where PbTx-2 and PbTx-3 represent the most abundant intra- and extra-cellular brevetoxin species, respectively.[20] In addition to PbTx-2 and PbTx-3 acting as the primary indicators of *K. brevis* as the culprit organism of a given HAB occurrence, the relative amounts of these brevetoxins offer insight into the relative age of these blooms as a general increase in the abundance of PbTx-3 relative to that of PbTx-2 is observed as the bloom ages.[20] While the reason for this shift in the relative amounts of PbTx-2 and PbTx-3 is not fully understood, the process is commonly attributed to the enzymatic activity driven reduction of the K-ring aldehyde of PbTx-2 into the K-ring reduced aldehyde found in PbTx-3.[20,21] As such, the ratio of PbTx-2 and PbTx-3 species reflect the developmental stage or age of a *K. brevis* HAB where high ratios of PbTx-2:PbTx-3 are observed throughout the growth phase of the bloom while high ratios of PbTx-3:PbTx-2 are observed when the bloom has matured and cell death begins to occur.[20]

Extracellular brevetoxins contaminate waterways, spread as aerosols in sea spray, and enter the food chain of affected areas through the ingestion of *K. brevis* cells by filter feeding bivalves, fish, and other marine organisms.[22] The resulting bioaccumulation and upward transfer of brevetoxins throughout the food chain act as a route of exposure to higher order organisms and human beings, meaning that even minor red tides can be dangerously toxic events. These toxins are responsible for large-scale mortalities of wildlife in affected areas. They are also responsible for a syndrome called neurotoxic shellfish poisoning (NSP) in humans when people contact brevetoxins through ingestion of contaminated seafood[8] or the inhalation of contaminated aerosols.[23] Brevetoxins induce NSP by binding to receptor Site 5 of the voltage

gated-sodium channel (VGSC), which normally mediates the transfer of sodium ions across the cell membrane and causes an influx of sodium ions.[24] The high-affinity binding of brevetoxins keeps the channel continually open and the nerve membrane, and in some reported cases the entire muscle, depolarized, resulting in neuro-excitation.[24] Although no fatalities due to NPS have been reported, there is no known antidote and the symptoms can be severe and include gastrointestinal and neurological effects ranging from mild cases of nausea and vomiting to hospitalization due to respiratory distress and partial limb paralysis.[24]

Just as the waterways, atmosphere, food chains, and human populations are infiltrated with brevetoxins released during a *K. brevis* HAB event, so too are the sediments underlying affected areas. Once released into the environment, sediments become contaminated through the death and decomposition of *K. brevis* cells and contaminated marine wildlife, as well as the entrainment and settling of extra- and intracellular brevetoxins through the flocculation and sedimentation of organic and clay matter.[3,25–31] The flocculation of *K. brevis* cells and extracellular brevetoxins with clays and organic flocculants has been well documented and explored as a means of mitigating the spread and severity of HABs.[3,25,27,28] While the algae cell and toxin removal varies with bloom cell concentration, water mixing, and salinity, clay flocculation has been shown to remove more than 80% of *K. brevis* cells and up to 70% of extracellular brevetoxins with the use of clay-rich sediments.[27] The interaction between these flocculants and brevetoxins has been purposefully exploited to mechanically control blooms[3], but the process also occurs naturally.

This mechanism acts not only as a means of introduction of lipophilic marine toxins to underlying sediments, making them the final sink for brevetoxins, but also suggests that brevetoxins sorb directly onto the sediment matrix.[25,26,31] Although PbTx-2 and PbTx-3 have the ability to persist within the sediments of a *K. brevis* HAB area up to 8 months after the termination of a bloom,[32] the timeframe for the long-term persistence of brevetoxins in sediments has not been definitively constrained. The potential for their direct sorption and long-term persistence within the sediment matrix points strongly towards the possibility of these toxins acting as a proxy for historical *K. brevis* events.

1.1.2. *Karenia Brevis* Sedimentary Record

Historically, reports of HABs and the massive fish kills associated to *K. Brevis* HAB events are commonplace and have been occurring in the GOM since the seventeenth century and as early as 1648 in Yucatan, Mexico.[8,33] While many of the characteristics and negative effects of these blooms reflect those of *K. Brevis* red tides, the earliest identification of *K. brevis* as the causative organism of HAB wildlife mortalities in the GOM was reported by Davis in 1947.[8,18] This identification was made following one of the worst HAB occurrences, in terms of duration and fish kills, occurring in 1946, with cell concentrations of 5.6×10^7 cells L^{-1}.[7] Earlier red tides have also been attributed to *K. brevis* owing to their geographic location in the GOM, the scale of marine wildlife mortalities and reports of NPS symptoms by nearby communities,[8] but they are not officially confirmed. Current water sampling, satellite monitoring programs, and cyst palynological analyses offer nothing in the way of retroactively identifying, localizing, and measuring HABs caused by the unarmored *K. brevis* dinoflagellate, another source of the information must be established.

The sequestration and persistence of brevetoxins in sediments underlying *K. brevi*s HAB affected areas result in these toxins being a valuable molecular probe for evaluating past bloom occurrences. With the temporally-stratified deposition of material and the millions of square kilometers of sediments covering the Earth's surface, this sedimentary record accounts for the only historical account of the spatial and temporal evolution of past *K. brevis* events. The extraction and quantification of brevetoxins from sediment samples collected from various locations and depths allow for the determination of the frequency, intensity, dynamics, and geographic distribution of *K. brevis* HABs after their termination. Further, this information allows retrospectively assessing the impacts of climate change and anthropogenic activity on the occurrence and apparent increase of these blooms in the GOM.

The coupling of this historical reconstruction with sediment dating and palynological analyses also allows improving our understanding of the environmental conditions that lead to the initiation of *K. brevis* blooms. Paleo-environmental reconstruction databases, where dinocyst identification and counting allow for the determination of sea-surface parameters like temperature, salinity, and primary production, have become more widely available for the GOM research area[34]. These databases present an opportunity, along with the historical reconstruction,

to evaluate the key parameters controlling the occurrence of *K. brevis* HABs beyond the 69 years since the species was identified. A prognostic tool of this nature would ultimately allow for the prediction and control of these HABs through preemptive, rather than reactionary, responses to natural and anthropogenic environmental conditions favorable to their occurrence. These preventative measures, and potentially the prevention of these blooms, would be invaluable to preserve the health of coastal ecosystems, the populations in proximity to HAB hot zones, and the economic interests of affected industries and conservation authorities.

1.2. Brevetoxin Recovery

Given the widespread benefits gained from the interpretation of the sedimentary record of past *K. brevis* red tides occurrences, efforts towards developing a robust determination method for sedimentary brevetoxins are underway. While several approaches have been developed for the analysis of brevetoxins in water, aerosols, vegetation, tissue, and cells, none have been successfully applied routinely to marine sediments. In addition to the challenge of fully extracting the brevetoxins from the sediment matrix, such a method also has to be robust enough to overcome the limitations stemming from variations in the composition and complexity of the sediment matrix as *K. brevis* blooms occur worldwide above highly contrasting sedimentary environments. Only a few studies reporting the development of a brevetoxin extraction method from marine sediments are available in the literature until now.[26,32]

1.2.1. Soxhlet

Soxhlet (SOX) is an extraction method allowing the recovery of an analyte from a solid sample matrix insoluble in common solvents.[35] The extraction takes place in a SOX apparatus where constantly refluxing solvent flows through a porous thimble containing the sample, dissolving and collecting the analyte through continuous extraction.[35]Although the extraction takes place unattended and SOX is traditionally viewed as the high recovery standard method to which other solid extraction methods are compared, disadvantages of the extraction method include the need for hundreds of millilitres of high purity solvent and long extraction times of 12 to 24 hours.[36,35]

The high thermal stability of brevetoxins makes SOX a common extraction method for the recovery of brevetoxins from filtration material used on water samples and aerosol samples.[37,38,39] To the best of our knowledge, no literature exists on the SOX extraction of brevetoxins from sediment samples but, it has been used successfully in the extraction of a vast array of lipophilic biomarkers from sediment samples.[31,40,41]

1.2.2. Sonication

Sonication (SON) is an extraction method also used in the extraction of an analyte from a solid sample matrix, but does so through physical disruption of the sample and dissolution of the analyte using ultrasonic radiation.[35] SON extraction involves the placement of the finely divided sample in an extraction solvent, where the solution is either submerged in an ultrasonic bath or exposed to an ultrasonic probe for repeated static cycles. The solvent from each cycle is then separated from the solid sample via centrifugation, and pooled in order to maximize analyte recovery. While the SON extraction method is less time consuming and requires less solvent than the SOX method, SON is still a long and high solvent consumption method where the repeated cycles and solvent pooling involve increased sample manipulation and opportunity for experimenter error, as these steps cannot be automated or proceed without supervision.[36]

Widely used in the assessment of lipophilic molecular markers in sediment samples,[31,42] SON is the most commonly used brevetoxin extraction method for solid samples, and is employed in the recovery of aerosolized brevetoxins accumulated on filters as well as brevetoxins from *K. brevis* cell cultures, tissue, vegetation, and sediments samples.[26,32,38,43,44] The implementation of the SON extraction method to recover brevetoxins from GOM mairne sediments detected brevetoxin concentrations ranging from 0.81 and 89.0 ng/g sediment [26,32] and demonstrated recovery efficiencies of only about 40% of brevetoxin spikes.[26] To the best of our knowledge, this is the highest recovery reported for any method for brevetoxins in a marine sediment matrix. The low brevetoxin recovery was attributed to the binding of brevetoxins to the sediment particles, as the extraction efficiency was independent of sonication time.[26]

1.3. Accelerated Solvent Extraction - Solid Phase Microextraction: A Novel Brevetoxin Extraction Method

The need for a high efficiency method for the total recovery of brevetoxins from the sediments has prompted the evaluation of new extraction methods, used until now only for lipid and lipophilic analytes extracted from solid samples.

1.3.1. Accelerated Solvent Extraction

Unlike the extraction methods discussed above, accelerated solvent extraction (ASE) utilizes high temperature and pressure conditions to improve the extraction of the analyte from the solid sample.[31,35,36] These conditions allow for the facilitated diffusion of the extraction solvent throughout the sample matrix and result in the more complete dissolution and recovery of the analyte.[31,35,36] The sample to be extracted is placed in a sealed metal cell that is then placed into a heated oven chamber and filled with the extraction solvent, as shown in the extraction process scheme in Figure 1-3. The extraction cell is then pressurized, allowing for an increase in the boiling point of the extraction solvent, and for the solubilisation of the analyte at a temperature higher than would be possible at atmospheric pressure. The sample is then extracted and collected by the automated filling and voiding of the cell through repeated static cycles. Compared to other solid sample extraction techniques, ASE requires less time, consumes less solvent during the extraction and with the added benefit of automation, it has proven effective for several environmental solid samples.[31]

ASE has never been exploited for the analysis of brevetoxins in marine sediments, but has demonstrated its effectiveness in the extraction of other hydrophobic, lipophilic marine sediment contaminants, like polycyclic aromatic hydrocarbons,[36] and marine toxins including yessotoxins, pectenotoxins and okadaic acid.[31] ASE is thus a promising approach to improve the recovery of brevetoxins from marine sediments.

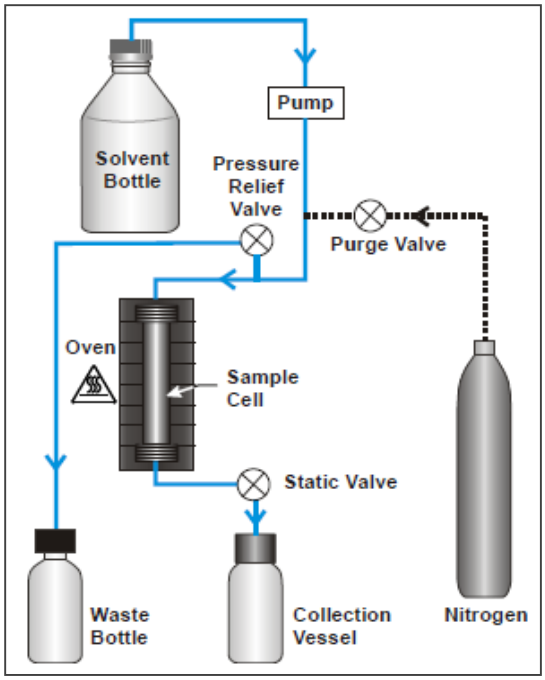

Figure 1-3. Schematic of ASE process. See text for description.[45]

1.3.2. Solid Phase Microextraction

As the recovery of brevetoxins from tissue samples is improved by the implementation of sample preparation methods,[43,44] a solid phase microextraction (SPME) treatment of the ASE sediment extracts was performed. This SPME sample preparation step could lead to an improvement in brevetoxin recoveries through the exclusion of interferences from the final sample matrix, thereby reducing the potential for ionization suppression and improving the accuracy of analyte peak area integration during LC-MS analysis. Although SPME products are typically designed for thermal desorption and gas chromatographic analysis of volatile and semi-volatile compounds, a SPME fiber coating suitable for solvent desorption and liquid chromatographic analysis of a wide range of polar and nonpolar analytes has recently been developed.[35,46] These SPME products are available in both multi-use fiber assemblages and single-use fiber probe formats, which consist of a fused silica, stainless steel, or titanium-nickel metal alloy fiber core coated in a thin polymeric stationary phase.[35,46] The SPME preparation of a liquid sample consists of the partitioning of the analyte between the fiber coating and an aqueous sample matrix. Extraction is a function of the analyte distribution coefficient and exposure to the sample matrix.[35,46] Given the small mass of the SPME material, this results in the

non-exhaustive recovery of the analyte. Extraction is considered complete when the concentration of the analyte between the sample matrix and fiber coating has reached distribution equilibrium.[46,47]

The equilibrium condition is described by the equation:

$$C_o V_s = C^{\infty}_s V_s + C^{\infty}_f V_f$$

where C_o is the initial concentration of the analyte in the sample, V_s and V_f are the volume of the sample and fiber coating, respectively, and C^{∞}_f and C^{∞}_s are the equilibrium concentrations of the analyte on the fiber and in the sample matrix, respectively. The distribution constant of the analyte between the two phases is described by the equation:

$$K_{fs} = C^{\infty}_f V_f / C^{\infty}_s V_s$$

Once the absorption of the analyte onto the fiber is complete, the fiber is removed from the sample and the analyte is desorbed from the fiber coating using strong solvent through either the use of an online SPME/HPLC interface or offline into a desorption solvent.[35,46]

SPME boasts the advantages of reduced sample manipulation, a final analyte matrix that is HPLC ready, and targeted analyte selectivity based on sorbent material choice. An additional advantage, specific to the extraction of brevetoxins from marine sediments, comes from the fiber format of this single-step, solid-liquid extraction, sample preparation method. This SPME fiber probe format, and the desorption of the analyte into a vial containing clean solvent, eliminates the physical particulate filtration that result from chromatographic sample preparation methods, such as solid phase extraction (SPE) discs or cartridges. This is an important consideration for the recovery of brevetoxins from marine sediments owing to the ability of organic and clay matter flocculates within the sediment extract matrix to entrain and settle brevetoxins out of solution, making their physical exclusion from the sample matrix detrimental to the recovery of brevetoxins. To this day, the implementation of SPME for the sample preparation of sediment extracts in the recovery of brevetoxins has yet to be reported.

1.4. Scope of Thesis

As the brevetoxin sedimentary record represents the single greatest source of information on the spatial and temporal history of *K. brevis* blooms, a robust extraction method for brevetoxins in marine sediments is required. This extraction method would not only allow for the paleo-reconstruction of past *K. brevis* events, but would also aid in the development of a prognostic tool for prediction and mitigation of these harmful blooms through the correlation of past occurrences to the sea-surface parameters and anthropogenic influences that contributed to their initiation.

This thesis will report on the development and optimization of a protocol for the extraction and quantitative measurement of brevetoxins from marine sediments using a novel ASE-SPME-HPLC-MS method for implementation within the GOM and other *K. brevis* blooms affected areas. The high pressure and temperature extraction conditions of the ASE will be exploited in combination with the SPME sample preparation method to improve upon the low brevetoxin recovery reported for the SON extraction method currently in use. The developed ASE-SPME-HPLC-MS method will then be applied to a series of marine sediment samples obtained from the Laguna Mecoacán in the GOM in order to assess the effectiveness of the developed method on brevetoxins preserved in marine sediments.

2. Development of Accelerated Solvent Extraction - Solid Phase Microextraction Method for the Analysis of Brevetoxins in Sediment Samples

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2.1. Introduction

Harmful algal blooms (HAB), caused by the rapid proliferation and accumulation of one or more dominating species of algae, are a common phenomenon in aquatic environments and occur worldwide.[1] Aggregations of the organisms often cause visible water discoloration, turning waterways shades of red, brown, green, or yellow depending on the pigmentation of the prevalent algae species, and result in a myriad of adverse effects to the surrounding ecosystem.[2] In addition to their many negative environmental and economic effects, such as the formation of hypoxic or anoxic zones in waterways and the annual expenditure of millions of dollars towards environmental monitoring programs, certain HABs owe their harmfulness to the synthesis and dispersal of biotoxins when in bloom.[1,2,17]

One toxin-producing type of HAB, commonly referred to as red tides, are caused by the photosynthetic marine dinoflagellate *Karenia Brevis* and are characterized by the production of a suite of neurotoxins known as brevetoxins.[8,12] Ranging in molecular weight from 867 to 953 g/mol, these lipid soluble, cyclic, methylated polyether compounds exist as a group of derivatives of two structural backbones, A (PbTx-1) and B (PbTx-2), as shown in Figure 2-1.[18,19] *K. brevis* red tide events are dominated by the presence of two brevetoxin species: brevetoxin-2 (PbTx-2), the unaltered type B backbone form of the toxin, and brevetoxin-3 (PbTx-3), the aldehyde-reduced derivative of PbTx-2.[18]

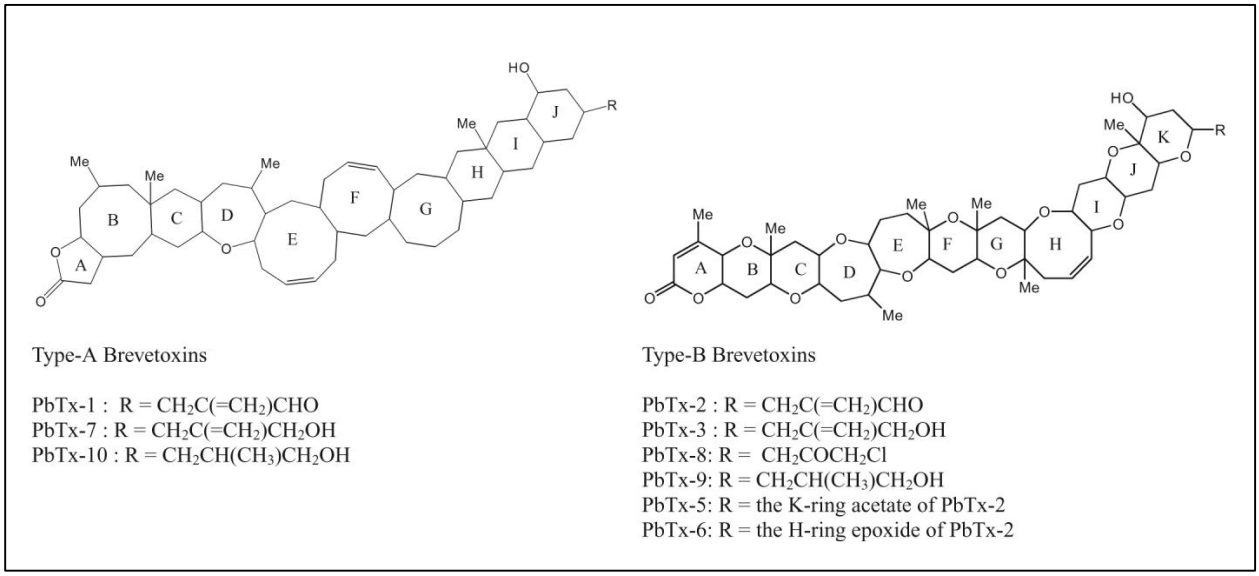

Figure 2-1: Brevetoxin backbone and derivative structures. Modified from ref. [25].

Brevetoxins contaminate waterways, spread as aerosols in sea spray, and bioaccumulate in marine organisms, allowing for their introduction and upward transfer throughout the food chains of affected areas. Not only do these brevetoxin dispersal mechanisms result in the large-scale mortalities of wildlife within red tide events, but they also act as routes of exposure to human beings where the inhalation of contaminated aerosols or ingestion of contaminated seafood results in a syndrome called neurotoxic shellfish poisoning (NSP). Although *K. brevis* red tides occur in coastal waters all over the world,[11] nowhere are blooms as frequent and long lasting as those that take place annually within the Gulf of Mexico (GOM) and along the West Florida Shelf (WFS),[8] where increases in the occurrence, duration, seasonality, maximum intensity, cellular abundance, as well as on and off-shore geographic distribution of blooms have been recorded over a 50-year period.[12]

Though references to many of the health and ecological effects that characterize *K. brevi*s red tides have been recorded as early as 1648 in the GOM, its identification as the causative organism for these toxic HABs only occurred in 1947.[8,18] Because modern-day water sampling, satellite monitoring programs, and cyst palynological analyses offer nothing in the way of retroactively identifying, localizing, and measuring past *K. brevis* HABs, the sediments underlying bloom affected areas are being assessed as a potential historical record of these events. The brevetoxins produced by *K. brevis* blooms become incorporated into marine sediments through their entrainment and sedimentation from the water column by the flocculation of organic and clay matter, making them the final sink for the bloom born toxins.[3,25–31] The temporally-stratified deposition and possible long-term persistence of brevetoxins within marine sediments demonstrate their potential to act as molecular probe for evaluating the spatial and temporal evolution of past *K. brevis* events.

While several approaches have been developed for the analysis of brevetoxins in water, aerosols, vegetation, tissue, and cells, the need for an extraction method that can be routinely applied for the recovery of brevetoxins from marine sediments still exists. This extraction method must not only allow for high efficiency recovery of the analyte from the complex sediment matrix, but must also be robust enough for its implementation on marine sediments from a wide range of geographic settings and with highly contrasting sedimentary compositions. The currently used sonication extraction method, which utilizes ultrasonic radiation to physically

disrupt the sample and facilitate dissolution of the analyte, results in the recovery of approximately 40% of spiked brevetoxins and, to the best of our knowledge, is the highest reported recovery of brevetoxins from a marine sediment matrix.[26]

An extraction method that has yet to be implemented in the recovery of brevetoxins from marine sediments, but has demonstrated its effectiveness in the extraction of other lipophilic, marine sediment contaminants, is accelerated solvent extraction (ASE).[31,36] ASE allows for the automated extraction and recovery of an analyte through repeated static extraction cycles at high temperature and pressure, in order to facilitate the diffusion of the extraction solvent throughout the sample matrix and allow the more complete dissolution and recovery of the analyte.[31,35,36] The high temperature and pressure conditions of the ASE allow for improved recoveries of analytes from marine sediments and should provide a means to assess the *K. brevis* bloom history from the sedimentary record. Due to the complexity of the marine sediment matrix and potential for the unintended co-extraction of non-analyte, hydrophobic compounds by the ASE, a sample preparation step was implemented in order to clean-up the ASE extracts. Not only would the introduction of a sample preparation step improve the robustness of the extraction method, but the use of these techniques, like that of solid phase extraction (SPE) in the recovery of brevetoxins from tissue samples, improves recovery efficiencies.[43] As such, the partition based, solid phase microextraction (SPME) treatment of the ASE extracts was performed before LC-MS analysis.

In this work, a method for the quantitative measurement of brevetoxins from marine sediments, based on a novel ASE-SPME-HPLC-MS approach, was developed and optimized. It was applied to sediments of the GOM and other areas affected by *K. brevis* blooms. This extraction method will allow assessment of the long-term persistence of brevetoxin in sediments, as well as deciphering the spatial and temporal sedimentary record of past *K. brevis* red tide occurrences.

2.2. Materials and Methods

2.2.1. Method Development, Test Sediment

Several sediment samples were used during the development and optimization of the extraction method due to the limited amount of GOM sediment available. These test sediments were chosen from geographic regions known to be unaffected by *K. brevis* HAB events and the lack of naturally contained brevetoxins, as confirmed in this work by the analysis of blank sediment extractions. The sediments were obtained from the Saguenay Fjord, Canada (Station 30, 48°18.28 N, 70°15.44 W), the Franklin Bay, Canada (70°02.73 N, 126°18.06 W), and the Argentina Esperanza Station in Antarctica (62°14.80 S, 58°40. 05 W). The organic carbon (OC) concentration varied between 0.38 and 2.30% in these samples. The OC concentration was determined for each freeze-dried sample using elemental analysis (see Appendix A for details of elemental analysis, OC concentration analysis).

2.2.2. Sediment Sample Collection

Sediment samples were collected from the Laguna Mecoacán (18.42°N - 93.15°W) saltwater lagoon in south-western GOM during the R/V *El Puma* cruise of November 2011.[34] This lagoon was chosen because of its central location along the Tabasco Coast, a region known to be cyclically affected by *K. brevis* born HABs,[22] with June to September bloom peak concentrations reaching 10^6 cells L^{-1}.[1] This cell abundance is high enough to cause water discoloration and to produce brevetoxins, which remain at the surface of underlying sediments for up to eight months after an HAB event,[3] at concentrations high enough to force the closure of shellfish harvesting, result in fish kills and to cause respiratory distress.[2]

The samples were taken from the surface (0-1 cm) of sediment collected using box cores, which were subsampled with pushcores, sliced onboard and transported back to the lab. The wet sediments were then stored at 4°C and freeze-dried before being ground and homogenized using a mortar and pestle.

Table 2-1. Location and depth of sediment samples analyzed.

Lagoon	Sample Number	Site	Depth (cm)	Latitude N	Longitude W
Laguna Mecoacán	1	MEX-11-12	0-1	18° 26' 01.7"	93° 07' 44.0"
	2	MEX-11-13	0-1	18° 25' 46.2"	93° 08' 55.3"
	3	MEX-11-14	0-1	18° 25' 22.8"	93° 08' 49.2"

2.2.3. Chemicals

Brevetoxin standards of PbTx-2 and PbTx-3 were purchased from MARBIONC (Wilmington, USA). Acetonitrile (HPLC grade) was purchased from VWR (Montreal, Canada), while formic acid (Optima LC/MS grade) and methanol (Optima grade) came from Fisher Scientific (Ottawa, Canada). Nanopure, 18 MOhm water was used throughout this work.

2.2.4. Accelerated Solvent Extraction

The extraction of brevetoxins from the sediment samples was performed using an ASE (Model ASE 150, Thermo Scientific Dionex) in a 66-mL cell, at 103.5 bar (1500 PSI) and 100 °C, using 3 extraction cycles of optima grade methanol, 5 min each. About 1.5 g of precisely weighed sediment was loaded into the cell and the remaining void volume was filled with pre-combusted Ottawa Sand (20-30 mesh, Fisher Scientific) to minimize solvent volume. During method development, test sediment samples were spiked with 30 µL of a 1 ng/µL methanolic solution of PbTx-2 and PbTx-3 prior to extraction. The resulting ASE sediment extract was then concentrated using a rotary evaporator, transferred to a 2mL sample vial, and evaporated to dryness while in a sand bath heated to 80°C, under a gentle stream of N_2 gas. The dried residue was re-solubilized in 1800 µL of a 25% (v/v) methanolic solution in water by sonication and vortex mixing in preparation for the solid phase microextraction sample preparation step. The use of polypropylene vials should be avoided throughout all steps of the extraction method as decreases in brevetoxin recovery efficiency have been observed resulting from sediment extract solution contact with the material (data not shown, see Appendix C for additional details on effect of polypropylene vials on brevetoxin recovery).

2.2.5. Solid Phase Microextraction

Determination of SPME fiber probe recovery - The ASE sediment extracts were cleaned by SPME using C-18 coated SPME-LC fiber probes (film thickness (d_f) 45 µm, reversed-phase, Sigma-Aldrich). Before SPME fibers were introduced to sediment extracts, analyte recoveries for each fiber was determined using a solvent matrix spiked with known amounts of standard PbTx-2 and PbTx-3. The SPME fibers were conditioned in a 50% (v/v) methanolic solution in water for 30 minutes before use. The conditioned fibers were then introduced to 1800 µL of a brevetoxin spike, 25% (v/v) methanolic solution in water for 12-hr with orbital shaking to ensure equilibrium conditions. The SPME fibers were then rinsed with Milli-Q water and introduced to vials containing 300 µL of 100% methanol for offline brevetoxin desorption for 1-hr with orbital shaking. The desorption solution was then evaporated to dryness in a sand bath heated to 80°C under a gentle stream of N_2 gas and stored at 4°C until analysis. Before HPLC-MS analysis, the dried spiked residues were re-solubilized in 100 µL of a 70% (v/v) methanolic solution by sonication and vortex mixing. The recovery efficiency (in percent) of each fiber was then used to calculate the total brevetoxin content of the test and sediment extracts. Variations in recovery efficiency between individual fibers arise from variations in the coating of the fibers with sorbent material.

SPME fiber probe sample recovery - Once the recovery efficiency for each fiber was measured, the conditioning, absorption, desorption, drying, and resolubilization sequence was repeated with the re-dissolved ASE extracts obtained from sediment samples. The concentration of the brevetoxins in the extracts was obtained using Multiple Reaction Monitoring, High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MRM) Analysis.

2.2.6. High Performance Liquid Chromatography-Mass Spectrometry Analysis

The HPLC-MS setup consisted of an Agilent Technologies, model 1200 series liquid chromatographic system and a Waters Micromass, Quattro triple quadrupole mass analyzer. The reversed-phase separations were performed on a 30 mm x 2.1 mm x 1.7 µm Kinatex C18 column, with a 2 µm SecurityGuard ULTRA C18 guard column (both from Phenomex Inc., CA, USA) at room temperature. The separation was carried out under gradient conditions, with an injection volume of 10 µL and a flow rate of 300 µL/min. The mobile phase consisted of (A)

water containing 0.1% formic acid and (B) acetonitrile containing 0.1 % formic acid where the mobile phase composition was immediately increased from 30 to 45% B, then to 95% B over the next 4 min, maintained at 95% B for 1 min to wash the column, then reduced to the initial conditions of 30% B over the next 3 min with each injection made 15 min after the previous one.

Mass spectra data were acquired in positive electrospray ionization mode (ESI^+) using a z-spray source at a block temperature of 80°C. The analyte identities and retention times were confirmed with analytical standards of PbTx-2 and PbTx-3 using daughter ion spectrum scan mode from *m/z* 100 to 900. Nitrogen gas was used as both the drying and nebulizing gas, with flow rates of 90 and 350 L/hr, respectively, and a nebulizing gas temperature of 350°C. The optimized instrument parameters include the use of nitrogen as the collision gas at a pressure of 3.6×10^{-6} bar, cone voltages of 30V used for both brevetoxins, and collision energies of 20 and 25V were used for PbTx-2 and PbTx-3, respectively. The multiple reaction monitoring (MRM) of brevetoxins occurred with dwell times of 0.5 s and transitions set to 895.5 *m/z* to 877.5 *m/z* and 897.5 *m/z* and 725.5 *m/z* for PbTx-2 and PbTx-3 respectively. Brevetoxin quantification was performed using an 8-point external calibration curve with solutions of 0.5, 1, 2, 4, 8, 16, 32, and 64 ng/mL made by serial dilution with analytical standards of PbTx-2 and PbTx-3 and duplicate measurements made for each standard. The LC-MS method within-run precision, based on 14 measurements of the same standard solution throughout a 15.5 hour period, was 6.3% for PbTx-2 and 7.3% for PbTx-3, while the limits of detection (3σ) were 1.0 ng/mL for PbTx-2 and 0.5 ng/mL for PbTx-3 and limits of quantification (10σ) were 2 ng/mL for both PbTx-2 and PbTx-3.

2.3. Results and Discussion

2.3.1. Method Optimization

Preliminary ASE Brevetoxin Extraction Method – A preliminary assessment of the ASE method to evaluate the recovery of brevetoxins from marine sediments. The parameters and solvent used for the development of the ASE brevetoxin extraction method were selected based on similar methods developed for the extraction of lipophilic analytes from marine sediments.[31] The ASE method framework mirrored the only other method used for brevetoxins, which consists of the SON extraction, evaporation, resolubilization and LC-MS analysis, where the SON extraction step was replaced by ASE. The preliminary extractions consisted of three sets of

samples that were spiked with the two brevetoxins (*i*) before the ASE extraction, (*ii*) before the evaporation, and (*iii*) before the analysis. The extractions resulted in recoveries of 1.9% for PbTx-2, 3.1% for PbTx-3 for the pre-ASE spike, 3.3% for PbTx-2, 0.6% for PbTx-3 for the pre-evaporation spike, and 51.4% for PbTx-2, 53.7% for PbTx-3 for the pre-analysis spike. The pre-evaporation brevetoxin recovery, when determined in a solvent-only rather than sediment extract matrix, resulted in the recovery 97.3% for PbTx-2 and 99.3% for PbTx-3 of the spiked brevetoxins.

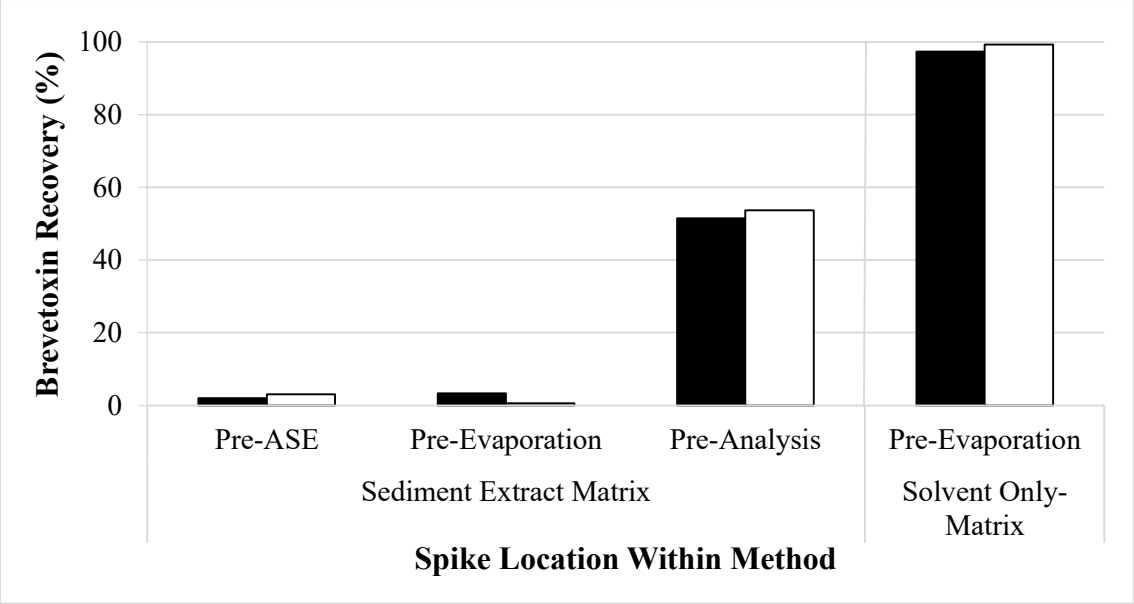

Figure 2-2. Variations in recovery of 30 ng/ml spiked PbTx-2 (black) and PbTx-3 (white) depending on spike location when using the ASE extraction method without sample pre-treatment, See text for details. Replicates agree within the error of the method (n=2).

The data indicates that independent of the efficiency of the ASE to extract the PbTx-2 and PbTx-3 brevetoxins from the sediment matrix, less than 55% of the spiked brevetoxins are quantifiable when the ASE-extracted solution is analyzed without sample pre-treatment. The solvent-only spiked sample, which underwent the same procedural manipulations, shows that the loss of brevetoxins is not a result of the handling of the sample and that the sediment extract matrix likely is responsible for the low brevetoxin recoveries. The high temperature and pressure conditions used during the ASE extraction results not only in the solubilization of the target analytes, but also in the co-extraction of interfering organic compounds with chemical

characteristics similar to those of brevetoxins. The presence of organic matter interferences in the sediment extract leads to the formation of flocculates that accumulate and increase in size with time. These flocculates may trap the extracted brevetoxins and remove them from solution, making them unavailable for detection. This hypothesis is supported by the observation that the spikes added at the beginning of the extraction sequence, with a longer contact time with the sediment extract matrix, show greater brevetoxin losses upon analysis. An additional sample pre-treatment step designed to reduce the effect of these interferences on recoveries, and to assess the link between organic carbon and recovery efficiency was thus developed.

Solid Phase Microextraction Sample Pre-Treatment - SPME is a selective and rapid sample pre-treatment method in which the targeted analytes are recovered in an HPLC-compatible final solution. In addition, the single use C-18 SPME fiber probes used in this work allow for high throughput treatment of multiple samples simultaneously while keeping the cost of each analysis reasonable.

Influence of Solvent Composition on SPME Extraction Recovery - Although the composition of typical SPME absorption solution range from 1 to 5% (v/v) organic phase in water, the solubility of brevetoxins increases with increases in the proportion of methanol in solution. For this reason, SPME absorption solutions with organic compositions of 1, 5, 25, 40, 55, and 70% (v/v) methanol were evaluated in order to determine the composition that allowed for the optimal balance between favorable sample – C18 sorbent material partitioning of brevetoxins and their solubility in the absorption solution. The evaluation was carried out in both a solvent-only and ASE extract of the Argentina Esperanza Station in Antarctica, a sediment sample with a low OC concentration, to allow determination of SPME recovery efficiency in samples with low concentrations of interfering compounds. The best results were obtained for an absorption solution composition of 25% (v/v), with spike recoveries varying between 66.7 and 71.1% for PbTx-2 and PbTx-3 (Figure 2-3).

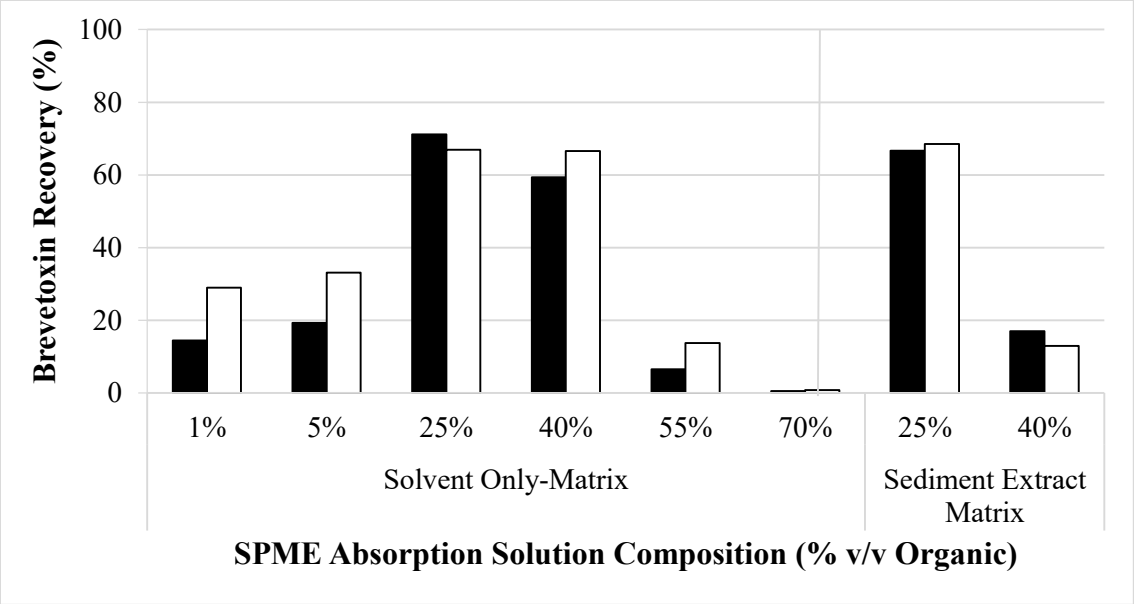

Figure 2-3. Recovery of PbTx-2 (black) and PbTx-3 (white), spiked at 30 ng/ml, for different SPME absorption solution compositions in a solvent-only and sediment extract matrix. Replicates agree within the error of the method (n=2).

These findings suggest that an absorption solution of less than 25% (v/v) of methanol in water leads to the incomplete solubilization of brevetoxins, making them unavailable for absorption onto the SPME fiber. On the other hand, a solution above 40% (v/v) results in a low solid-liquid partitioning of brevetoxin and thus on a low recovery of the analytes through absorption on the solid C-18 fiber. In addition to allowing for optimal analyte partitioning and recovery, the 25% (v/v) absorption solution composition also leads to statistically similar recoveries of brevetoxins for the sediment extract and solvent-only matrices (95% confidence interval). All further method optimizations and sample analyses were performed using these SPME absorption conditions.

SPME Brevetoxin Partitioning Coefficient - The SPME brevetoxin partitioning coefficient and equilibrium conditions of the extraction, which are dependent on the nature of the analyte, its concentration in solution, and the volume of sorbent material available, were determined through the replicate, solvent-only matrix brevetoxin recovery of 20 SPME fibers (Figure 2-4). These SPME extractions resulted in brevetoxin recoveries of 68.9 ± 10.7% for PbTx-2 and 66.0 ± 8.9% for PbTx-3. The recoveries ranged from as low as 48.5% for PbTx-2, 49.1% for PbTx-3 to as high as 86.0% for PbTx-2, for 78.4% PbTx-3, suggesting large fiber-to-fiber variations in

sorbent material volume or accessibility to sorbent absorption sites. As all SPME fibers were obtained from the same production lot, the high fiber-to-fiber variability in brevetoxin recovery suggests that large variations exist in the SPME fiber probe manufacturing process. The extraction efficiency for each fiber must thus be assessed separately prior to its application to real samples in order to correct for this potential bias. Such assessment and corrections were applied throughout this work.

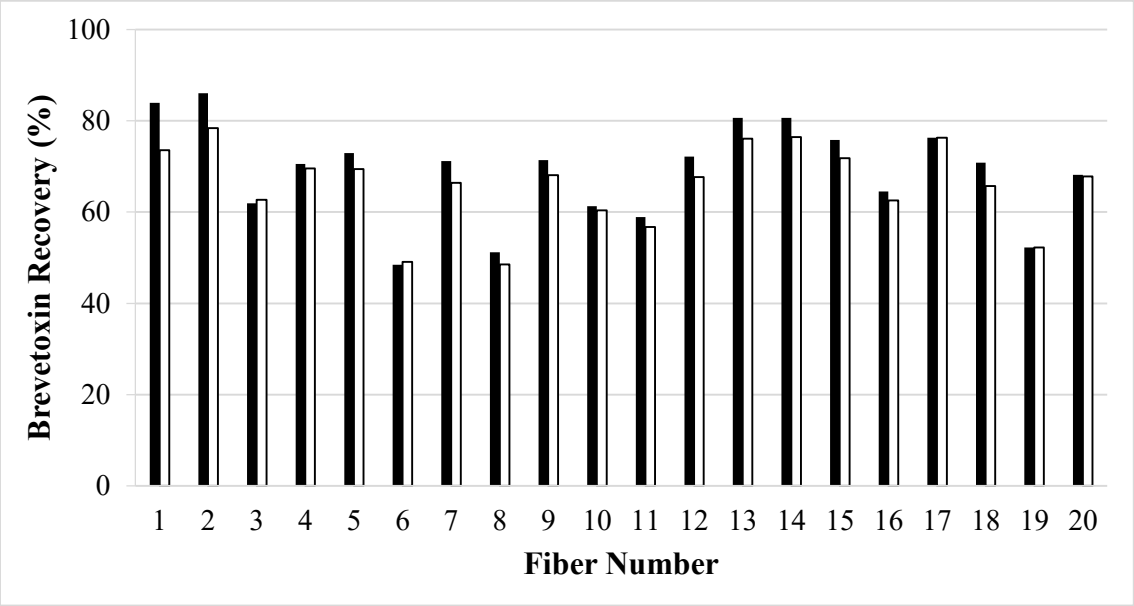

Figure 2-4. Variation in the recovery of PbTx-2 (black) and PbTx-3 (white), spiked at 30 ng/ml, in a solvent only matrix for 20 SPME fiber probes. Each measurement is based on an n of 2, where the instrument precision is 6.3% for PbTx-2 and 7.3% for PbTx-3.

It is important to note that the contamination of samples by polyethylene glycol (PEG) results in a decrease in the recovery efficiency of the SPME fibers through the partially irreversible absorption of the contaminant to the SPME fiber (data not shown). The introduction of PEG at any point in the ASE-SPME-HPLC-MS method should be avoided at all costs to maximize the recovery of brevetoxins and accurately quantify their concentration (see Appendix B for additional details on PEG contamination).

SPME Fiber Re-Use - The extraction of several spiked sediment extracts, using a single SPME fiber, was performed in order to determine whether individual fibers could be used more than

only once. The assessment revealed that the recovery efficiencies of individual SPME fibers diminished by 3.1 ± 2.9% for PbTx-2 and 1.9 ± 1.6% for PbTx-3 upon its second use and by 30.0 ± 8.7% for PbTx-2 and 13.0 ± 9.9% for PbTx-3 upon its third use.

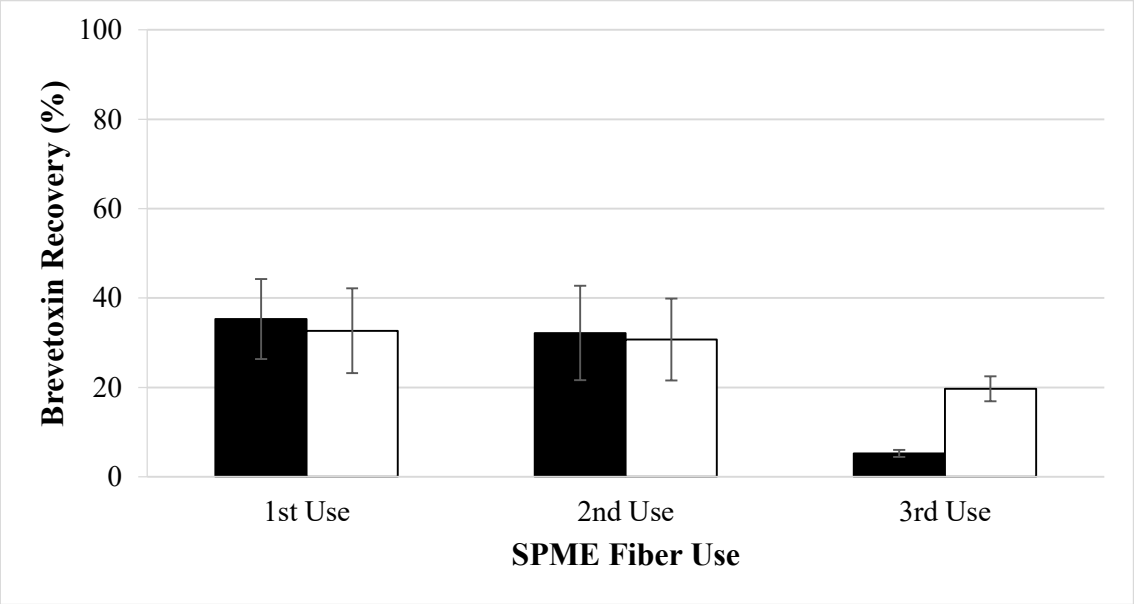

Figure 2-5. Recovery of PbTx-2 (black) and PbTx-3 (white), spiked at 30 ng/ml, from a sediment extract matrix as a function of multiple extractions performed using a single SPME fiber in a sediment extract matrix (n=3).

Since the re-use of SPME fibers and the brevetoxin recovery in both the sediment extract and solvent-only matrices are not significantly different (95% confidence interval), the extraction efficiency of each SPME fiber can be determined through a solvent-only matrix test extraction before its use with a real sample. The carry over after the 1st and 2nd use of SPME fibers, determined by repeating the desorption procedure after the initial desorption of the sample, resulted in recoveries of no more than 0.41% for PbTx-2 and 0.52% for PbTx-3, which is much less than the precision of the LC-MS method used.

2.3.2. Final Accelerated Solvent Extraction- Solid Phase Microextraction Brevetoxin Recovery Method

Effect of Sample Organic Carbon Concentration - The effect of organic matter on the recovery of brevetoxins was assessed as the flocculation of organic matter has been used to mitigate the spread and impact of *K. brevis* blooms through the entrainment and sedimentation of brevetoxins from the water column to the underlying sediments. While this mechanism is one of the possible explanations for the preservation of brevetoxins in the marine sediments underlying *K. brevis* HAB affected areas, the other being the direct sedimentation of the source organism, *K. brevis*, it could also lead to a loss of brevetoxins from the extraction solution as flocculates form and sequester other lipophilic organic compounds. The effect of organic matter content was evaluated through the analysis of three sediment samples of varying OC concentrations using the finalized ASE-SPME-HPLC-MS method. The recovery efficiencies shown in Figure 2-6 for sediment samples with low (0.38 wt%), mid-range (1.01 wt%), and high (2.30 wt%) OC concentrations result in brevetoxin recoveries of 62.0 ± 9.5% (PbTx-2) and 61.6 ± 6.5% (PbTx-3), 20.4 ± 3.0% (PbTx-2) and 20.3 ± 3.8% (PbTx-3), and 10.7 ± 2.5% (PbTx-2) and 10.0 ± 2.3% (PbTx-3), respectively.

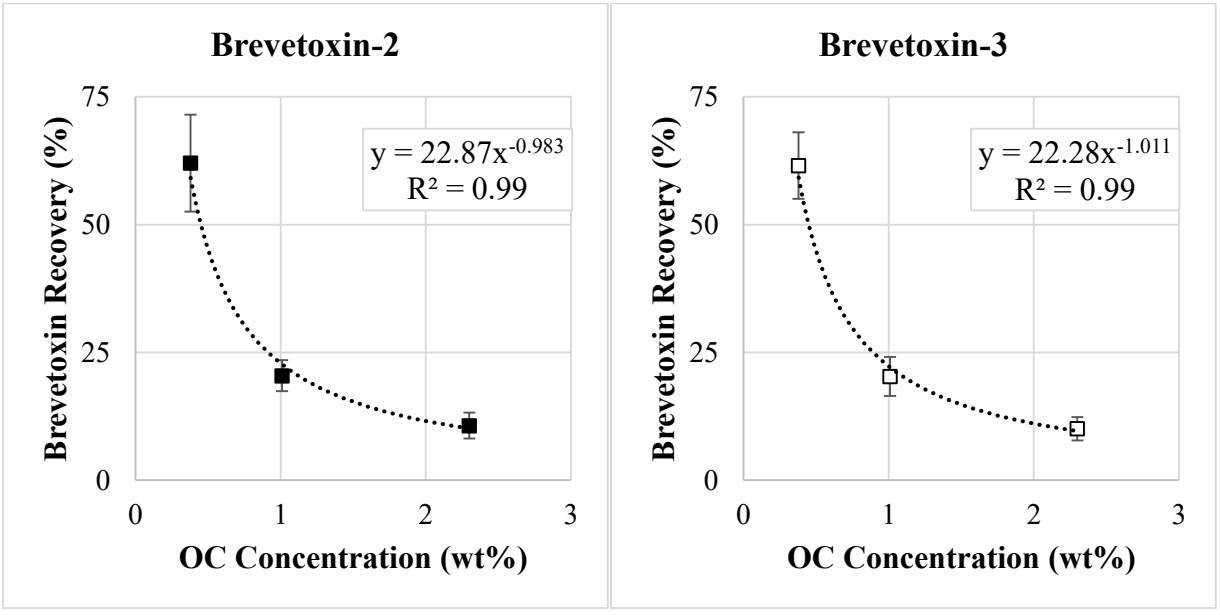

Figure 2-6. Relationship between sediment OC concentration and PbTx-2 (black) and PbTx-3 (white) recovery (n=3).

This trend in brevetoxin recovery supports the hypothesis that the co-extracted organic matter from the sediment matrix interferes with the absorption of brevetoxins on the SPME fiber through the flocculation and sequestering of the brevetoxins from solution. While these results reveal an inverse relationship between OC concentration and brevetoxin recovery, additional data points are needed to confirm the accuracy of the mathematical relationship. It is important to note that the majority of sediments around the globe have organic carbon concentrations around 1 wt%,[48] a level at which the interferences are less problematic. The recovery-OC concentration relationship should however be better defined before applying this approach on sediments with OC concentrations above this threshold and for correcting brevetoxin concentration data for the effect of OC flocculation.

Brevetoxin Quantification in GOM Sediment Samples - The ASE-SPME-HPLC-MS extraction and quantification of three samples obtained from the GOM resulted brevetoxin concentrations shown in Table 2-2.

Table 2-2. Corrected, measured brevetoxin concentration of Laguna Mecoacán sediment samples (n=4).

Lagoon	Sample Number	Site	PbTx-2 (ng/g sediment)	PbTx-3 (ng/g sediment)	Organic Carbon Conc. (%)
Laguna Mecoacán	1	MEX-11-12	1.39 ± 0.38	0.876 ± 0.15	1.54
	2	MEX-11-13	0.612 ± 0.092	0.450 ± 0.059	0.87
	3	MEX-11-14	0.428 ± 0.033	0.298 ± 0.052	0.67

This data shows that the ASE-SPME-HPLC-MS method is suitable for the extraction, recovery, and quantification of brevetoxins from marine sediment samples with relative standard deviations (RSD) better than 17%. The only exception is for PbTx-2 in the MEX-11-12 sample for which the RSD is 27%. Although the recoveries appear low for samples with OC concentrations above 1 wt%, it is worth noting that the overwhelming majority of sediments from the world ocean have OC concentrations ranging between 0.5 and 1.5%,[48] and that the maximum values found in low dissolved oxygen environments is about 8 wt%. At such high OC levels, the brevetoxin recoveries would be slightly less than 5%, and while the measured

brevetoxin concentrations would be less precise, the data would still be informative if replicate analyses were run. Further efforts into improving this method should be devoted to the understanding and, if possible, the mitigation of the effect of organic matter flocculation on brevetoxin recoveries. The data obtained for the GOM samples shows that brevetoxins from a past *K. brevis* HAB in the sample region are preserved in sediments, and that they can vary by almost two-fold between samples. The fact that the highest brevetoxin concentrations are found in the sample with the highest OC content agrees with the hypothesis of a strong interaction between brevetoxins and sedimentary organic matter, providing clues on a potential mechanism explaining their preservation in sediments.

2.4. Conclusion

The recovery efficiency of the ASE-SPME-HPLC-MS method for the extraction of brevetoxins from marine sediment samples is dependent upon the complexity of the sample and the effect of co-extracted organic matter from the sediment matrix. While the ASE was expected to more effectively extract brevetoxins from the sediment matrix and result in a more complete recovery of the analyte, the formation of organic flocculate in the extract solution reduced analyte recovery. Flocculate formation proved unavoidable throughout the extraction and sample preparation steps and reduced brevetoxin recovery even after short periods of contact with the sediment extract. Although a SPME sample pre-treatment step was introduced to mitigate the effect of these interferences, the recovery efficiency of the extraction method remains dependent on the OC concentration of the sediment sample. However, the strong inverse relationship between OC concentrations and brevetoxin recovery allows correcting for the effect of flocculation and allows quantifying with confidence PbTx-2 and PbTx-3 in sediments. The ASE-SPME-HPLC-MS method will prove very useful for deciphering spatial and temporal variations in *K. brevis* HAB, which in turn could be instrumental in understanding the causes for the recent expansion of affected areas.

3. General Conclusion

Although more work is needed to make the ASE-SPME-HPLC-MS method of extraction of brevetoxins from marine sediments as robust as needed for optimally deciphering the sedimentary record of past *K. brevis* HABs, what was learned through its development and optimization is invaluable to future attempts to improve brevetoxin recoveries. The most important problem to overcome is the effect of OC content on brevetoxin recovery efficiency as the analytes are entrained and sediment out of solution through organic matter flocculation.

The inverse relationship between OC concentration and brevetoxin recovery suggests that the short and long-term interaction between brevetoxins and hydrophobic organic matter should be investigated in an effort to understand whether this interaction leads to enhanced persistence of brevetoxins in marine sediments and provide clues on the best approaches to eliminate their effect on the recovery of brevetoxins from the sediment matrix. The presence of hydrophobic organic matter in the sediment extract is due to its co-extraction at the high temperature and pressure used during the ASE treatment and likely is unavoidable. Further optimization of the ASE extraction parameters, with the dual purpose of optimizing brevetoxin extraction and minimizing the recovery of organic matter interferences, to reduce flocculation and improve brevetoxin recovery may prove difficult as brevetoxins constitute a class of hydrophobic species similar in composition to the co-extracted organic matter; any improvement in the extraction of the former would lead to an improvement in the extraction of the latter, and vice versa. The addition of a SPME sample preparation step post-extraction, although successful for the recovery of brevetoxins from solvent-only and low OC concentration samples, did not allow efficiently overcoming the low brevetoxin recoveries induced by organic matter flocculation or competitive absorption of other hydrophobic compounds on the SPME fibers low recoveries in organic-rich sediments. More work should be done to reduce the effect of flocculation and competitive absorption on brevetoxin recoveries, or alternative sample clean-up methods should be explored.

In addition to the effect of the sample OC concentration on the extraction efficiency of brevetoxins from the sediment matrix, several other conclusions were derived from the development and optimization of the ASE-SPME-HPLC-MS method. While the primary source of organic matter responsible for the decreases in brevetoxin recovery originates directly from

the sediment sample, our findings also demonstrate the importance of avoiding contamination from external sources of organic compounds through the pre-combustion of glassware or base rinsing of heat sensitive materials used throughout the extraction method. An example of a contaminant that is problematic is polyethyleneglycol (PEG), owing to its competitive absorption on the SPME fibers, thus decreasing brevetoxin recovery and the number of extractions for which a single fiber can be used. As this contaminant has many laboratory and everyday sources and is at least in part irreversibly absorbed by the SPME fiber sorbent material, extra care must be taken and periodic verifications made to ensure that the contaminant is not present.

The ASE-SPME-HPLC-MS method presented here is well suited for the recovery of brevetoxins from sediment matrices. Additionally, it may serve as a framework in the development of extraction methods for analytes unaffected by sample organic matter flocculation, including the more than 200 other HAB marine toxins and their derivatives.[31] As more than 90% of these biological toxins are lipophilic in nature and are found in highly diverse sedimentary environments worldwide, the trends and relationships established throughout this work will provide a promising starting point for future sediment ASE extractions as well as serve as a cautionary tale of the pitfalls faced when recovering such analytes from sediments.

Future Work

The data and observations presented within this work have identified several aspects of the extraction of brevetoxins from marine sediments that require further investigation in order to better understand the interactions at play. In addition to the need for more data points to validate the brevetoxin recovery-OC concentration relationship and its implementation as a correction to account for the effect of OC flocculation, the effect of the variation in the chemical composition and complexity of the organic matter of the sample should also be assessed. This would be best performed through the extraction of sediment samples bearing similar OC concentrations but originating from varying geographic locations and environments as well as the extraction of sediment samples devoid of background organic matter content that have been spiked with organic matter of varying types.

Another aspect of the developed method that merits further examination is that of ionization suppression due to the sediment extract sample matrix. As ionization suppression

results in a change in the efficiency with which the spray droplet forms or evaporates, thus affecting the mass to charge ratio of the ion that reaches the detector, the quantification of the brevetoxin analytes can be greatly affected by the coelution of interferences, cross-talk, or the sample matrix.[49] The ionization suppression of the brevetoxin analytes would be identified by comparing the instrument response for the analyte when: 1) injected within a solvent-only matrix to obtain its relative 100% response, 2) injected within a pre-extracted sediment extract matrix to determine the effect of the sample matrix, and 3) injected as part of a sediment sample having undergone the complete extraction method to determine whether any signal loss is attributable to the extraction process.[49] Any ionization suppression identified can then be addressed by adjusting the sample preparation protocol, altering the HPLC method, or corrected for by use of a co-eluting, isotopically labeled or structural analog internal standard.[49] Note that in addition to the benefit aiding in the identification of and correction for ionization efficiency issues during LC-MS analysis, the incorporation of an internal standard to the SPME portion of the extraction method would also allow for the correction of the fiber to fiber variability observed throughout the method optimization.

The recovery efficiency and robustness of the extraction method may also be improved through the use of alternative sample preparation techniques that offer greater analyte selectivity, the exhaustive recovery of the analyte, or are better suited to the non-homogeneous, flocculate bearing sediment extract matrix. One sample preparation method that allows for the complete recovery of the analyte and allows for the mixing of bulk amounts of sorbent material throughout the sample extract, is the use of the "QuEChERS" (the quick, easy, cheap, effective, rugged, and safe) method, dispersive SPE.[50] The most promising of sample preparation techniques involves the use of molecularly imprinted polymers (MIPs) to increase analyte selectivity through the artificial generation of recognition sites complimentary to the shape, size, and functionality of the target molecule.[51] Several modes of MIP sample preparation techniques, including molecularly imprinted solid-phase microextraction (MI-SPME) and matrix solid phase dispersion (MSPD), are well suited to the clean-up of brevetoxin containing sediment extracts and warrant the development of brevetoxin template based sorbent materials.[51]

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Appendix A: Elemental Analysis OC Concentration Analysis

The OC concentration of the GOM and method development test sediments were measured using an elemental analyzer (EuroVector). Each sediment sample, after having been freeze-dried, ground, and homogenized, was weighed into open silver capsules and exposed to 12 N HCl vapor for a 12-hour period in order to remove all inorganic carbon. Following the removal of residual water and acid by a 1-hour period at 50°C, the silver boats were sealed, wrapped in a tin capsule, and stored in a desiccator until ready for elemental analysis. Instrument calibration and OC concentration determination was performed in-house using a pre-calibrated β-alanine standard while instrument stability was monitored using interspersed standard measurements.

Appendix B: Polyethylene Glycol Contamination

Throughout the method development and optimization, a polyethylene glycol (PEG) contaminant was discovered. Polyethylene glycols, a family of synthetic polymers that exist in a wide range of molecular weights, are a common LC-MS system contaminant with several potential sources, such as ESI organic solvents and glassware detergents, as well as everyday sources external to the laboratory environment like hand creams.[52] PEGs have the ability to contaminate not only samples but also the MS instruments used for their analysis, resulting in a loss in signal of the analyte of interest.[53]

The PEG contamination of the sediment extracts was detected as high intensity signals repeating every 44 Da of the PbTx-2 and 3 mass spectra acquired for the samples, as seen in Figure B-1. This spectral pattern, which is characteristic of PEG contamination, was observed in several sediment extract solutions undergoing the ASE-SPME-HPLC-MS extraction method, independent of the sediment geographic origin. As the PEG contaminant was not observed throughout the preliminary assessment and early optimization tests of the extraction method, we assume that it was introduced through a change in the method designed to increase brevetoxin recovery and remove other interferences. In order to pinpoint where the introduction of PEGs into the sample was occurring, an MS evaluation of sediment extract solutions from various steps of the sample extraction and treatment was performed.

PEG contamination of the samples was found to originate from the PTFE lined vial caps used to store samples throughout the extraction process. Although no further investigation to pinpoint the direct source of the PEG was made, we believe that trace amounts of an ethelyne oxide containing glass cleaning product named Sparkleen^{TM} remained trapped underneath the PTFE liner of the caps and was introduced into the vials as they were base and solvent rinsed in preparation for use.

Once the PEG contaminant was eliminated, the brevetoxin recovery efficiency of the SPME sample preparation step in a solvent-only matrix increased by 13.8% and 12.8% for PbTx-2 and 3, respectively. This increase in brevetoxin recovery suggests that the PEGs present in solution were acting as a competitor to the brevetoxins for the fiber coating of the SPME fiber probe. The decrease in fiber coating absorption site density available solely for the recovery of brevetoxins in solution, as well as potential decreases in the ionization efficiency of the MS quantification of the samples, resulted in the apparent low recovery yield of the SPME.

A large increase in the recovery efficiency upon elimination of the PEG contamination was also observed for repeat uses of the SPME fibers. The initial assessment suggested that SPME fibers could only be used once as the recovery efficiencies of individual SPME fibers diminished by 45.8 ± 7.8% for PbTx-2 and 38.9 ± 1.6% for PbTx-3 upon their second use. These recovery efficiencies were dramatically improved, with differences of only 3.11 ± 2.90% for PbTx-2 and 1.94 ± 1.65% for PbTx-3 following the elimination of PEG contaminants, suggesting that PEG was not only competing with the brevetoxins for absorption sites on the SPME fiber but also that it was at least partially irreversibly retained on the fiber coating. The introduction of PEGs to any part of the ASE-SPME-HPLC-MS extraction of brevetoxins from sediment samples should be avoided at all costs to maximize brevetoxin recoveries and accurately quantify them in sediment extract solutions.

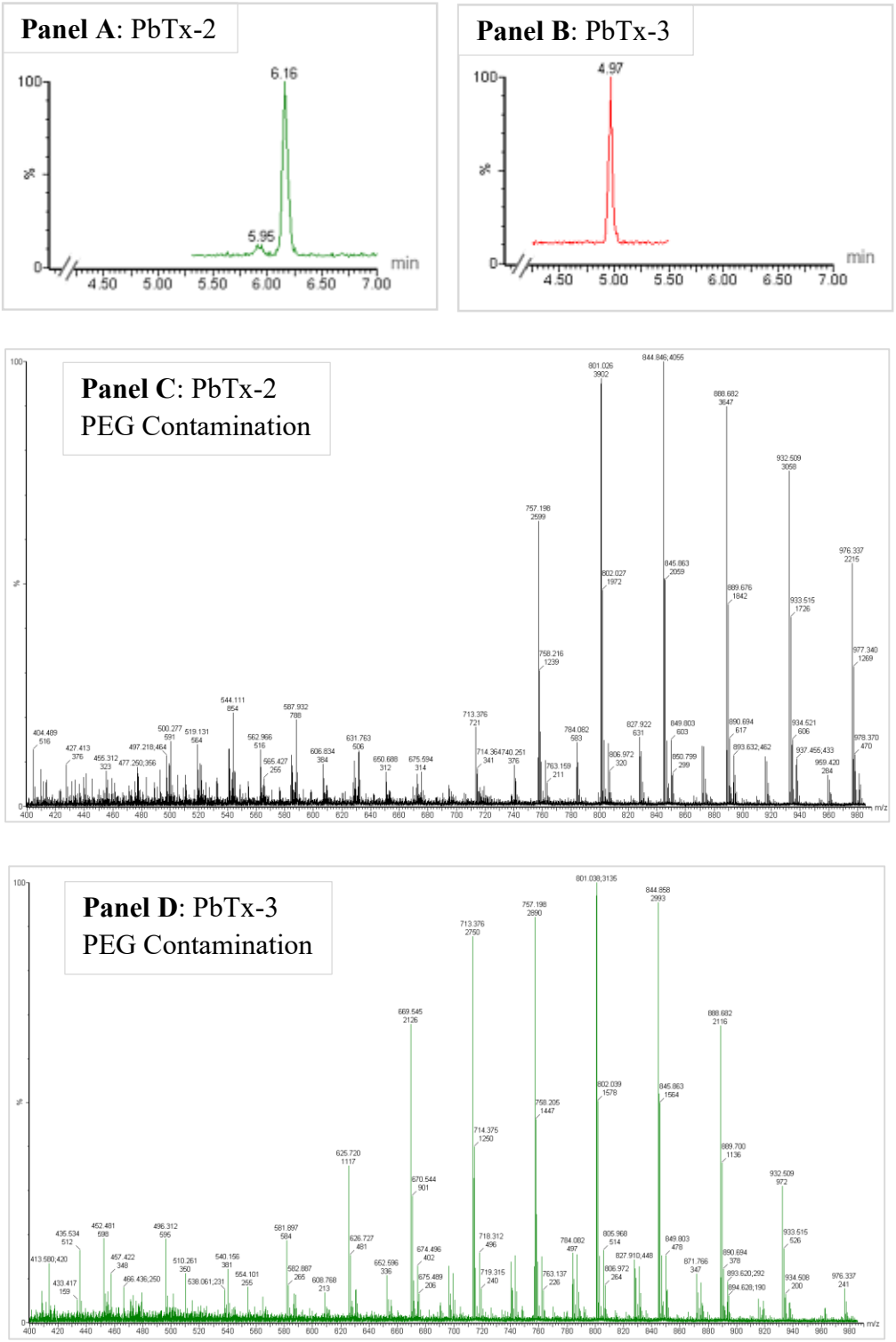

Figure B-1: Chromatogram of PbTx-2 (Panel A) and PbTx-3 (Panel B) as well as mass spectra of PEG contaminated sediment extract at the time of elution time of each analyte (PbTx-2 in Panel C and PbTx-3 in Panel D).

Appendix C: Polypropylene Vial Use

Throughout the development of the ASE-SPME-HPLC-MS method, several vials and vessels were used to contain and prepare the sediment extracts for final analysis, including the stainless steel ASE extraction cell, and borosilicate glass vials among others. The choice of one vial type in particular, the 300-µL polypropylene (PP) micro vials used for the desorption of the SPME fiber probes, was made in an effort to minimize solvent use and ensure a high MS signal by limiting sample dilution. An unexpected side effect of using these polypropylene vials was the immediate decrease in the peak areas associated with PbTx-2 and PbTx-3 upon contact of solvent-only matrix brevetoxin standard solutions. This decrease in signal occurred uniformly at any brevetoxin concentration and took place after only 2-3 minutes of contact with the polypropylene vials. Table C-1 shows the change in peak area associated with a brevetoxin standard solution made in a glass vial, quantified using the HPLC-MS portion of the developed extraction method, then transferred to a 300-µL PP vial with a burnt Pasteur pipet, and re-analyzed. No standard solution analyzed after its contact with the PP vial resulted in peak areas higher than $21.4 \pm 0.0_3$% for PbTx-2 and $23.3 \pm 0.0_5$% for PbTx-3 of the original peak areas determined when the standards were contained in a glass vessel.

It is unclear what attributes of the PP micro vial are responsible for the decrease in brevetoxin signal upon contact with standard solutions and why the phenomenon is not observed with the use other PP materials, such as the autosampler vial inserts used. The MS scan of the standard solution in contact with the PP micro vials revealed two high intensity peaks at 918.5 m/z for PbTx-2 and 920.5 m/z for PbTx-3, indicative of the sodiated adduct of each brevetoxin present. An examination of solvent-only and sediment extract matrix samples having gone through the SPME sample preparation method, where the desorption of brevetoxins from the SPME fiber took place in the PP vials, showed no sign of these sodiated adduct signals. This result suggests that an equilibrium based or time sensitive mechanism for the sodium adduct formation may be taking place.

Any potential loss in signal attributed to the SPME desorption in PP vials was integrated into the brevetoxin recoveries calculated for each individual SPME fiber during the fiber test stage, as all fiber tests and sample SPMEs were performed with the same PP micro vials and as recoveries

were not significantly different for the solvent-only and sediment extract matrices. The interaction of brevetoxins with PP materials in reference to sodium adduct formation as well as the effect of the length of the exposure to this material require further investigation. Its effect on the recovery efficiency of the ASE-SPME-HPLC-MS has been accounted for in this work and is not a detriment to the ability of the method to recover and quantify brevetoxins from sediment samples except at low brevetoxin abundances. Standard solutions analyzed after being transferred from one glass vial to another, or after contact with the PP vial inserts and pipet tips used throughout the method, resulted in changes in peak area of no more than 3.08% for PbTx-2 and 6.99% for PbTx-3, which is much less than the precision of the LC-MS method used.

Table C-1. Effect of 5-min contact of brevetoxins with 300-µL PP micro vials on LC-MS quantification

Standard Solution Concentration (ng/mL)	Glass Vial		PP Vial		PP Vial / Glass Vial	
	PbTx-2 Peak Area	PbTx-3 Peak Area	PbTx-2 Peak Area	PbTx-3 Peak Area	PbTx-2 Peak Area	PbTx-3 Peak Area
0.5	9.4	4.0	N/A	N/A	N/A	N/A
1	15.3	8.5	N/A	N/A	N/A	N/A
2	30.3	17.4	N/A	N/A	N/A	N/A
4	67.1	36.4	11.8	5.4	0.175	0.147
8	137.2	66.8	28.9	17.0	0.211	0.254
16	249.2	139.4	63.3	38.4	0.254	0.276
32	516.7	277.9	121.6	72.3	0.235	0.260
64	1152.3	595.9	227.0	137.0	0.197	0.230

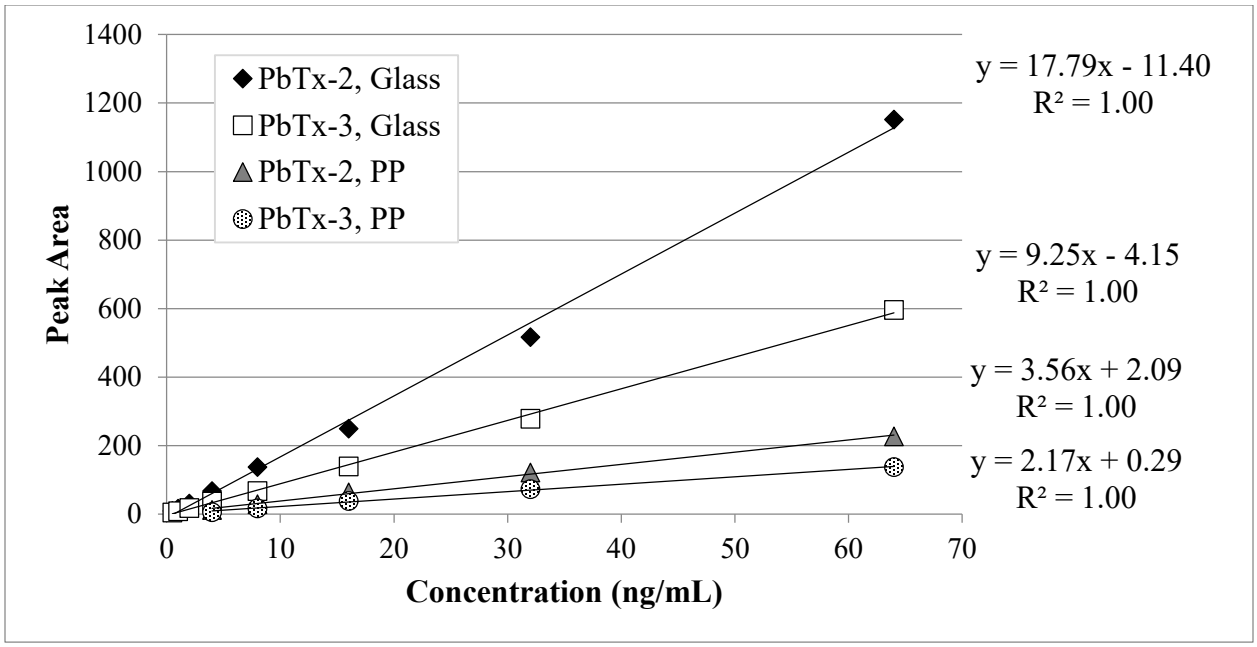

Figure C-1. Effect of 5-min contact of brevetoxins with 300-µL PP micro vials on calibration curves.