THE ROLE OF DISSOLVED ORGANIC MATTER IN STRUCTURING MICROBIAL COMMUNITY COMPOSITION

by

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ABSTRACT OF THE DISSERTATION

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Dr. Sybil Seitzinger and Dr. Oscar Schofield

Dissolved organic matter (DOM) is an important source of nutrients in aquatic systems contributing to the growth of phytoplankton and bacteria. The overall response appears to be driven by the phytoplankton and bacteria species present as well as the composition of DOM provided. This dissertation explores the bioavailability of allochthonous and autochthonous DOM sources to, and its affect on, the growth of marine phytoplankton and bacterial community abundance and composition.

This research utilizes advanced chemical (electrospray ionization mass spectrometry (ESI-MS)) and molecular (terminal restriction fragment length polymorphism (TRFLP)) techniques to characterize the DOM and microbial community. To investigate the role of allochthonous DOM in phytoplankton growth, DOM from two different riverine sources from watersheds with different land use practices, was supplied to a natural cyanobacteria population. The bioavailability of autochthonous DOM was investigated by supplying DOM produced by a single culture of cyanobacteria to a natural dinoflagellate bloom community. The potential negative effect of an individual autochthonous DOM

compound was investigated through the addition of marine toxin, brevetoxin, to three different natural bacterial communities.

This dissertation resulted in the first ESI-MS characterization spectra of the DOM associated with three different natural phytoplankton blooms, a culture of cyanobacteria, and two different South Florida rivers. It was also the first study to identify previously uncharacterized allochthonous and autochthonous DOM masses bioavailable to natural marine phytoplankton communities. Bulk level analyses within these experiments quantified lower limits for the bioavailability of allochthonous and autochthonous DOM sources and the relative community response to each of these sources. This dissertation also represents the first molecular evaluation of the bacteria associated with a bloom and the first investigation of the allelopathic properties of brevetoxin. It has discussed and applied the use of ESI-MS to investigate the bioavailability of complex DOM, identified and quantified potential nutrient sources and linked marine toxin production to changes in bacterial community composition.

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Chapter 1: Introduction

1.1 Dissolved Organic Matter in Marine Systems

Dissolved organic matter (DOM) is the largest pool of reduced carbon in the ocean; it is one of the major reservoirs of carbon on earth, and equals the amount of CO_2 present in the atmosphere (Siegenthaler and Sarmiento, 1993; Benner 2002). Research of DOM is diverse in both the topic and application. Investigations of DOM can be found in all fields of oceanographic research including chemical, biological, geological and physical. Areas of current DOM research include but are not limited to investigations of global nutrient cycles, its role in microbial food webs, the identification and classification of specific compounds, and its origins and fates (Hedges 2002; Benner 2002; Carlson 2002).

In the past, DOM was considered to be mostly refractory meaning that it was biologically unavailable; however, we now know that DOM is an important component of the microbial loop (Azam et al.1983). Aquatic DOM serves as source of nutrients and energy for phytoplankton and bacterial communities, attenuates light, chelates metals and through its production and remineralization plays a larger role in atmospheric CO_2 concentrations (e.g., Benner 2002; Bronk 2002; Carlson 2002; Hedges 2002). These different properties reflect the diversity in composition of the numerous DOM compounds in aquatic systems. Work has shown that differences in compound composition play a role in their bioavailability, with some phytoplankton species preferentially using certain compound or groups of compounds over others (e.g., Berg et al. 1997; Gobler and Sañudo-Wilhelmy 2001a). This preferential usage in turn affects their respective removal rates and role in the nutrient cycle (Carlson 2002). With the immense amount of research currently being performed on questions pertaining to DOM

dynamics it is amazing that as much as 85% of DOM compounds have still not yet been chemically characterized at the molecular level (Hedges et al. 2000; Benner 2002). To understand DOM dynamics in aquatic systems, we must first be able to identify compounds and assess their respective bioavailabilities to different microbial populations.

There are two primary approaches to the study DOM, the holistic approach, studying the dynamics and characteristics of the entire DOM pool, and the reductionist approach, studying individual compounds or classes of compounds within the DOM pool (Hedges 2002). The holistic approach encompasses investigations of bulk DOM fractions, colored dissolved organic matter spectra and isotopic signatures (Hedges 2002). Traditionally, holistic investigations have been used to determine DOM concentrations. Changes in the concentration of DOM components have been used to assess bioavailability or degradation of a source. This approach provides useful mass balance information however; holistic observations can be biased toward the large refractory fraction of the DOM pool. Interpretations of holistic measurements are also limited in oligotrophic regions where concentrations of DOM are low and nutrient cycling is believed to be efficient thus revealing little or no change in bulk nutrient concentration. The observed static concentrations have lead to mistakenly identifying some DOM pools as refractory (Krogh 1934; Menzel 1964).

The reductionist approach investigates a smaller portion of the DOM pool but at higher resolution (compound or class level) showing subtle differences that can not be observed with bulk analyses. This approach fills in the gaps of knowledge left by holistic investigations. With the ability to identify and track changes in a single compound or groups of compounds we are better suited to investigate the dynamic nature of marine

DOM. This dissertation uses both the holistic and reductionist approaches to advance our understanding of the role DOM in marine ecosystems.

1.1.1 DOM dynamics

The term DOM represents all organic compounds no matter what other elements are also present. Dissolved organic carbon (DOC), dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) are often thought of as separate organic entities however, these are all subgroups of the same DOM pool. All DOM compounds can be classified as DOC, however, DON and DOP compounds are those DOC compounds that also contain N and/ or P. One compound can be represented in all three (DOC, DON and DOP) classifications.

Early investigations suggested that oceanic DOC was a static pool of refractory compounds (Krogh 1934; Menzel 1964). This assessment was based on vertical profiles of DOC that showed a relatively constant concentration with depth (Krogh 1934; Menzel 1964). This notion was further supported by radiocarbon assessments which indicated that the DOM within the Pacific Ocean had an average age of approximately 3400 years (Williams et al. 1969). While these and other investigations provided significant evidence that deep oceanic DOC was refractory, other investigations concluded the exact opposite for surface DOM. One such investigation showed that DOC varied seasonally, increasing in concentration with phytoplankton abundance (Duursma1963), indicating that phytoplankton may serve as significant source of DOC in the surface ocean.

In the mid 1980's a new method for DOC and total dissolved nitrogen (TDN) quantification appeared to challenge the old concentrations and perspectives of DOC in marine systems (Suzuki et al. 1985; Sugimura and Suzuki 1988; Hedges 2002). The high

temperature catalytic oxidation (HTCO) method (Suzuki et al .1985) revealed concentrations significantly higher than previously recorded with the then commonly used persulfate oxidation method (Menzel and Vaccaro 1964). This new method and the implications of the higher DOC concentrations sparked a great deal of interest in the DOM community. Although the initial Suzuki method was later determined to be flawed, the DOM community had come to a consensus on a revised version of the HTCO method and within this time period of trial and error a great deal of research on marine DOM dynamics had occurred re-energizing the field of marine DOM research (Hedges 2002; Sharp 2002).

Historically, there has been a greater emphasis on DOC dynamics than either DON or DOP (Sharp 2002), however, with increasing evidence of the importance of DON and DOP as nutrient sources in aquatic systems the bias toward DOC is quickly fading (Bronk 2002; Karl and Bjorkman 2002; Berman and Bronk 2003). DON concentrations often exceed DIN concentrations in aquatic systems representing between 18 and 85% of the total nitrogen pool in coastal and open ocean surface water (Antia et al. 1991; Bronk 2002). Like DOC, DON was once believed to be primarily refractory but is now considered an important nutrient source in aquatic systems (Berman and Bronk 2003). DON concentrations decrease with increasing distance from the coast with rivers generally having the highest mean concentration (34.7 ± 20.7 μM DON) and open ocean waters having the lowest mean concentration (5.8 ± 2.0 μM DON; (Bronk 2002)). The major source of DOM also changes from terrestrial to marine produced farther away from coast (Murphy et al. 2008). Although they differ in sources, DOM serves an important role in both coastal and oceanic food webs. Despite a great deal of evidence, like DOC,

DON remains largely (> 85%) uncharacterized (Benner 2002; Bronk 2002) and dynamics associated with the production and cycling of these different pools is still not understood.

1.1.2 DOM and the microbial loop

Nearly simultaneously to the DOC revolution, the microbial community was embracing the new concept of a microbial loop (Pomeroy 1974; Azam et al. 1983). The premise of the traditional aquatic food web is that phytoplankton access inorganic nutrients autotrophically binding them into particulate organic matter (POM) and DOM. The phytoplankton (in this case also considered the POM) is then consumed by zooplankton passing the nutrients on to the next trophic level. The difference between the microbial loop and the traditional aquatic food web is that bacteria are present to remineralize the DOM and POM from planktonic excretions and dead cells. These bacteria then remineralize the DOM and POM freeing the bound nutrients, converting them into their inorganic form making them once again available for uptake by phytoplankton and thus allowing those once bound nutrients to re-enter the system. This method of nutrient regeneration would enable essential elements like N and P to potentially be used by a community several times before it was bound into a refractory component of the DOM pool. This is an extremely important process because in many estuarine and coastal systems nitrogen is the nutrient most limiting primary production (Ryther and Dunstand 1971; Oviatt et al. 1995). Recycling bound nutrients would increase the nutrient efficiencies and productivity in nutrient limited regions.

Microbial communities act as both sources and sinks of DOM in aquatic systems. DOM can be released by healthy cells during growth, natural cell death or through predation of phytoplankton or bacterial cells by viruses, bacteria, phytoplankton or

zooplankton (Fogg and Boalch 1958; Lampert 1978; Glibert and Bronk 1994; McCarthty et al 1998; Carlson 2002; Berman and Bronk 2003). DON released by phytoplankton and bacteria can be significant (Carlson 2002). DON release associated with N_2 fixation by diazotroph *Trichodesmium* sp. has been shown to increase background DON concentrations in the Gulf of Mexico by a factor of 3 or 4 (15-20 μM N; Lenes et al. 2001). Seasonal trends in DON concentrations have been observed with average concentrations increasing in spring and summer and decreasing in fall and winter (Butler et al. 1979; Bronk et al. 1998). This increase in DOM concentration correlates with primary production within these regions (Duursma 1963) further supporting the notion that phytoplankton are a significant sources of DON. However, this correlation is not true for all blooms under all conditions (Bronk 2002) and understanding the variability in DOM production remains an active area of research.

The majority of phytoplankton growth in the ocean is limited by nutrients (Hecky and Kilham 1988). DOM has the potential to relieve nutrient stress as organic nutrients are one of many factors considered to be important in the growth and proliferation of phytoplankton (Antia et al. 1991; Bronk et al. 2007). Some phytoplankton species grow better on these organic compounds than on inorganic forms (e.g.,Berg et al. 1997; Gobler and Sañudo-Wilhelmy 2001a). The classic interpretation of phytoplankton growth depicts a strict photoautotroph thriving only on inorganic carbon and inorganic nutrients, however, many phytoplankton species are able to supplement their nutrition with organic nutrients in the form of DOC, DON and/or DOP. The ability for photosynthetic species to supplement their nutrition through the consumption of POM or DOM is called mixotrophy (Smayda, 1997; Stoecker et al. 1997; Granèli et al. 1999; Taylor et al. 2006).

Approximately 20% of all phytoplankton are believed to have mixotrophic capabilities and this nutrient strategy is believed to be common in low light and low nutrient conditions (Stoecker 1991).

1.1.3 *Investigating DOM and microbial dynamics*

Early work has demonstrated that DOM provides an energy, carbon and nutrient source planktonic populations in aquatic systems and can be labile on time scales of hours to years (Azam et al. 1983; Keil and Kirchman 1991; Carlson and Ducklow 1996; Rich et al. 1997; Carlson et al. 1999). This was confirmed by field observations and controlled laboratory studies. These studies often investigated the uptake of individual known organic compounds such as glucose, urea and amino acids by various phytoplankton species and populations (examples include Carpenter et al. 1972; McCarthy 1972; Wheeler et al. 1977; Flynn and Butler 1986; Gobler and Sañudo-Wilhelmy 2001a). Although studies of individual compounds have provided evidence that phytoplankton are capable of using specific fractions of DOM, known compounds generally only represent a small portion (11-14%) of natural DOM (Benner, 2002; Bronk, 2002). These known compounds may not reflect the bioavailability of the thousands of organic compounds that comprise the largely uncharacterized bulk DOM pool.

Several experimental strategies have been employed to investigate DOM bioavailability. These strategies include bioassay experiments, enzyme activity evaluations and nutrient uptake kinetics experiments. The use of bioassay experiments to investigate nutrient bioavailability is relatively common. A nutrient source (i.e. a single compound or suite of compounds) is added to a subsample of a natural population and changes in population abundance and/ or composition are used to assess bioavailability.

If populations increase in abundance the source is considered bioavailable. By measuring the response of one community to multiple sources, nutrient limitation and preference can be assessed. Alkaline phosphatase and urease activities are a measure of the enzymes capable of degrading DOP and urea, a form of DON, respectively into their inorganic forms. Investigations of the activities of these enzymes revealed that a number of phytoplankton species have the ability to use organic nutrients (Remsen et al 1972; Rose and Axler 1998; Dyhrman and Anderson 2003; Fan et al. 2003). Uptake experiments trace isotopicly labeled (e.g.^{15}N) compounds into the cell by measuring incorporation rates of these isotopes from the dissolved to the particulate fraction (Dugdale and Wilkerson 1986). Using these methods, several complex multi-compound DOM sources such as humic acids, river water concentrates, rainwater and storm water runoff have also been shown to increase phytoplankton abundance in both cultures and natural populations (Prakash and Rashid 1968; Peierls and Paerl 1997; Seitzinger et al. 2002; Boyer et al. 2006; See et al. 2006). However, the response to DOM additions vary based on the microbial community present. While bulk DOC, DON and DOP concentrations indicate that nutrients are present, these classifications do not indicate if these sources are available to a given microbial community. Therefore, the quality of the compounds present appears to play a larger role in bloom dynamics than simply the quantity/concentration of the total DOM pool.

The details of the relationship between phytoplankton and DOM in nature are not well known. This reflects the gap in our understanding of the complexity of phytoplankton physiology, phytoplankton ecology as well as a lack of analytical methods to characterize the DOM in complex samples at the compound level. Identifying the

bioavailable compounds within a source will help us identify the properties that make a compound or source bioavailable. Analytical limitations as well as new methodological and analytical approaches used to investigate complex marine DOM at the compound level are discussed further in chapter 2.

In contrast to enhancing growth as a nutrient, organic compounds can also be toxic to marine biota including phytoplankton and bacterial populations. Allelochemicals and marine toxins are naturally produced organic compounds that have negative affects on marine communities. Allelopathic chemicals are secondary metabolite (toxins) produced by plants, phytoplankton, bacteria and fungi that biochemically inhibit the growth of co-occurring species (Molisch 1937; Muller 1969). Allelopathic relationships are believed to be common among aquatic microbial populations (Maestrini and Bonin1981; Legrand et al. 2003) and may play a role in maintaining species diversity in the ocean (Roy and Chattopadhyay 2007). This form of chemical warfare may also aid in the distortion microbial populations and elimination of competitors (Granéli 2006). The affects of one such marine toxin, brevetoxin, on bacterial community abundance and composition is discussed further in chapter 5.

1.1.4 Sources and sinks of DOM in coastal systems

DOM in coastal systems comes from both allochthonous and autochthonous sources. Allochthonous sources include atmospheric deposition, rivers, overland flow, advection and ground water. The main autochthonous sources of DOM in the coastal ocean include phytoplankton and bacteria. Understanding the bioavailability of DOM from rivers and DOM produced through phytoplankton growth to marine microbial populations remains an open question (Fig. 1-1)

Land derived DOM from riverine and overland flow is very complex. Terrestrial soils, plant matter as well as runoff from urban, agricultural and industrial sources are all potential contributors to riverine DOM. It is estimated that between 20 and 70% of DON supplied by rivers is bioavailable to aquatic microbial populations (Manny and Wetzel 1973; Seitzinger and Sanders 1997; Stepanauskas et al 2000; Seitzinger et al 2002). This difference in bioavailability is likely due to a difference in DON chemical composition. Land use practices within a given watershed are reflected in the DOM of the subsequent rivers and streams, rivers and runoff from different land use practices have different DOM signatures (Seitzinger et al 2005). Those DOM signatures from similar land use practices have similar DOM signatures (Seitzinger et al 2005; reviewed in chapter 2). The effects of these differences on microbial bioavailability is not known.

While land-derived DOM serves as a significant source of nutrients to estuarine and coastal populations, those species that thrive in offshore oligotrophic waters attain the majority of their nutrients from other sources including autochthonous production. As discussed earlier, phytoplankton and bacteria can be a significant source of DOM in aquatic systems. Unlike riverine DOM which can have numerous contributing factors, autochthonous DOM sources are limited to those species that are able to convert inorganic nutrients into organic nutrients. While heterotrophic bacteria do produce DOM compounds from DOM compounds they are more accurately converting the DOM pool but not adding "new" DOM to it.

Although the DOM produced by one species is a simple point source, the suite of compounds produced by any one species can be very complex (e.g. Capone et al. 1994; Gruber et al 2006). As discussed earlier, phytoplankton can act as both producers and

consumers of DOM. It is also believed that this production of nutrients by one species may fuel the growth of another, simultaneously or sequentially (Walsh and Steidinger 2001; Walsh et al 2006). Therefore, it is important to understand the roles that DOM and mixotrophy play in phytoplankton productivity and community composition.

1.2 Objectives of Dissertation

This dissertation seeks to address the following questions:

1.) How will electrospray ionization (ESI) mass spectrometry enable our understanding of DOM and phytoplankton ecology in nature?

2.) Are *Synechococcus* blooms in Florida Bay stimulated by different land based DOM sources and what masses within each source are bioavailable to this community?

3.) Does the DOM produced by *Trichodesmium* sp. enhance the growth of red tide *Karenia brevis*, what masses within this DOM are bioavailable this population and how does this nutrient source and the subsequent growth of *K.brevis* affect the associated bacterial community composition?

4.) Does brevetoxin affect bacterial community abundance and composition?

1.2.1 Dissertation Overview

This dissertation documents the response of natural phytoplankton and bacterial communities to and the bioavailability at the bulk nutrient and unit mass level of allochthonous and autochthonous DOM source. Chapter 2 addresses the analytical limitations associated with compound level reductionist approaches by providing an overview of the benefits and caveats of using a relatively new instrumental application, electrospray ionization (ESI), to investigate DOM dynamics in natural freshwater and marine systems. It also provides the first compound level ESI-MS DOM characterization spectra of the DOM associated with a natural bloom of harmful algae raphidophyte *Chattonella cf verruculosa*.

Chapter 3 investigates the affect of allochthonous DOM on coastal marine phytoplankton populations by assessing composition and bioavailability of two riverine DOM sources to a natural *Synechococcus* bloom in Florida Bay. DOM from two different Everglades riverine sources, Shark River Slough (SR) and Taylor Slough (TS), was supplied to a *Synechococcus spp*. dominated phytoplankton community in 96 hour bioassay experiments to determine their respective availabilities. These two sources represent areas of different land use practices and differed in chemical compositions at the bulk nutrient (μM) and compound (unit mass) level. *Synechococcus* spp. responded positively to both nutrient sources. DON from TS was found to be at least 25% available to this *Synechococcus* spp. community compared to SR which was at least 10% available. Through the use of ESI-MS previously uncharacterized bioavailable masses within natural land derived DOM sources were identified.

The bioavailability of phytoplankton produced autochthonous DOM was investigated in chapter 4. DOM from laboratory cultures of *Trichodesmium sp*. was isolated, concentrated and then supplied as a nutrient source to a natural population of *Karenia brevis* collected from the Gulf of Mexico in a 216 hour bioassay experiment. *Karenia spp.* began to increase immediately after the *Trichodesmium* sp. cellular exudates (TCE) addition allowing populations to reach specific growth rates of 1 cell division day^{-1}. There was rapid and complete utilization of the TCE DON supplied. ESI-MS was used to investigate the bioavailability of *Trichodesmium* sp. produced DOM at the compound (unit mass) level. Changes in the bacterial community composition associated with the DOM addition and subsequent growth of *K.brevis* were assessed using terminal restriction fragment length polymorphism (TRFLP). This investigation revealed that

K.brevis can achieve growth rates of 1 cell division day^{-1} on complex DON sources, found that at least 53% of DON produced by *Trichodesmium* is bioavailable, identified previously uncharacterized bioavailable masses produced *Trichodesmium* to *K.brevis* and showed that bacteria community composition was affected by *K.brevis* growth.

Chapter 5 builds on the results of chapter 4 by exploring the relationship between *Karenia brevis* and bacteria through the investigation of the effects of brevetoxin, the neurotoxin produced naturally by *K.brevis* and raphidophyte *Chattonella cf. verruculosa* on bacterial community abundance and composition. Synthesized brevetoxin was added to bacterial communities from three different bay locations (1) Great Bay, New Jersey, (2) Rehoboth Bay, Delaware and (3) Sarasota Bay, Florida, with varying historical brevetoxin exposure from no documented exposure, occasional exposure, and frequent exposure, respectively in a 48 hour bioassay experiment. These bacterial communities differed in the concentration of brevetoxin required to observe a significant decreases in live bacterial number. Those populations with limited or no exposure were more susceptible to the effects of brevetoxin than those populations frequently exposed to brevetoxin. Assessments of bacterial community composition revealed that bacterial communities with limited or no previous exposure became more similar with increasing brevetoxin concentrations indicating that brevetoxin was selecting for specific species or groups of species and against others. The resilient species within the bay communities were compared to the bacterial communities associated with a natural *K.brevis* bloom from the Gulf of Mexico and two different *K.brevis* cultures with different levels of brevetoxin production. Six species were common among all of the bay samples, the

natural *K.brevis* bloom and both *K.brevis* cultures. Four of these represented greater than 2% of the total community in all of the above mentioned samples.

Understanding the role that DOM plays in the microbial loop is essential to understand population and bloom dynamics. These investigations together have advanced our knowledge of the role of DOM in the marine microbial loop. Through the use of ESI-MS and TRFLP higher resolution investigations of DOM and bacterial community dynamics can be observed.

1.3 Implications

The role that DOM plays in determining marine microbial community composition is still an open question. The objective of this research was to gain insight into the effect of DOM on phytoplankton and bacterial community abundance and composition. This objective was accomplished by investigating the effects of allochthonous and autochthonous DOM on natural phytoplankton and bacterial community abundance and composition and assessing the bioavailability of the DOM supplied at the compound (unit mass) level. Through the use of advanced chemical (ESI-MS) and molecular (TRFLP) techniques significant advancements have been made.

Florida Bay like many coastal systems is strongly influenced by allochthonous DOM sources (Boyer and Keller 2007; Madden in Press). Recent legislation has outlined a Comprehensive Everglades Restoration Plan to restore the freshwater resources of central and southern Florida (Perry 2004). This effort would increase the flow of freshwater through Everglades National Park and increase the volume of freshwater reaching Florida Bay. The restoration of flow through Everglades National Park has implications for the future of Florida Bay by potentially altering the rate, concentration and composition of

nutrients reaching this estuary. This change in flow may be significant to this system because riverine DOM is believed to at least in part support the large *Synechococcus* spp. dominated phytoplankton blooms plaguing this system (Glibert et al. 2004; Boyer et al. 2006; Madden in press).

This study has assessed the bioavailability of two different Everglades National Park riverine sources, Shark River (SR) and Taylor Slough (TS) which possess different bulk nutrient and unit mass signatures. To better understand the potential affects that increased input from these sources would have on the phytoplankton community, the percentage of the bioavailable DON within each of these systems evaluated. DON from TS was found to be at least 25% bioavailable as compared to SR which was found to be at least 10% bioavailable. These values will help predict and model the effects of increased flow from these sources to *Synechococcus* spp. communities.

Beyond holistic, bulk nutrient assessments of DOM, reductionist assessments of DOM at the unit mass level using ESI-MS were also performed. The ESI-MS evaluation represents the first chemical characterization of either of these rivers at the compounds (unit mass) level. Through the use of ESI-MS this study was also the first to assess the bioavailability of riverine DOM to a natural phytoplankton bloom at the unit mass level. The bioavailable masses within the TS treatment were found to be more similar to those within Florida Bay study site water than to SR bioavailable masses. This similarity in bioavailable masses reflects the influence of TS or sources of similar land use practices on the Florida Bay site. Through the use of ESI-MS previously uncharacterized bioavailable masses from land derived DOM sources were identified.

In the past, complex ecological models were required to explain what conditions might allow the "slow-growing" species *Karenia brevis* to thrive in the oligotrophic waters of the Gulf of Mexico (Bissett et al. 2008). Beyond the low observed growth rates, the sources of nitrogen required to sustain large blooms of *K.brevis* had also not been determined (Vargo et al. 2004). By quantifying the percentage of bioavailable DON produced by *Trichodesmium*, applying new stoichometric parameters for population growth and re-defining maximum achievable growth rates for *Karenia brevis* this dissertation has provided crucial information that can be applied to current and future models to better predict the growth and proliferation of this ecologically significant species. The TCE bioassay experiment described in chapter 4 was the first investigation to document growth rate of 1 division day^{-1} in a natural *K.brevis* bloom. It is also the first study to investigate the bioavailability of *Trichodesmium* produced DOM at the compound (unit mass) level and the first to study to investigate the changes in bacterial community composition associated with the growth *K.brevis*. Due to the diversity in known compounds used including urea, amino acids (Baden and Mende 1979; Bronk et al. 2004), and the complete utilization of *Trichodesmium* sp. derived DON in this study it is clear that both natural and anthropogenic N sources support and enhance growth within *K.brevis* blooms. This implies that *K.brevis* will thrive on both allochthonous and autochthonous DON sources and will become more abundant if either of these sources were to increase.

While the TCE experiments described in chapter 4 revealed that *Karenia brevis* affects bacterial community composition, the direct link between *K.brevis* and bacterial community composition remained unclear. In chapter 5 brevetoxin, a neurotoxin

produced by *K.brevis* and *Chattonella cf. verruculosa*, was found to directly affect natural bacterial community abundance and composition. This was the first study to investigate the direct allelopathic effects of brevetoxin on natural microbial populations. This study revealed that bacterial communities differed in the concentration of brevetoxin required to illicit a significant decrease in the number of live bacteria. Those populations with limited or no previous exposure were more susceptible to the effects of brevetoxins than those populations frequently exposed to brevetoxin. Frequently exposed communities showed no significant difference in bacterial abundance or community composition between any of the brevetoxin concentrations investigated identifying an apparent community tolerance to this toxin. Bacterial communities with limited or no previous exposure became more similar with increasing brevetoxin concentrations. This indicates that brevetoxin selects for specific species or groups of species and against others. Due to the diverse ecological niches that sustain blooms of *K.brevis* and *C. cf. verruculosa* understanding the effects of brevetoxin on previously unexposed populations is critical in understanding bloom dynamics and community composition of affected areas. Based on the number of species eradicated in the 200µg/L brevetoxin treatment after 48 hours, as much as 37% of the species present in a system could be lost through the introduction of brevetoxin to a previously unexposed community. This loss of almost 40% of the species would on larger scales be considered a mass extinction (Arens and West 2008). The implications of eliminating as significant number of species within a system range from alteration of local nutrient cycles, primary production and the composition of other genera. Therefore, if the presence of brevetoxin increases in frequency and concentration in systems with limited or no previous exposure to

brevetoxin, decreases in bacterial number and changes in community composition would be expected.

Species resilient against brevetoxin additions were compared to those detected in a natural *K.brevis* bloom transect and two *K.brevis* cultures known to produces different levels of brevetoxin. These resilient species represent populations unaffected by brevetoxin and may be species with algicial or symbiotic relationships with *K.brevis* or simply species that have developed a tolerance to this potent toxin. Each of these scenarios has implications for pharmacological applications and bloom or toxin mediation.

This dissertation provides a closer look at some of the sources, compounds and species that mediate microbial community composition. It has used both holistic and reductionist approaches to assess DOM dynamics within marine systems. This work has advanced our knowledge of the bioavailability marine DOM.

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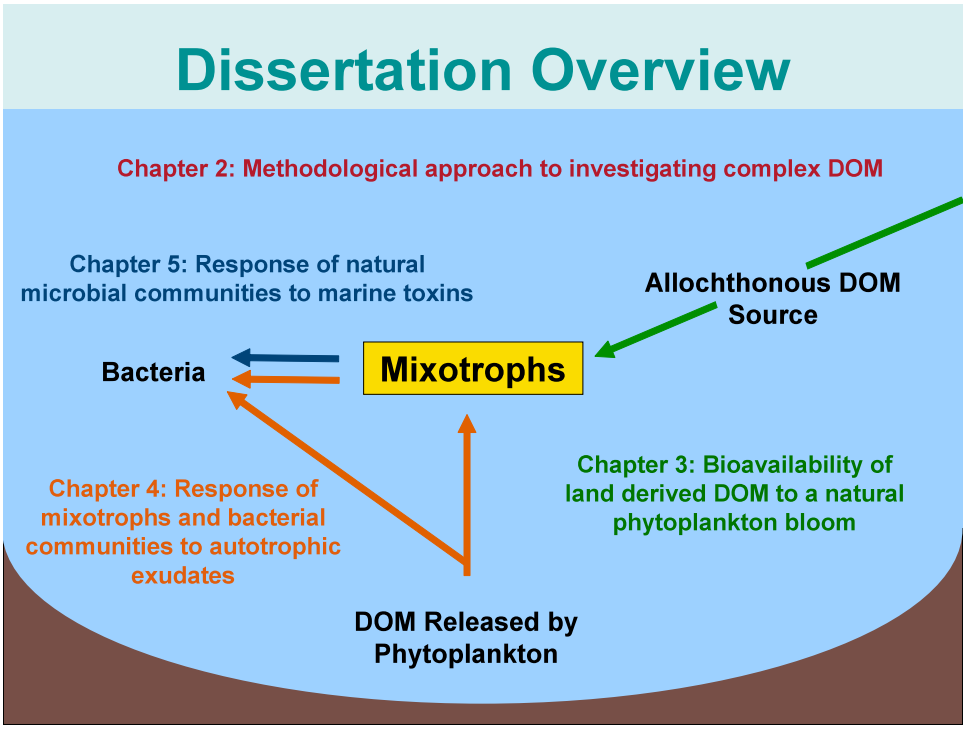


Fig. 1-1. Dissertation overview: How the research presented in this dissertation relates to marine ecosystem dynamics

Chapter 2. Use of Electrospray Ionization (ESI) Mass Spectrometry to Investigate Complex Dissolved Organic Matter (DOM) and its Potential Applications in Phytoplankton Research

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2.1 Abstract

Organic nutrients are one of many factors considered to be important in the growth and proliferation of phytoplankton including many species that cause harmful algal blooms (HABs). Several studies have investigated the effects of known organic compounds on phytoplankton growth, however, the role of natural dissolved organic matter (DOM) in phytoplankton nutrition remains understudied at the compound level. This lack of research is due in part to analytical limitations for the characterization of DOM compounds. Electrospray ionization (ESI) mass spectrometry provides an unprecedented level of chemical information on thousands of organic compounds that comprise the bulk DOM pool. In this paper we provide a brief overview of some of the benefits and caveats of using ESI to investigate DOM in natural freshwater and marine systems and show an example of ESI-MS DOM characterization for a natural bloom of the raphidophyte *Chattonella cf verruculosa*.

2.2 Introduction

Dissolved organic matter (DOM) is crucial to microbial production in aquatic systems. It accounts for greater than 97% of the total organic matter present in seawater and is one of the major reservoirs of carbon on earth, equaling the amount of CO_2 present in the atmosphere (Siegenthaler and Sarmiento 1993; Benner 2002). DOM is produced by all organisms and is used as a nutrient source by bacteria and some phytoplankton (Azam et al. 1983; Antia et al. 1991; Granèli et al. 1999; Berman and Bronk 2003; Glibert and Legrand 2006).

Numerous studies have investigated the uptake of individual known organic compounds such as glucose, urea and amino acids by various phytoplankton species and populations (examples include Carpenter et al. 1972; McCarthy 1972; Wheeler et al. 1977; Flynn and Butler 1986; Gobler and Sañudo-Wilhelmy 2001). Although these studies have provided evidence that phytoplankton are capable of using specific fractions of DOM, known compounds generally only represent a small portion of natural DOM (Benner 2002; Bronk 2002). These known compounds may not reflect the bioavailability to phytoplankton of the thousands of organic compounds that comprise the bulk DOM pool.

Several complex multi-compound DOM sources such as humic acids, river water concentrates, rainwater and storm water runoff have also been shown to increase phytoplankton abundance in both cultures and natural populations (Prakash and Rashid 1968; Peierls and Paerl 1997; Seitzinger et al. 2002; Boyer et al. 2006; See et al. 2006). Although it is known that phytoplankton can use some DOM compounds, the details of the relationship between phytoplankton and DOM in nature are not well known. This is

not only due to complicated physiology and community structure, but also to a lack of analytical methods to characterize the DOM in complex samples at the compound level. A number of analytical techniques (e.g., nuclear magnetic resonance (NMR; Simpson et al. 2001), gas chromatography mass spectrometry (GC-MS; Rowland et al. 2001), direct temperature- mass spectrometry (DT-MS; Simjouw et al. 2004) provide insight into the "black box" of DOM; however, approximately 75% of natural DOM still remains uncharacterized at the individual compound level (Benner 2002; Bronk 2002). Here we describe several analytical methods using Electrospray ionization (ESI) mass spectrometry to investigate the uncharacterized compounds that comprise the bulk DOM pool and we provide selected examples that illustrate the use of ESI mass spectrometry to investigate complex environmental questions and its potential use in the study of phytoplankton nutrition.

2.2.1 Electrospray Ionization – How it works

ESI mass spectrometry is a relatively new analytical tool for characterizing ionizable organic compounds. Electrospray is designed to convert liquids into aerosols using electricity, not gas, to form the droplets (described in Kebarle and Ho 1997). Unlike many other types of inlet systems that fragment compounds, ESI is a soft ionization inlet system that can allow for the detection of complete non-fragmented compounds (Marshall et al. 1998). To convert liquids into aerosols a positive or negative charge is applied to a liquid sample via a capillary tip. The liquid sample is destabilized by increasing amounts of charge. When the sample reaches the point when no more charge can be held by the liquid it disperses forming an aerosol of highly charged droplets. As the droplets flow through the system the carrier solvent evaporates leaving

behind the charged ions that can be detected using a variety of instruments including several different types of mass spectrometers (McEwn and Larsen 1997: Chernushevich et al. 1997; Bier and Schwartz 1997; Laude et al. 1997; These and Reemtsma 2003). Compounds are detected with mass spectrometers based on mass to charge ratios. Singly charged compounds represent their molecular weight MW +1 $(MW+H)^+$ in the positive ionization mode and MW-1 $(MW-H)^-$ in the negative ionization mode (McEwen and Larsen 1997). A number of more detailed reviews address the capabilities and caveats of ESI mass spectrometry including the use of ESI mass spectrometry to investigate both high molecular weight and low molecular weight compounds (Gaskell 1997; Marshall et al. 1998; Kebarle 2000; Kujawinski 2002).

2.2.2 Pairing ESI with other analytical instruments

There are a number of different instrumental applications that use ESI as an inlet system. Here we briefly review three instrumental pairings: ESI with a single quadrupole detector (ESI-MS), ESI with tandem mass spectrometry (ESI-Tandem MS or ESI-MS/MS) including ion traps, and ESI with Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS). Other applications not reviewed in this paper include time-of-flight mass spectrometry (Chernushevich et al. 1997) and liquid chromatography paired with ESI-MS (Voyksner 1997).

2.2.2.1 Single Quadrupole (ESI-MS)

The ESI-MS with single quadrupole detection has unit mass resolution and provides molecular weight information of detected compounds represented as a mass to charge ratio (*m/z*). Ion abundance is used as a measure of concentrations. With unit mass resolution one mass may represent one or more compounds (Kebarle and Ho 1997).

ESI-MS can be used to process a large number of samples per day, to determine which masses (*m/z*) are present and track changes in each of those masses via changes in the ion abundance and can be used quantitatively using authentic standards. Furthermore, characteristics of the functional groups associated with each compound can be determined by the detection of a compound in one or both of the two ionization modes, positive and negative (Kebarle and Ho 1997). A response in the positive ionization mode indicates basic functional groups (e.g., alcohols) while a response in the negative ionization mode indicates more acidic functional groups (e.g., carboxylic acids) as demonstrated by standards (Seitzinger et al. 2003). In order to further characterize individual compounds in terms of exact structure and chemical composition, ESI coupled with other detectors can be used.

2.2.2.2 Tandem MS (MS/MS) or Ion Trap

While the ESI inlet system does not fragment compounds, it does allow for the intentional fragmentation of individual compounds through the addition of MS/MS or ion traps (Gaskell 1997; McEwen and Larsen 1997). MS/MS uses multiple mass spectrometers in line with each MS adding another level of fragmentation. The ion trap has only one analyzer but is able to focus in on, or trap, one ion/compound for additional fragmentation. While these two instruments are different in terms of mechanical design, they are similar in that both provide information on the chemical structure of a given mass through the fragmentation of the parent ion (Bier and Schwartz 1997). Using the mass spectra obtained through either of these methods, one can begin to recreate the structure of a compound based on the functional groups cleaved during the fragmentation process (McLafferty and Turecek 1993). While this method may not allow one to

determine definitively if a compound contains elements like nitrogen, it can identify if a compound contains functional groups like nitrogen-containing amines or amides with well studied cleavage behavior (McLafferty and Turecek 1993). This is important information when addressing dissolved organic nitrogen (DON) or potentially dissolved organic phosphorus (DOP) bioavailability. For information on the molecular formula of a compound, ESI paired with FT-ICR-MS can be used.

2.2.2.3 FT-ICR-MS

FT-ICR MS provides higher mass resolution than ESI-MS. ESI-MS has unit mass resolution in that it can differentiate between compounds with an *m/z* of 889 vs. an *m/z* of 890. The FT-ICR MS has the capability to obtain sub ppm resolution allowing for a 0.0001 *m/z* or better detection and thus can differentiate between components like CH_4 and O, a difference undetectable with single quadrupole (Marshall et al. 1998; Kujawinski 2002; Koch et al. 2007). With increased mass resolution, FT-ICR MS also has increased mass separation. Unlike ESI-MS where each *m/z* can represent one or more compounds, with FT-ICR MS each *m/z* represents only one molecular formula. However, when trying to determine the molecular formula of that one compound, there may be multiple mathematical molecular formula possibilities. The higher the molecular weight of a compound, the greater the number of possible molecular formulas for that particular mass (Koch et al. 2007). Several papers have suggested "rules" for the accurate molecular formula assignment of natural DOM using ESI FT-ICR MS (Stenson et al. 2003; Koch et al. 2005; Kujawinski and Behn 2006; Koch et al. 2007).

Due to the high mass resolution obtained by FT-ICR MS, it is a great tool for the investigation of specific unknown compounds (*m/z*'s). However, because of the large

amount of data generated for each sample, even for each unit mass, and the large number of potential molecular formula assignments per each compound, ESI FT-ICR MS may not be the best tool to use for a first step assessment of a wide variety or larger number of samples. FT-ICR can not be used quantitatively and relatively few (ten or less) samples can be processed on the instrument per day. When instruments like ESI-MS or MS/MS are used prior to the use of ESI FT-ICR-MS, it may reduce the amount of time required to process samples and data and provide additional levels quantitative and structural information.

2.2.3 Use of the ESI mass spectrometry to study DOM dynamics

ESI mass spectrometry has been used in a wide variety of research fields including complex environmental investigations (examples include Leenheer et al. 2001; Kujawinski et al. 2004; Persson et al. 2005; Koch et al. 2005; Seitzinger et al. 2005a; Kim et al. 2006). ESI-MS with single quadrupole has been used to compare and contrast DOM signatures of different sites and sources; for example, rainwater and suburban streams, have different low molecular weight DOM signatures (Seitzinger et al. 2005a; Seitzinger et al. 2005b; Fig. 2-1). From these samples collected in New Jersey, almost twice as many masses (557 *m/z*'s) were present in the suburban stream sample compared to the rainwater sample (241 *m/z*'s). The rainwater sample also had a narrower molecular weight distribution with no masses greater than *m/z* 500 detected, while 24% of the masses detected in the suburban stream sample had molecular weights greater than 500 *m/z*.

As shown in the above example different sources, like the rainwater and suburban streams, have visibly different DOM signatures, sources with similar origins have similar

signatures. In a comparison of two different suburban streams with similar land uses, 88% of masses detected in the positive mode occurred in both streams (Seitzinger et al. 2005b). ESI-MS is able to detect changes in the presence of individual *m/z*'s as well as the production and consumption of compounds based on changes in their ion abundance. It is this ability to observe differences and similarities among samples that enables us to compare and contrast DOM signatures from various sources and sites including potential comparisons of phytoplankton blooms.

ESI-MS has been useful in observing the biological production of new organic compounds by freshwater bacteria (Gruber et al. 2006). They observed that when a single organic compound, glucose, was initially supplied and consumed by a single species culture of freshwater bacteria (*Pseudomonas chlororaphis*), a number of new organic compounds were rapidly produced. Some of these compounds were consumed (e.g. *m/z* 233) while others persisted throughout the course of the experiment (e.g., *m/z* 517 and 152; Fig. 2-2A). In this experiment over 100 compounds were produced from a single organic precursor, glucose. The production of a large number of organic compounds by even a single microbial species provides insight into the complexities of natural DOM pools. While changes in general ion abundance (concentration) were observed for a number of *m/z*'s in this experiment, the exact molecular formula of the changing *m/z*'s could not be determined with ESI-MS. Compounds identified using ESI-MS that showed significant changes in ion abundance were further evaluated using ESI FT-ICR MS (9.4-T Fourier transform ion cyclotron resonance mass spectrometer equipped with an ESI source at the National High Magnetic Field Laboratory (NHMFL))

to determine the chemical composition. *m/z* 517 from Fig. 2-2A is used as an example for higher resolution FT-ICR MS investigations.

In this experiment with freshwater bacterial DOM, both data from ESI-MS and FT-ICR MS show negligible ion abundance for compounds represented by *m/z* 517 on day zero (Fig. 2-2B). By day two of the experiment both ESI-MS and FT-ICR MS data show a significant increase at m/z 517. FT-ICR MS validated the data obtained through the ESI-MS investigation and provided increased resolution, allowing for molecular formula assignment. The day two peak was identified as *m/z* 517.1432 (Fig. 2-2C). Using MIDAS Formula Calculator Software (v1.1), 144 molecular formulas were possible for *m/z* 517.1432. Following the suggested guidelines outlined by Koch et al. (2007), the list of 144 possible molecular formula assignments were reduced to seven possible molecular formulas for that one compound. With additional statistical and chemical evaluation or through the application of MS/MS or ion trap the number of possible molecular formulas could be further reduced to one and structural information obtained.

In addition to observing the production of unknown compounds from simple known compounds, the ESI-MS has also been used to investigate the bioavailability of complex land derived DOM from a suburban stream to natural assemblages of bacteria (Seitzinger et al. 2005b). Approximately 40% of the land derived DOM compounds supplied to the bacterial assemblage decreased in ion abundance (e.g., *m/z* 265) throughout the course of the experiment (Fig. 2-3), while other masses either increased (5%) or remained unchanged (55%). Duplicate flasks showed good replication in the ion

abundance changes of specific compounds, indicating that equal amounts of the same compound(s) were consumed in both flasks.

ESI FT-ICR MS has been used in similar experiments to investigate the biodegradability of DOM from temperate and tropical streams (Kim et al. 2006). Stream water concentrates were supplied to natural microbial communities as a nutrient source. There was a 22% decrease in bulk dissolved organic carbon (DOC) concentrations in the tropical stream sample compared to a 42% decrease in DOC in the temperate stream sample. This decrease in DOC concentration correlated with a shift to a lower mass region after being exposed to the microbial population based on the FT-ICR analysis. Molecular formulas were also evaluated, the microbial community in this experiment preferentially degraded oxygen-rich molecules while hydrogen deficient molecules were generally refractory, thus providing insight into the properties that make a compound bioavailable.

2.3 Application of salt removal method for use with ESI mass spectrometry

The examples presented for the use of ESI mass spectrometry in molecular level characterization and studying DOM dynamics have thus far been in freshwater environments. As with many other techniques, the salts in brackish and seawater samples tend to interfere with the detection of compounds (Gaskell 1997). As much as 70% of DOC can be recovered from saline samples when ultra filtration (UF) and solid phase extraction (SPE) are used in tandem (Simjouw et al. 2005). Both UF and SPE have been used independently to recover approximately 30% of DOC in saline systems. This new tandem approach to salt removal has doubled the recovery and allows for the investigation of a larger portion of the DOM pool. While SPE has been used to extract

DOM for analysis using ESI FT-ICR MS (Koch et al. 2005), the tandem UF/SPE salt removal method has not been used to date with ESI mass spectrometry to our knowledge.

The analysis of two replicate field samples of a natural *Chattonella cf. verruculosa* bloom was used to demonstrate the precision of the salt removal method when used with ESI-MS. Bloom samples were collected from Russell Canal, a tributary of Jefferson Creek (38^{o} 31.250'N, -75^{o} 3.668'W) in Bethany Beach, Delaware, on August 25, 2006, and had a salinity of 28.1. This bloom was identified and monitored by the Citizens Monitoring Program, affiliated with the University of Delaware, Lewes. While cell counts of *C. verruculosa* are not available for the date sampled, the bloom abundance was 1.11 x10^{6} *C. verruculosa* cells l^{-1} at the same site the previous day, August 24, 2006.

DOM from duplicate *C. verruculosa* samples was extracted using the UF and SPE salt removal method (Simjouw et al. 2005). The DOM of these samples was then characterized using ESI-MS (Agilent 1100 Liquid Chromatograph/ Mass Spectrometer with ESI source) in the positive ionization mode (see Seitzinger et al. (2005b) for details of instrument operation conditions). Good replication between *C. verruculosa* samples was found, with 95% of the masses present in both replicates. Of the remaining masses, 3% were unique to replicate 1 and 2% were unique to replicate 2. The ion abundance also showed a high degree of similarity; of the 95% of masses present in both samples, only 11% were statistically different (95% CI) in ion abundance (concentration). The data from the *C. cf verruculosa* duplicate samples demonstrate the reproducibility in both the presence and ion abundance of the compounds retained during the salt removal process.

To ensure that the similarity of the *C.cf verruculosa* replicates was not due to contamination, procedural blanks from the salt removal process were run using deionized

water (DI). DOC concentrations were determined via high temperature combustion using a Shimatzu 5000 total organic carbon analyzer (Sharp et al. 1993). The DI water blank had a DOC concentration of less than 5μM C and the *C. cf verruculosa* sample had a DOC concentration of 735μM C, indicating a relatively small contribution by contaminants (Fig. 2-4). Therefore, the masses observed represent natural compounds present in samples taken during this *C. cf verruculosa* bloom.

2.4 Future applications of the ESI in phytoplankton research

Understanding the nutritional requirements and preferences of different phytoplankton species is essential to understanding growth dynamics. To fully investigate phytoplankton nutrition and the bioavailability of a DOM source, we need to know more than simply that DOM can be used but what compounds are used. ESI coupled to mass spectrometry is a step forward in determining which compounds play a role in phytoplankton growth.

Although ESI is not yet widely used in the natural sciences, there is growing evidence that it is well suited for complex environmental investigations (examples include Leenheer et al. 2001; Kujawinski et al. 2004; Koch et al. 2005; Seitzinger et al. 2005b; Kim et al. 2006). ESI has a variety of instrumental applications and each application provides different information and varied levels of resolution for molecular weight determination, molecular formula assignment and structural composition assessment.

ESI mass spectrometry has the potential to provide new insights into the role of DOM in phytoplankton dynamics. Although organic nutrients appeared to play an important role in phytoplankton growth, prior to the use of ESI mass spectrometry, there

was no direct way to asses which specific compounds from complex natural mixtures were produced and consumed by phytoplankton. The salts in saline samples also made analysis difficult. Through the use of tandem UF/SPE we can investigate a larger portion of the natural DOM pool. This ability to investigate brackish and saline samples is essential to understanding marine microbial dynamics.

In this paper we have briefly reviewed the use of ESI mass spectrometry to investigate both fresh and saline systems from field and laboratory samples and have used ESI-MS to characterize the DOM of duplicate *C. cf verruculosa* bloom samples, which had not previously been characterized. The tandem UF/SPE salt removal method paired with ESI mass spectrometry has the potential to compare and contrast DOM signatures from various bloom sites and species, evaluate which DOM compounds are being used and produced throughout the course of a bloom, explore the origin of bioavailable compounds, assess the role of DOM in species progression, and investigate DOM cycling within microbial communities. ESI mass spectrometry with other analytical techniques will help us look deeper into the black box of uncharacterized DOM.

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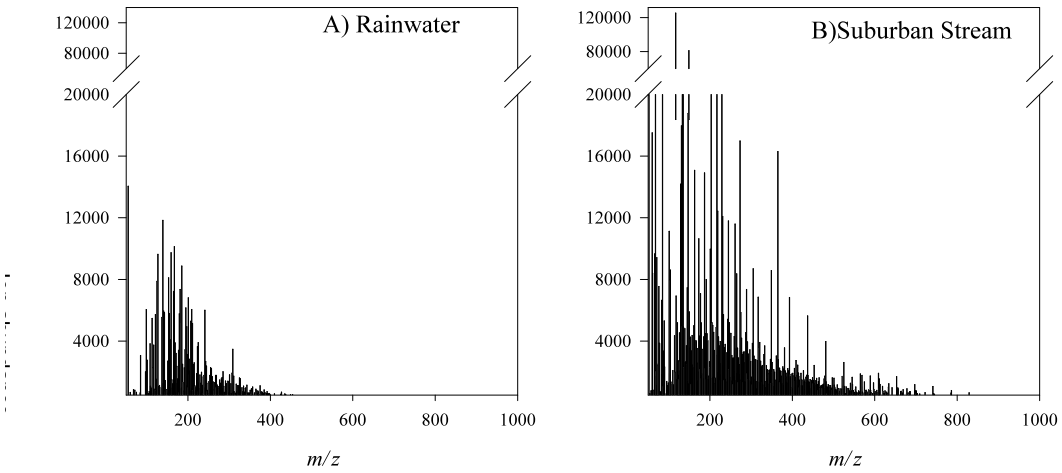


Fig. 2-1. ESI-MS positive mode spectra of A) rainwater sample collected on June 6, 2002 in the New Jersey Pinelands (re-plotted from Seitzinger et al., 2005a) and B.) suburban stream sample collected on April 1, 1998 in New Brunswick, NJ(plot created using data from Seitzinger et al., 2005b).

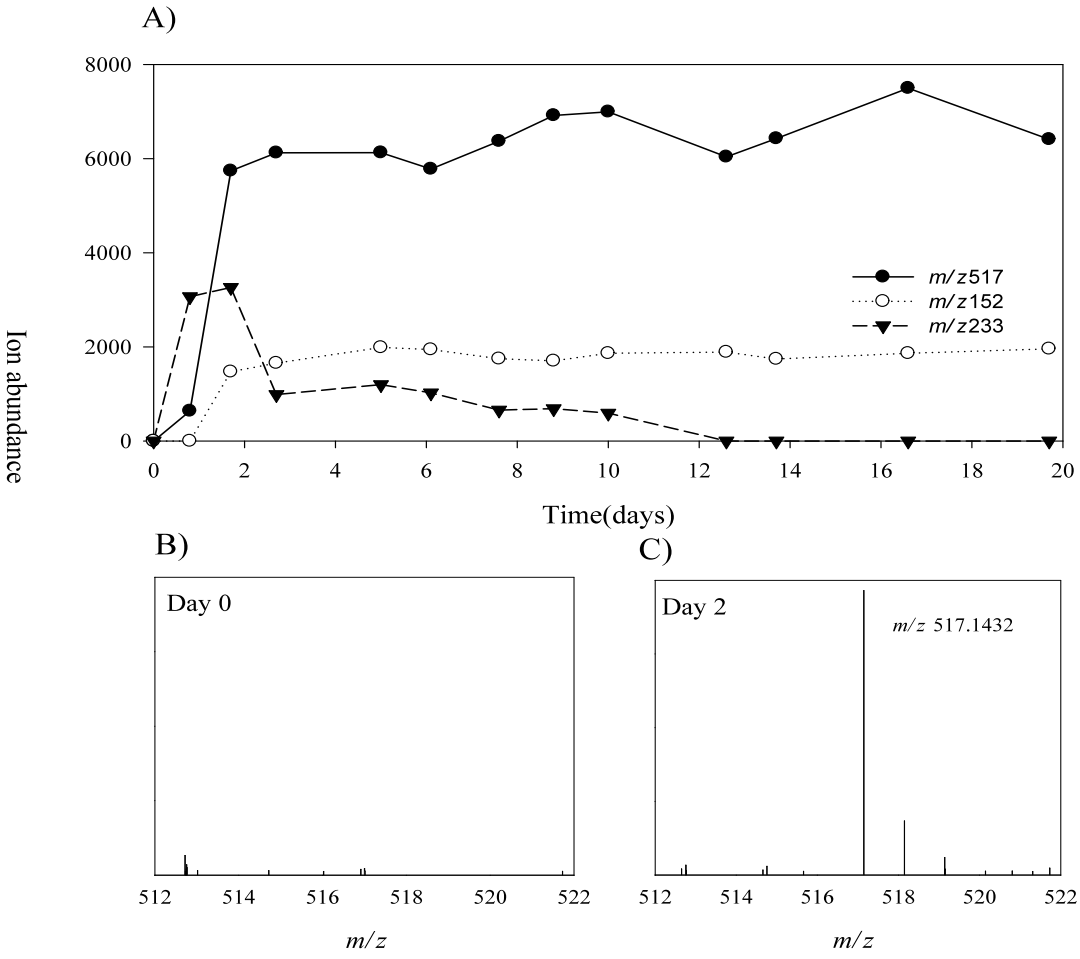


Fig. 2-2. Investigation of biological production of new organic compounds by freshwater bacteria; A) time series plot of compounds produced by a single species of bacteria using ESI-MS (plot created using data from Gruber et al., 2006), B.) ESI FT-ICR MS spectra of day 0 sample and C) ESI FT-ICR MS spectra of day 2 sample showing increase in *m/z* 517.1432.

Fig. 2-3. Examples of the use of ESI-MS to study the consumption of land derived DOM by a natural population of bacteria (re-plotted from Seitzinger et al., 2005) a and b are results from duplicate flasks.

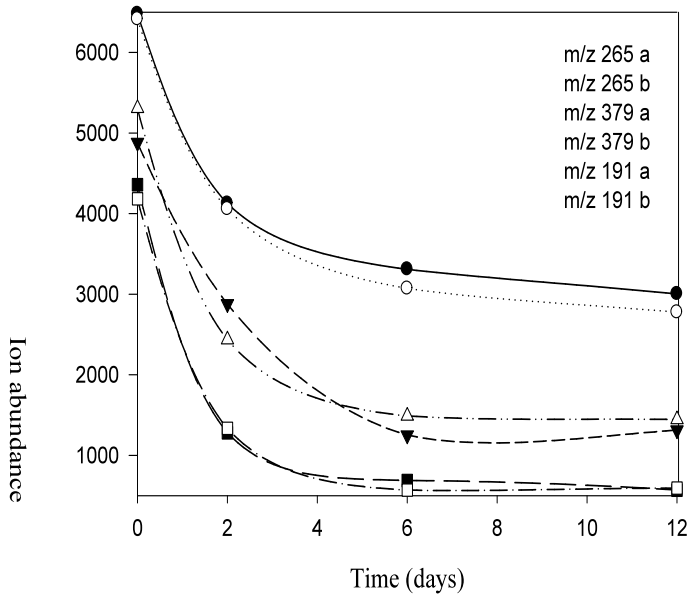
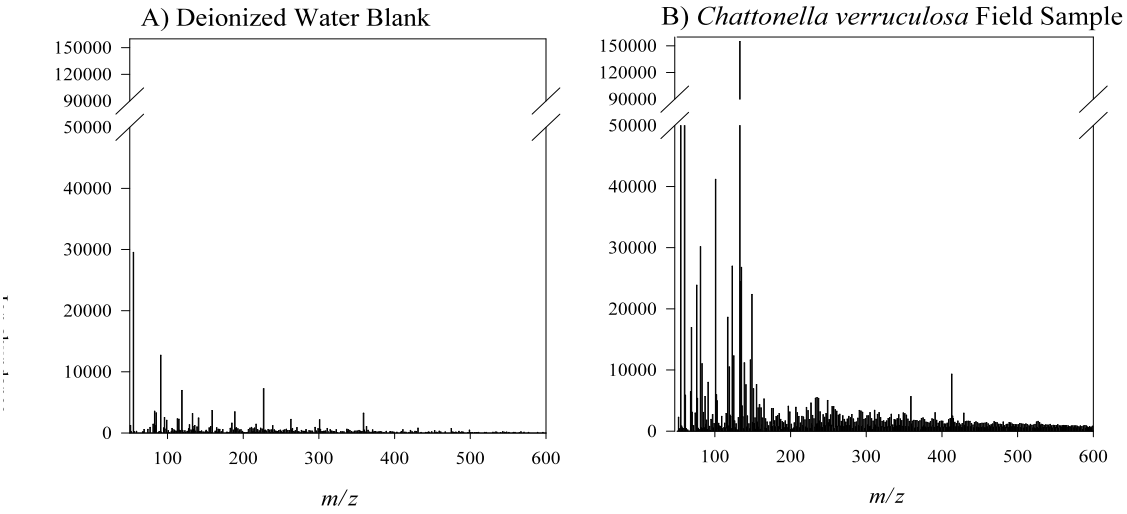


Fig. 2-4. Ion abundance of masses (*m/z*) detected by ESI-MS in the positive ionization mode after salt removal using UF/SPE: A) deionized water and B) field sample from a *C. verruculosa* bloom.



Chapter 3. Chemical characterization of bioavailable dissolved organic matter from two land based sources to a natural *Synechococcus* spp. bloom in Florida Bay

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Sipler, R.E., S.P. Seitzinger, R.J. Lauck, P.M. Glibert, C.A. Heil, A.T. Barlikas. Chemical characterization of bioavailable dissolved organic matter from two land based sources to a natural *Synechococcus* spp. bloom in Florida Bay.

3.1 Abstract

This study is a comparative analysis of the composition and bioavailabilty of two riverine dissolved organic matter (DOM) sources to a *Synechococcus* bloom in Florida Bay. DOM from two different Everglades riverine sources, Shark River Slough (SR) and Taylor Slough (TS), was supplied to a *Synechococcus spp*. dominated phytoplankton community in 96 hour bioassay experiments to determine their respective bioavailabilities. SR and TS represent areas of different land use practices. Differences in chemical compositions between the two sources were observed at the bulk nutrient (μM) and compound (unit mass) level. Only 32% of masses detected using electrospray ionization mass spectrometry (ESI-MS) were present in both sources. *Synechococcus* spp. abundance as size fractioned (<3μm) chlorophyll *a* (Chl *a*) doubled when SR DOM was added and increased by 50% when TS DOM was added. Dissolved organic nitrogen (DON) and dissolved organic phosphorus (DOP) decreased in concentration in both the SR and TS bioassays. DON from TS was at least 25% bioavailable compared to SR which was at least 10% bioavailable. Bioavailability is determined as a significant ($p<0.05$) loss in nutrient concentration or ion abundance. At the unit mass level, the time series bioassay treatment TS was more similar to the Florida Bay study site water than to SR based on masses that significantly ($\alpha=0.05$) decreased in ion abundance. This

similarity in bioavailable masses may reflect the influence of TS or sources of similar land use practices on the Florida Bay site. Through the use of ESI-MS previously uncharacterized bioavailable masses within natural land derived DOM sources were identified.

3.2 Introduction

Florida Bay is a sub-tropical lagoonal estuary located at the southern tip of Florida (USA). Bounded by the mainland to the north and the island chain of the Florida Keys to the east and south, it is a dynamic system with a complex history. Changes in ground and surface water flow as well as oceanic exchange have altered the dynamics and productivity of this system causing increased economic, social as well as environmental concern (Fourqurean and Robblee 1999; Hunt and Nuttle 2007).

Recent legislation has outlined a Comprehensive Everglades Restoration Plan to restore the freshwater resources of central and southern Florida (Perry 2004). This effort would increase the flow of freshwater through Everglades National Park and increase the volume of freshwater reaching Florida Bay. The restoration of flow through Everglades National Park has implications for the future of Florida Bay by potentially altering the rate, concentration and composition of nutrients reaching this estuary. Therefore, an indirect result of returning freshwater flow through Everglades National Park may be increased eutrophication of Florida Bay.

In the early to mid 1900's, the Florida everglades were targeted for "reclamation," which included large scale dredging efforts to drain and clear land for urban and agricultural development (Perry 2004). The reclamation effort effectively reduced the original size of the Everglades by 50% and reduced the fresh water flow through this region to Florida Bay by as much as 70% (USFWS 1999). By the 1980's, the historically

clear waters of Florida Bay began experiencing increased turbidity and dense, nearly mono-specific phytoplankton blooms (Fourqurean and Robblee 1999). These blooms are dominated by *Synechococcus* sp. and reached sufficiently high biomass to affect water quality resulting in negative impacts on aquatic flora and fauna (Thayer et al. 1994; Butler et al. 1995; Thayer et al. 1999; Fourqurean and Robblee 1999). Such blooms continued intermittently throughout the next 2 decades. The most severe blooms occur in late summer through fall and until recently were located nearly exclusively in the central region of Florida Bay (Philips et al. 1999; Glibert et al. 2004). In the past, the phytoplankton community of eastern bay consisted of a diverse assemblage of non-bloom forming phytoplankton including diatoms, dinoflagellates, cyanobacteria and microflagellates (Hitchcock et al. 2007). In 2005 however, large blooms of *Synechococcus* were identified in eastern bay (Madden in press; Glibert et al. in press). *Synechococcus* spp. bloomed nearly continuously for several years in the eastern bay region and was the dominant species during this study (Glibert et al. in press).

Although the cause of these blooms remains unclear, one potential contributing factor may be an influx of anthropogenic nutrients from riverine and overland freshwater discharge (Glibert et al. 2004; Boyer et al. 2006). Due to its wedge like shape, long flushing rates and low tidal influence (Wang et al. 1994; Nuttle et al. 2000), the northeastern and central regions of Florida Bay are greatly influenced by anthropogenic freshwater sources (Boyer and Keller 2007; Madden in press;). The observed increase in phytoplankton blooms correlate with eastern Florida Bay sediment analyses that indicate nutrient enrichment in this region over the past 30 years (Wingard et al. 2007). Terrestrial DOM influence via drainage through Taylor Slough (TS) is high in the eastern

section of the Florida Bay basin (Boyer et al. 2006). Shark River Slough (SR), another potentially important source of organic and inorganic nutrients to Florida Bay, is of greater influence in the western regions of the Bay. SR and TS drain different regions of the everglades and agricultural (sugar cane fields and cattle ranches) land in southern Florida and represent DOM from proportionally different land use sources.

Due to the potential role of SR and TS in shaping the nutrient pool and thus the ecosystem, these sites have been the focus of several investigations (Rudnick et al. 1999; Glibert et al. 2004; Boyer et al. 2006; Heil et al. 2007). Nutrients from TS were once believed to play a minor role in bloom formation (Wingard et al. 2007); however, more recent investigations have shown the nutrients in their organic form may be significantly adding to the overall nutrient pool (Boyer et al. 2006). Bioassay experiments, observed nutrient concentrations, along with alkaline phosphatase and urease activity measurements support the importance of DOM as a nutrient source for phytoplankton and bacterial communities in Florida Bay (Glibert et al. 2004; Williams and Jochem 2006) and more specifically, that some portion of DOM from TS is bioavailable to these communities (Boyer et al. 2006).

Although organic nutrients are often higher in concentrations than inorganic nutrients and are known to be bioavailable to native bacterial and phytoplankton populations (Glibert et al. 2004; Boyer et al. 2006), the characteristics that make a source or compound bioavailable to a system or species is still unknown. It is also unclear what role the DOM from each of these rivers plays in sustaining these nuisance *Synechococcus* spp. blooms. Due to analytical limitations, the majority of DOM compounds in coastal and oceanic waters remain largely unidentified with more than 85% remaining

uncharacterized at the compound level (Benner 2002). Electrospray ionization (ESI) mass spectrometry now enables us to take the next step forward in investigating what masses within a source may be bioavailable to phytoplankton (Sipler and Seitzinger 2008).

The ESI uses a soft ionization inlet system that allows for the detection of complete non-fragmented compounds (Gaskell 1997; Kebarle 2000). This capability has improved our ability to investigate and chemically characterize polar DOM compounds at the molecular level (Kujawinski 2002; Altieri et al. 2008). ESI has been used to assess a variety of complex environmental samples (Seitzinger et al. 2003; Kujawinski et al. 2004; Koch et al. 2005). The ESI- MS with single quadrupole detection has unit mass resolution and provides information on the molecular weight represented as a mass to charge ratio (*m/z*) and concentration as ion abundance of compounds detected. One mass may represent one or more compounds (Kebarle and Ho 1997) and thus compounds cannot be identified in terms of exact structure and chemical composition. However, ESI does allow us to detect what masses are present within a sample and to track changes in each of those masses via changes in the ion abundance of each specific mass.

The goal of this study was to begin chemically characterizing the DOM entering Florida Bay, assess the bioavailability of DOM from SR and TS to a natural *Synechococcus* bloom in Florida Bay, and investigate what masses are bioavailable to this plankton community. To achieve these goals, DOM concentrates from two different land use sources, SR and TS, were concentrated and supplied as a nutrient source for an eastern bay phytoplankton community. The growth responses of the population as well as changes in nutrient concentrations were used to determine the bioavailability of each

source. ESI-MS was used to further investigate the bioavailability of each source at the mass level.

3.3 Methods

3.3.1 Overview

DOM from two rivers in southern Florida, SR and TS, was collected, concentrated and subsequently supplied as nutrient sources in a 96 hr bioassay experiment. The water sample used in the bioassay treatments was collected from Blackwater Sound in eastern Florida Bay and contained a natural phytoplankton population dominated by the cyanobacteria *Synechococcus* spp. The treatments: control, SR DOM addition, and TS DOM addition, were run in duplicate and varied in total quantity of inorganic and organic nutrients added. Population growth (as Chl *a*) and DOM bioavailability (investigated through nutrient stoichiometry and ESI mass spectrometry) were assessed over the 96 hr interval (Table 3-1). This study represents an in-depth investigation of the response of a natural phytoplankton community from a single site to complex land derived DOM. It is a snapshot of the bioavailability potential of two DOM sources to a given community.

3.3.2 DOM source collection and concentrates

River water samples were collected at the end of the wet season on October 24, 2006. The SR collection site was located at 25^{o} 09.015' N, 80^{o} 55.334'W and the TS site was located at 25^{o} 22.950' N, 80^{o} 36.565'W (Fig. 3-1). The source water was filtered on site through tandem 1.0µm and 0.5µm canister filters that had been pre-rinsed with deionized water (DIW). Source water was frozen within 3hrs of collection in acid washed (12% H_2SO_4 followed by multiple DIW rinses) 20L cubitainers. Filtered samples were

transported frozen to Rutgers University in New Brunswick, New Jersey for DOM concentration. At the time of DOM concentration, each source (SR and TS) was additionally filtered through precombusted (500^{o}C for 4 hours) 0.7µm Whatman GF/F filters, divided into 18L aliquots, sub-sampled for nutrient analysis (dissolved organic carbon (DOC), total dissolved nitrogen (TDN), dissolved inorganic nitrogen (DIN (nitrate + nitrite and ammonium)) and orthophosphate), and frozen at -20^{o}C.

The riverine sources were concentrated and de-salted using a tandem tangential flow ultra filtration (UF) and solid phase extraction (SPE) method (modified from Simjouw et al. 2005). The DOM retained in the UF step of the extraction process represents compounds with masses greater than 3kD. Additional compounds present in the UF filtrate are retained using SPE (Simjouw et al. 2005; Sipler and Seitzinger 2008). This tandem method allows the majority of salts and inorganic nutrients to past through as filtrate but retains a portion of the DOM.

DOM was first extracted using a High Flow Watson-Marlow (Model #603S/R) peristaltic pump and a Pall-Tincor (Filtron) tangential flow assembly fitted with a 5ft^{2}, 3kD suspended screen omega Centrasette (OS003C07). The Centrasette was initially conditioned with approximately 100L of DIW followed by 7L of 0.2N NaOH and then a final rinse with an additional 120L of DIW. A subsample of the DIW rinse was checked for DOC contamination prior to sample extraction. Approximately 2L of sample was then used to pre-rinse the Filtron assembly and Centrasette.

Water samples were defrosted immediately prior to concentration. During the concentration process, sample water and the filtrate from the UF extraction were kept on ice to reduce degradation. Filtrate and retentate were both immediately frozen upon the

completion of each 18L aliquot. The retentate was further concentrated through low temperature (60^{o}C) vacuum evaporation (Timperley et al. 1985).

The DOM in the UF filtrate was thawed and then acidified with 1.7mL 6N HCl per 1L sample and additionally extracted using a 90mm 3M Empore C18 SPE disk to retain additional compounds. The SPE disks were conditioned with (2) 40mL aliquots of methanol (MeOH) and (2) 40mL aliquots of DIW. Samples were filtered onto the C18 disks in 4L aliquots and eluted from the disks with (2) 40mL MeOH aliquots. The eluent was then heated at 55^{o}C until all MeOH had evaporated. The DOM was then reconstituted with DIW and re-combined with the UF fraction of the DOM to its original proportions based on the concentration factors of each component. A total of 155L of SR water and 96L of TS water were concentrated using the tandem UF/SPE method.

3.3.3 Field sample collection and bioassays

A bloom dominated by *Synechococcus* spp. was visually observed then microscopically identified in Blackwater Sound near Cross Key on the northeastern portion of Florida Bay at 25^{o} 11.100' N, 80^{o} 23.784'W (Fig. 3-1). A sample was collected on December 2, 2007, via bucket at the surface and was gently filtered through a 153µm mesh to remove larger zooplankton. The water was returned to the Florida Fish and Wildlife Conservation Commission Marathon Key Station in Marathon, Florida, within 3hrs of collection and was evenly divided into six acid washed (12% H_2SO_4 followed by multiple DIW rinses) 2.5L polycarbonate bottles. Duplicate control treatments received no additional DOM. Duplicate SR bioassays received concentrated DOM at a final addition concentration of 92µM DON, 4.6µM DIN, 0.2µM DIP and 3.8µM DOP. Duplicate TS bioassays received concentrated DOM at a final addition

concentration of 32µM DON, 2.4µM DIN, 0.2µM DIP and 1 µM DOP. The difference in nutrient concentrations between the two sources reflects the proportional difference in DON between the two sites at the time of collection. Bioassay bottles were incubated at ambient light, temperature and agitation (tidal mixing) tethered in sample floats located in a sheltered lagoon.

All treatments were sub-sampled for biological parameters (Chl *a*), nutrient analysis (DOC, TDN, nitrate + nitrite, ammonium, total dissolved phosphorus (TDP) and orthophosphate), and chemical characterization (using ESI mass spectrometry), at time 0, 24, 48, 72, and 96 hours. Bacterial count samples were collected but degradation prior to analysis precludes an analysis of bacterial response. Total Chl *a* concentrations were determined by filtering whole water samples through Whatman GF/F filters. To obtain the concentration of Chl *a* within the nanoplankton population (<3µm), a whole water sample was initially filtered through a 3.0 µm Nuclepore filter then filtered onto a Whatman GF/F. Samples were immediately frozen and remained frozen until analysis. All nutrient and chemical characterization samples were filtered through 0.7µm Whatman GF/F filters and stored frozen in acid washed 50mL Falcon tubes (nutrient analysis) or 500mL high density polyethylene bottles (chemical characterization). Water samples remained frozen until analysis.

3.3.4 Analyses

DOC and TDN concentrations were measured via high temperature combustion using a Shimadzu 5000A TOC analyzer operating in tandem with an Antek 7000B Total Nitrogen Analyzer (Sharp et al. 1993; Sharp et al. 2004). Ammonium, nitrate + nitrite and orthophosphate were analyzed using a Lachat 8500 Nutrient Analyzer (Lachat

Instruments). TDP was measured via sector field inductively coupled plasma mass spectrometer (SF-ICP-MS; Field et al. 2007). DON and DOP were determined as the difference between the inorganic and TDN or TDP concentration, respectively. All Chl *a* samples were analyzed within 1 week of collection according to Holm-Hansen et al. (1967).

3.3.5 Salt removal

Time series samples from the bioassay experiment were concentrated and de-salted using an approach similar to that used for the concentration of DOM from SR and TS, however, the procedure was modified for smaller volumes. A 100mL aliquot was taken from each of 12 bottles (4 time points from 3 different treatments). Samples were processed through a stirred cell ultra filtration cell (Millipore model 8200) fitted with a 1kD Millipore regenerated cellulose ultra filtration membrane under nitrogen gas at 25psi pressure to reduce microbial degradation and increase filtration flow rate. The filtrate from the stirred cell extractions was then acidified with 1.7mL 6N HCl per 1L sample and filtered onto a 47mm 3M Empore C18 SPE disk to extract additional compounds.

3.3.6 Chemical characterization of DOM

An ESI-MS with single quadrupole detection (Agilent 1100 Liquid Chromatograph/ Mass Spectrometer with ESI source) was used to characterize DOM from TS and SR source waters as well as the time series bioassay experiments. ESI- MS provides molecular weight information represented as a mass to charge ratio (*m/z*). All samples in this study were analyzed in the positive ionization mode. Compounds detected in the positive mode contain basic functional groups including N heterocycles, alcohols, amines, and amides (van Berkel 1997; Seitzinger et al. 2005). In the case of

singly charged compounds, the *m/z* detected in the positive mode represents the molecular weight of the compound +1 $(MW+H)^+$ (McEwen and Larsen 1997). ESI-MS allows for the assessment of masses present and tracks changes in each of those masses via changes in the ion abundance (Sipler and Seitzinger 2008). Due to its unit mass resolution, each mass (*m/z*) represents one or more compounds (Kebarle and Ho 1997).

In this study, samples were run under the conditions described in Seitzinger et al.(2005) with the exceptions of the mobile phase, which was a 50:50 v:v methanol: DIW and the mass range scanned was 50-1000 Da. Six replicate injections were analyzed per sample to obtain a statistically significant replication for interpretation of *m/z* ion abundance changes. DIW was used for blank correction.

3.3.7 Statistical analyses

During ESI-MS analysis, raw mass spectra data were recorded on Chemstation (version A.7.01). The raw data was statistically analyzed using a custom program designed in C++ where the average ion abundance (± SD) was determined for each mass within the replicate injections. A t-test with 0.05 confidence level was performed on all *m/z*'s allowing for the retention of all masses with ion abundances significantly different from zero. Instrumental and analytical noise was addressed through blank correction. Ion abundances of *m/z*'s found within the DIW blank were subtracted from that same *m/z* detected in each sample.

After salt removal and DOM concentration, the two DOM source waters were chemically characterized and compared statistically using a standard paired t-test with 95% confidence based on ion abundance between two samples (i.e. SR DOM and TS DOM) for each individual *m/z*. SR and TS sources were normalized by DIW dilution to a

DOC concentration of 500μM C prior to ESI-MS analysis. To assess temporal changes in the average ion abundance (± 95% of the SD) of individual masses during the bioassay experiments, the slope of the line connecting each time point in series was determined. By applying a positive, negative or zero slope to each line throughout the time series a total time series trend could be applied to each individual mass.

The Sorensen similarity index was used to assess the relationship between two samples based on masses present. It is calculated as twice the number of masses shared between two samples divided by the sum of the total number of masses detected in each sample. The Simpson's index of diversity was also calculated and differs from the Sorensen similarity index in that it takes into account not only the number of masses but the relative abundance of each mass. Both tests have the same interpretational range of 0-1 with 1 indicating the greatest degree of similarity or diversity, respectively.

3.4 Results

3.4.1 Source water comparison

Initial physical and chemical properties of the SR and TS field samples varied between the two source water sites (Table 3-2). SR had higher concentrations of all nutrient components analyzed (DOC, DON, DIN and PO_4) than TS. Initial DOP concentrations were not determined. The total DOC recovered from the salt removal and DOM concentration process was 51% in SR and 79% in TS. The total DON recovered from the same process was 34% in SR and 58% in TS.

ESI-MS data indicated a number of differences in chemical composition of the DOM recovered from the two different sources. Although masses (a mass or *m/z* represents one or more compounds) were observed through the entire range analyzed (50-

1000 Da) in both samples, more masses were detected in the SR (210 *m/z*'s) source than in the TS (186 *m/z*'s) source. Of the *m/z*'s detected, 70% (146 *m/z*'s) were detected only in SR and 65% (122 *m/z*'s) in TS (the masses unique to each source are shown in Fig. 3-2). The two also differed in the median of their unique masses (622 in SR and 468 in TS). Fifty-nine percent of *m/z*'s observed in TS and 43% in SR were less than *m/z* 500.

3.4.2 Bioassay site characteristics

Blackwater Sound was chosen as the site for the bioassay tests because it represented a high Chl *a* bloom region in eastern Florida Bay. While eastern bay is typically characterized by low Chl *a* concentrations averaging 0.85µgL^{-1} Chl *a* (Boyer et al. 1999) and clear water (Hunt and Nuttle 2007), during this study Chl *a* concentrations of 5.9µg/L were observed. Ninety-seven percent of the total Chl *a* was in the < 3µm size fraction. The water depth at this site was 2 meters and temperature and salinity were 25.5^{o}C and 25.6, respectively. Nutrient concentrations were 447 ±7µM DOC, 0.1±0.1 µM DIN, 29.1 ±1.8 µM DON, 0.4 ± 0.1 µM PO_4 and 1.9± 0.2 µM DOP.

3.4.3 Biological and chemical response to DOM additions

Chl *a* concentrations significantly increased in treatments receiving either SR or TS source water additions. Within the first 48hours of the experiment Chl *a* doubled in concentration in the SR treatment and increased by 50% when TS DOM was added (Fig. 3-3). A less than 4% increase in Chl *a* concentration was observed in the control treatment. The picoplankton (< 3µm size fraction) dominated throughout the times series experiment accounting for 93% of the total Chl *a* at the 48hr time point in all treatments.

Nutrient concentrations decreased in SR and TS treatments (Fig. 3-4). Almost the entire amount of DIN supplied in each treatment was consumed within the first 48 hours

of the experiment, 91% in SR and 99% in the TS treatment. In the SR treatment 18% of DON supplied in the addition was consumed while 44% of the DON added in the TS treatment was consumed. SR and TS both consumed approximately 16μM DON within the first 48 hours. However, SR used an additional 9μM DON between 48 and 72 hours. The additional loss of DON in the SR treatment between 48 and 72 hours did not correspond to an additional increase in Chl *a*.

DOP also decreased in all treatments within the first 48 hours. Although twice as much DOP was consumed in the SR treatment compared to TS, both treatments showed the same percent change (32%) of the total DOP present. Phosphate concentrations ranged between 0.4 and 0.6μM P within all three treatments, however, the total change in PO_4 concentration was less than (±) 0.1μM PO_4. DOC concentrations fluctuated in both the SR and TS treatments resulting in a net increase in DOC in the SR treatment and a net decrease in DOC in the TS treatment. Increases in Chl *a* concentration correlate with observed decreases in DIN, DON and DOP concentrations. While DIN, DON and DOP all show a decrease in concentration (positive percent change Fig. 3-4) there is an inverse relationship between the change in DOC and the change in PO_4 concentration in all three treatments.

3.4.4 Compound level assessment

The total recovery of the initial DON and DOC from time series bioassay samples ranged between 40% and 56% for both analytes in all treatments. Fewer masses were detected in the control (381*m/z*'s) than in the SR (515 *m/z*'s) or TS (479 *m/z*'s) treatments (Table 3-3). Significant changes in ion abundance and increases and decreases of individual masses (*m/z's*) occurred in all treatments, including the control during the first

72 hours of the experiment. Therefore, although there was no measurable change in the total DON or DOC concentrations within the control treatment (Fig.3-4), significant changes did occur at the compound level.

The time series trends of individual masses reflect the highly dynamic nature of DOM (Fig. 3-5). There were 4 general types of responses in individual masses during the time series: masses that first increased and then decreased (were produced and consumed), masses that decreased only (were consumed), masses that increased only (were produced) and those that were variable without any uniform pattern. Some masses also showed no change over the time series. Those masses that increased and then decreased in ion abundance represented the largest percentage of the masses detected in any treatment (Fig. 3-6). Of those that decreased only, twice as many masses (139 *m/z*'s) decreased in SR, and 50% more (91 *m/z*'s) decreased in TS, compared to the control. Forty-nine of the masses that decreased in TS and SR were shared between the two treatments but were not detected in the control. Those masses that significantly decreased in ion abundance are considered bioavailable to this community. Approximately 20% of the masses detected increased in concentration and retained high ion abundances over the course of the experiment in all three treatments; however, the total number of *m/z*'s that increased varied by nearly 50% (Table 3-3). Masses shown as variable are *m/z*'s that showed significant changes (95% confidence) in ion abundance at each time interval throughout the course of the bioassay experiments. Those masses that did not change in ion abundance represented the smallest percentage observed.

While all treatments had similar median *m/z*'s for total masses detected (approximately *m/z* 650), the treatments did vary when the median *m/z*'s of each time

point (0, 24, 48 and 72 hours) were compared. The median *m/z* at any given time point was *m/z* 350, 552 and 480 for the control, SR and TS, respectively, showing that SR has proportionately more masses at the higher end of the analyzed range than the control. The median *m/z* of bioreactive masses, those masses that increased or decreased in ion abundance, ranged between *m/z* 690 and *m/z* 738 (Table 3-3). The median *m/z* of masses showing no change in ion abundance ranged between *m/z* 124 and *m/z* 236 in all treatments. This difference in median *m/z* of bioreactive versus refractory masses shows that the higher end of the analyzed range (500-1000 Da) is more dynamic than the lower range (50-499 Da).

SR and TS were more diverse than the control, based on ion abundances of m/z's detected. The Simpson's index of diversity values for SR, TS and the control were 0.78, 0.76 and 0.52, respectively. Masses consumed in the TS bioassay are more similar to those masses consumed in the control than those consumed in SR. This similarity is reflected in the Sorenson similarity index of the bioavailable masses present in each treatment with TS and the control having a similarity index value of 0.28 compared to 0.10 and 0.12 for SR and TS or SR and the control, respectively.

3.5 Discussion

This study provides a comparative analysis of the composition and bioavailabilty of two riverine sources from areas of different land use practices to a natural *Synechococcus* bloom in Florida Bay. The two riverine DOM sources, SR and TS, differed chemically at both the bulk and compound level. TS and SR were chosen as DOM sources based on their potential influence on the Florida Bay phytoplankton community. Both sources originate from upstream areas characterized by different land

use practices and this difference is reflected in the chemical composition of their DOM. TS drains a region with more farms while SR is more influenced by overland flow through the everglades (Solecki et al. 1999; Solecki 2001; USGS 2000). The Sorensen similarity index (0.32) indicates that these two sites are dissimilar based on masses observed. Comparatively, two suburban streams with similar land use practices shared 88% of masses detected (Seitzinger et al. 2005). It is important to note that with the unit mass resolution capabilities of this method, a mass detected in one sample may not represent the same compound (or suite of compounds) in a second sample (Kebarle and Ho 1997). Therefore, two samples could be found to be even less similar with higher resolution.

In this study a *Synechococcus* spp. dominated phytoplankton bloom with a total Chl *a* concentration of 5.9μgL^{-1} was identified in eastern Florida Bay. This is in contrast to earlier observations where *Synechococcus* blooms were typically found in central not eastern Florida Bay (i.e. Phillips et al 1999; Glibert et al. 2004). Historical data show that phosphorus (P) has been the major nutrient limiting phytoplankton growth in the eastern region (Fourqurean et al. 1993; Boyer et al. 1997; Boyer et al. 1999; Glibert et al. 2004; Childers et al. 2006). This P limitation is in part due to low P inputs from source waters within Everglades National Park (Rudnick et al. 1999) and the PO_4 scavenging carbonate chemistry associated with the calcium carbonate sediments in this region (Kitano et al. 1978; Boyer and Keller 2007; Madden in press). Field nutrient concentrations reveal that the DIN:DIP ratio at the eastern bay (Blackwater) site was 4 μM DIN:1μM DIP at the time of collection. The low DIN concentrations (0.1μM DIN) and high DIP (0.4μM PO_4) at this site are atypical for eastern Florida Bay (Boyer et al. 1999; Glibert et al. 2004;

Madden in press). DIN:DIP ratios less than 16 imply N limitation of phytoplankton growth (Redfield 1963) indicating that growth at this location was N not P limited at the time of collection. Thus, the inorganic nutrient composition during this bloom appears to be more characteristic of the typically N limited *Synechococcus* blooms in central Florida Bay than historic eastern bay conditions (Glibert et al. 2004; Hitchcock et al. 2007).

Observed increases in Chl *a* appear to be stimulated at least partially by a combination of DIN, DON and DOP. Based on the total DON recovery during the salt removal process and the amount of DON consumed during the time series bioassays, the DON from TS was at least 25% bioavailable to this Florida Bay community. SR was at least 10% bioavailable to the same community. PO_4 concentrations remained stable at approximately 0.5μM P, indicating the either no PO_4 was used for phytoplankton growth or that the DOP was cycled at such a rate that no change in PO_4 concentration could be observed with current methods. The change in DIN, DON and DOP within the first 48 hours closely mirrored trends observed in Chl *a* concentration indicating that these nutrients may be directly affecting phytoplankton growth. DON concentrations also significantly ($\alpha = 0.05$) decreased throughout the course of this experiment and likely contributed at least partially to the observed Chl *a* increases. The decrease in DON concentration between 48 and 72 hours did not correlate to an additional increase in Chl *a*, so the loss of DON during this time interval was likely do to bacterial uptake. In a study by Glibert et al (2004), DON was found to yield a higher response in bacterial number than in Chl *a* in the eastern bay, while Chl a at that time was more responsive to PO_4.

Both inorganic and organic nutrient additions have been found to evoke positive biological responses within phytoplankton communities of Florida Bay (Tomas et al. 1999; Glibert et al. 2004; in press; Boyer et al. 2006). Various DOM sources including humic acids, urea, and DOM extracted from TS have been investigated as a potential nutrient source for natural phytoplankton and bacterial populations (Glibert et al. 2004; Boyer et al. 2006). Boyer et al (2006) found that the DOM from TS stimulated both cyanobacterial and diatom growth in central and eastern Florida Bay phytoplankton communities. This response was independent of the location or background nutrient concentrations and supports the results of this study, which show a positive response in cyanobacterial growth associated with DOM additions.

While changes in nutrient and Chl *a* concentrations were not observed in the control treatment, significant changes, including masses decreasing or being consumed and new masses being produced did occur at the *m/z* level. This shows that nutrient cycling can occur at the mass level that is not observed through bulk chemical analysis. Thus, bulk chemical analyses are not sufficient to fully investigate nutrient cycling within aquatic systems.

Earlier efforts to chemically characterize DOM in Florida Bay included fluorescence spectroscopic monitoring of colored dissolved organic matter (CDOM; Maie et al. 2006). These monitoring efforts of the southwest coastal waters of Florida include locations in SR and showed that the quantity and quality of the DOM was controlled by source strength, hydrology and geomorphology (Jaffé et al. 2004). CDOM data for Florida Bay data showed a shift in composition from marine derived to a more terrestrial signature in the wet season and is indicative of increased DOM input from

Everglades National Park (Maie et al. 2006). While fluorescence spectroscopic analyses do provide insight into the DOM pool, they do not allow an analysis of concentration or bioavailability at the compound (unit mass) level.

Calculations of the Sorenson similarity index values showed that at the mass level, SR and TS were more similar to each other than to the Florida Bay water from the study site based on total *m/z*'s detected. However, TS was more similar to the control bioassay than to the SR bioassay based on bioavailable masses (*m/z*'s that significantly decreased in ion abundance). This similarity based on bioavailable masses may reflect the influence of TS or sources of similar land use practices on the Florida Bay study site.

Comparative analyses of median *m/z*'s give insight into mass characteristics of bioreactive compounds. The median *m/z* observed for new masses or masses that decreased in ion abundance (Table 3-3) was higher than the overall median mass observed. This indicates that the higher end (650-1000 Da) of the analyzed range is more dynamic than the lower end of the analyzed range.

Through ESI-MS analyses of time series bioassay samples we have been able to identify previously uncharacterized bioavailable masses within land derived DOM sources. With the use of higher resolution (non-quantitative) analyses such as ESI with Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) and ESI with tandem mass spectrometry (ESI-MS/MS), a chemical composition and structural information could be obtained for each bioavailable mass (Sipler and Seitzinger 2008). This study represents a step forward in the effort to identify the compounds and characteristics that make a source bioavailable.

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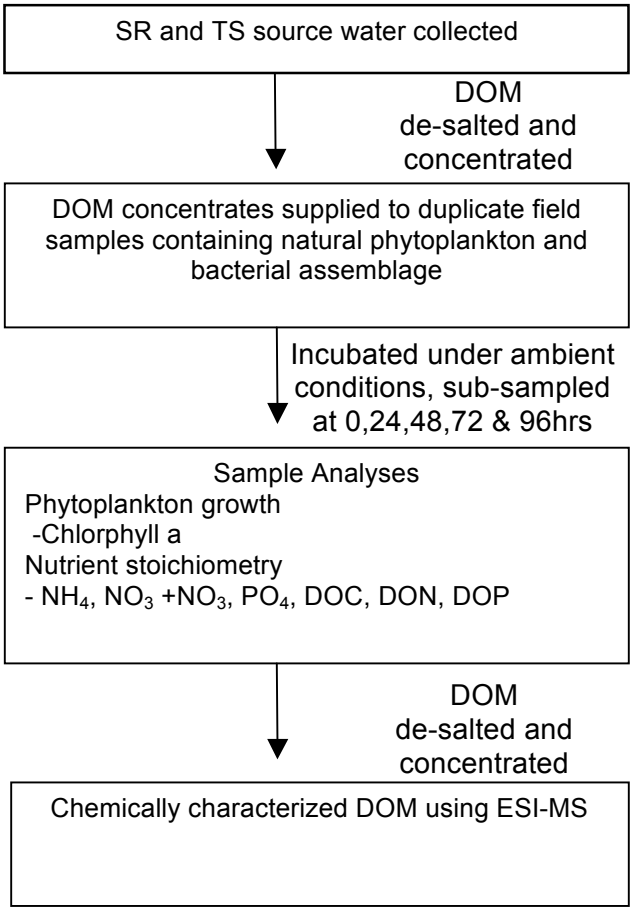


Table 3-1. Methods schematic for DOM bioavailability bioassay experiments

	Shark River	Taylor Slough
Salinity	9.4	0.2
Temperature (^{o}C)	26.7	26.2
DOC (μM)	1919	374
DON (μM)	96	28
DIN (μM)	8.7	4.9
PO_4 (μM)	1.4	0.1

Table 3-2. Initial physical and chemical parameters of SR and TS DOM source water at time of collection. DIN includes NO_2, NO_3, and NH_4.

	Control	+ Shark River DOM	+Taylor Slough DOM
No. *m/z* detected	381	515	479
median *m/z* detected	678	648	650
No. *m/z* that decreased in first 24 hours	64	139	91
median *m/z* of masses that decreased	738	700	690
No. new masses present at 24 hours	92	127	136
median *m/z* of new *m/z*	726	718	693

**m /z* = mass to charge ratio

Table 3-3. Summary of ESI-MS data for bioavailability experiments including: a control, SR (+SR) DOM addition and TS (+TS) DOM addition. The mass range of ESI-MS data was 50-1000 amu; number of masses based on unit mass resolution.

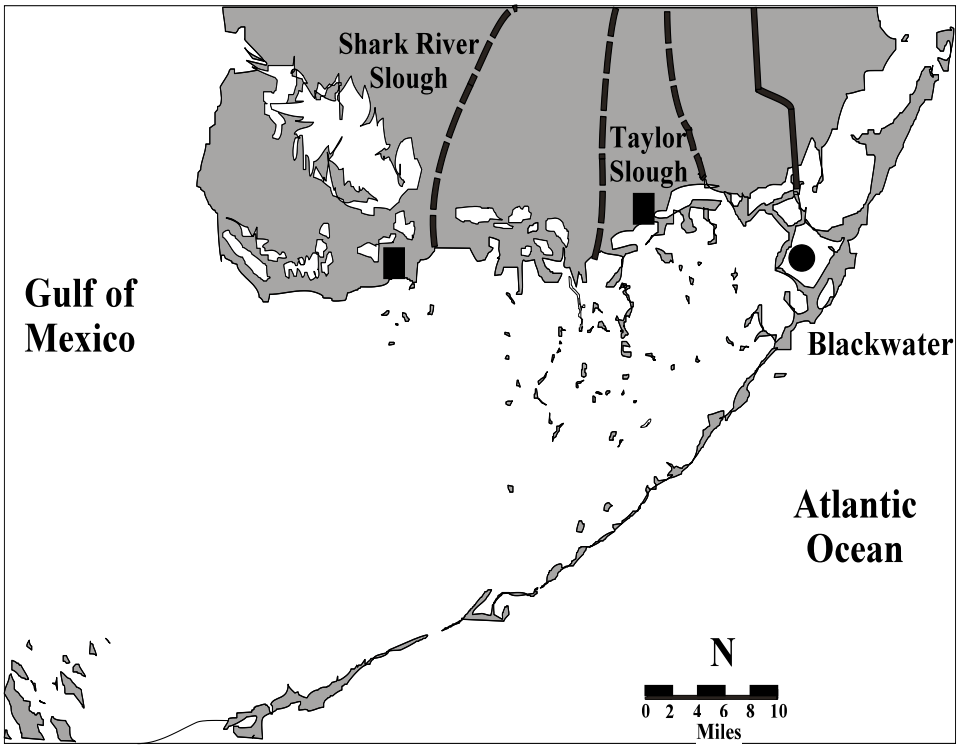


Fig. 3-1. Map of Florida Bay showing source water (Taylor Slough and Shark River (square)) and bioassay (eastern bay Blackwater Sound (circle)) sample sites.

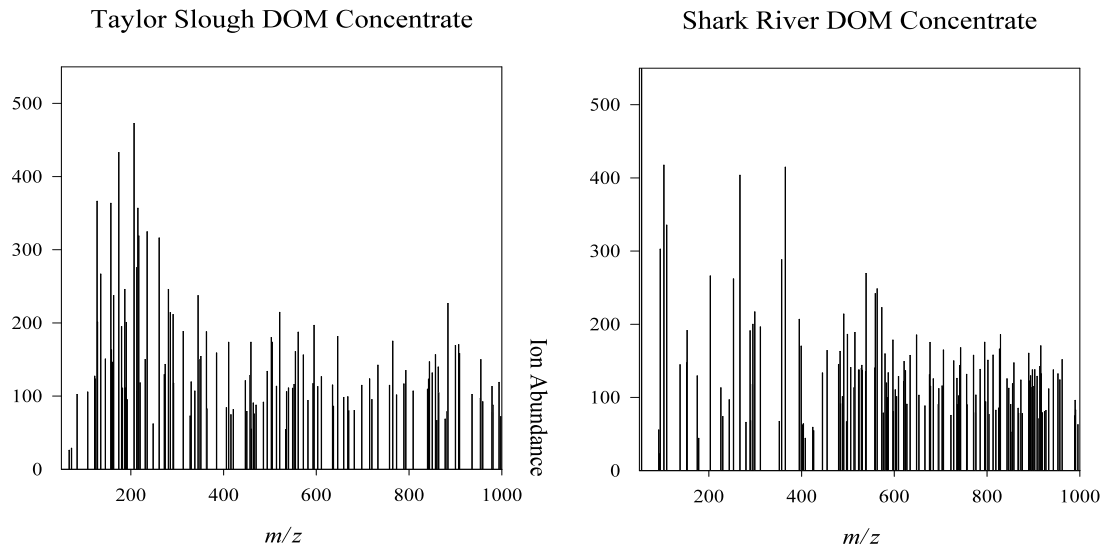


Fig. 3-2. *m/z*'s unique to Taylor Slough and Shark River DOM

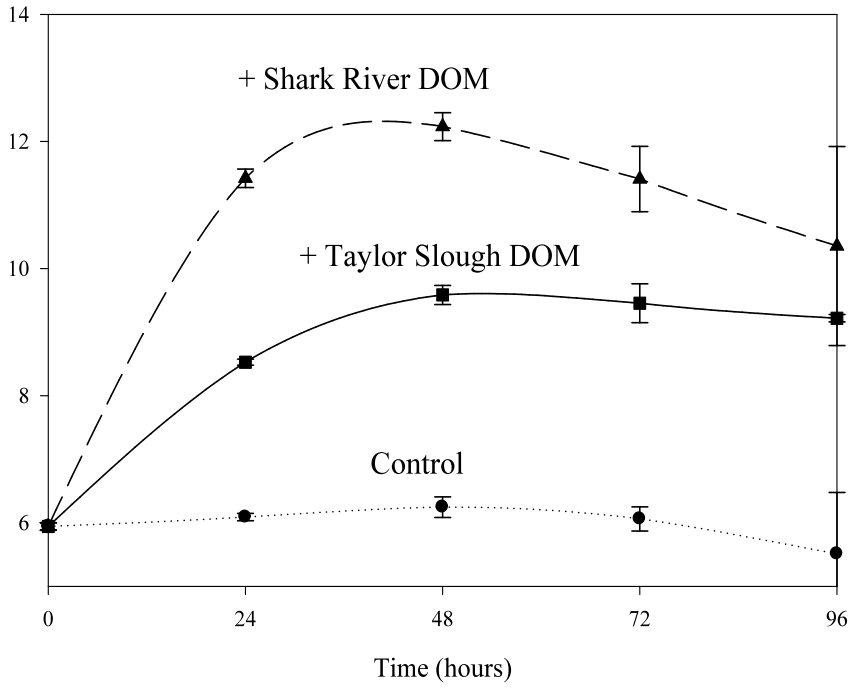


Fig.3-3. Response in Chl *a* to DOM additions (Control with no DOM added = dotted line, TS DOM added= solid line, SR DOM added= dashed line). Points represent the average concentration ± S.D.of duplicate incubations.

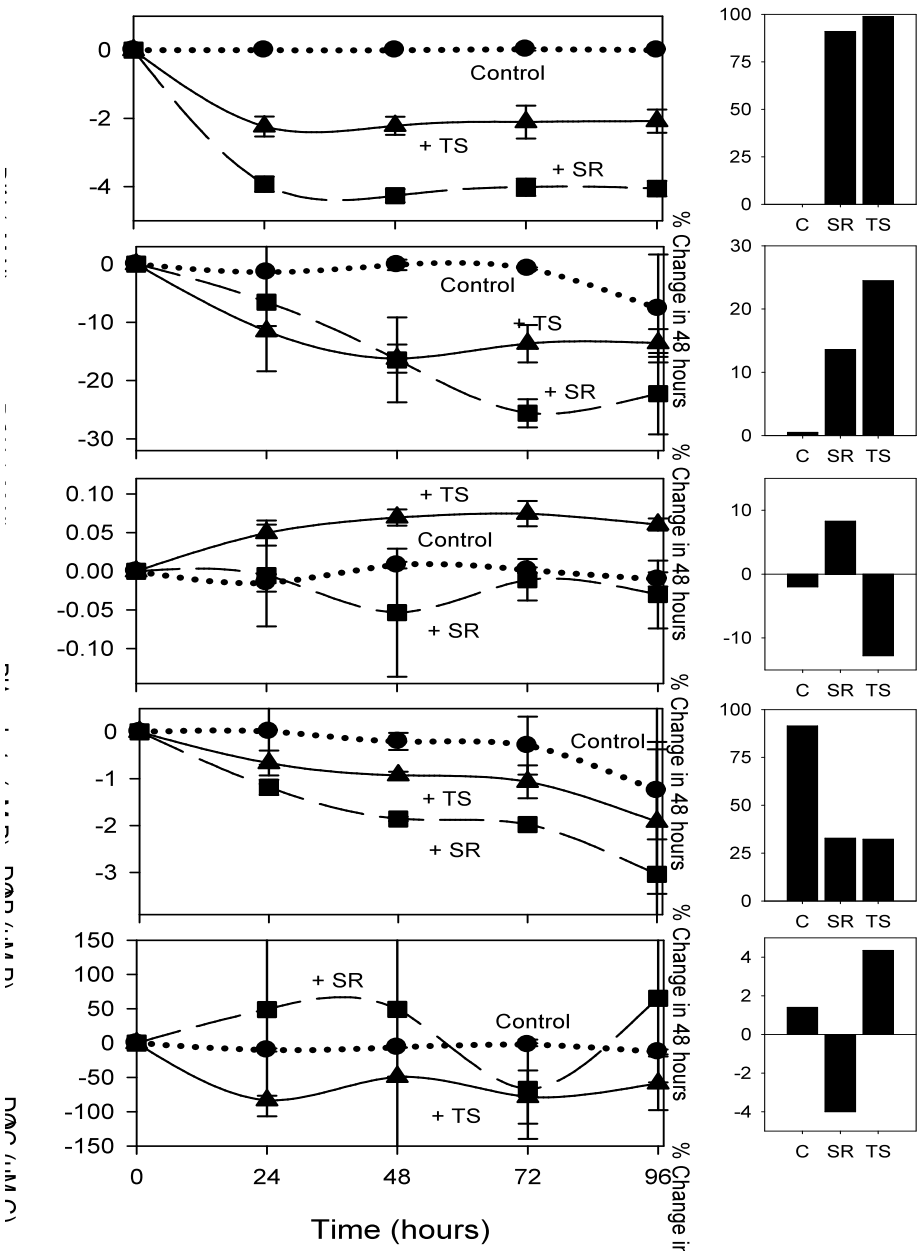


Fig. 3-4. Change in nutrient concentrations in bioassay treatments (Control= dotted line, TS DOM added= solid line, SR DOM added= dashed line). Points are the average difference in concentration ± S.D. of duplicate incubations (positive = nutrient increased in concentration, negative = nutrient decreased in concentration). Bar graphs are percent change in nutrient concentration in first 48 hours of bioassay experiments (positive = nutrient decreased in concentration, negative = nutrient increased in concentration).

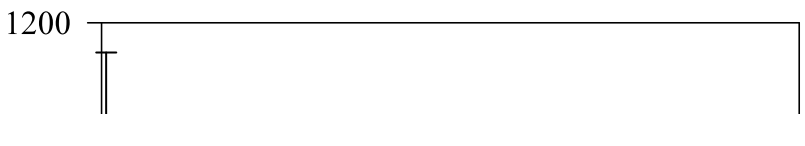


Fig. 3-5. Selected *m/z*'s from SR bioassay showing ion abundance patterns

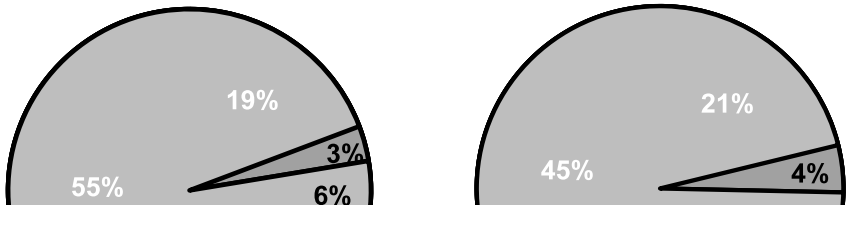


Fig. 3-6. Percent of total *m/z*'s detected displaying ion abundance time series trends during first 72 hours of the bioassay experiments.

Chapter 4. Relationship between dissolved organic matter, bacterial community composition and rapid growth of toxic red-tide *Karenia brevis*

Material in this chapter has been submitted to Marine Ecology Progress Series for publication as:

Sipler, R.E., S.P. Seitzinger, R.J. Lauck, L. McGuinness, G.J. Kirkpatrick, D.A. Bronk, C.A. Heil, L.J. Kerkhof, O.M. Schofield. Relationship between dissolved organic matter, bacterial community composition and rapid growth of toxic red-tide *Karenia brevis*.

4.1 Abstract

Natural populations of *Karenia brevis* were used to assess the potential nutrient dynamics associated with the co-occurring species, diazotroph *Trichodesmium* sp. Dissolved organic matter (DOM) from laboratory cultures of *Trichodesmium sp*. was isolated, concentrated and then supplied as a nutrient source to a natural population of *K.brevis* collected from the Gulf of Mexico. *Karenia spp.* abundance began to increase

immediately after the *Trichodesmium* sp. cellular exudates (TCE) addition allowing populations to reach division rates of 1 cell division day^{-1}. There was rapid and complete utilization of the TCE dissolved organic nitrogen (DON) supplied within the first 24 hours. Electrospray ionization mass spectrometry (ESI-MS) was used to chemically characterize the bioavailable DOM at the unit mass level. Approximately 60% of the masses (compounds or suite of compounds) added with TCE DOM addition were bioavailable as indicated by a decrease in ion abundance. Terminal restriction fragment length polymorphism (TRFLP) was used to assess bacterial community composition. The number of bacterial species initially increased when TCE DOM was added but decreased as *K.brevis* reached its maximum abundance. DON from *Trichodesmium* sp. provides an important source of nitrogen for *K.brevis* populations in the Gulf of Mexico. However, similar cellular responses to a single DON compound, urea, suggest that *K.brevis* is well suited to access a broad range of reduced nitrogen sources. This implies that *K.brevis* populations can be enhanced when exposed to DON from *Trichodesmium* sp., other natural sources or anthropogenic sources.

4.2 Introduction

Blooms of the toxic red-tide dinoflagellate, *Karenia brevis* Davis (previously *Ptychodiscus brevis and Gymnodimium breve* (Davis) Steidinger) occur frequently on the West Florida Shelf in the subtropical waters of the Gulf of Mexico. *K.brevis* blooms occur annually and account for a significant proportion of the annual carbon fixation within the Gulf of Mexico (Vargo et al. 1987). Non-bloom concentrations of *K.brevis* are ubiquitous throughout the eastern Gulf of Mexico and are also common in the South

Atlantic Bight (Gessey and Tester, 1993; Tester and Steidinger 1997). Bloom conditions (>1000 *K.brevis* cells L^{-1}) have been experienced during 28 of the 42 years between 1968 and 2000 (Walsh and Steidinger 2001). The frequency and cause of *K.brevis* blooms is important as this species produces a potent neurotoxin, brevetoxin. Brevetoxin has been associated with numerous negative water-quality impacts including marine mammal mortalities, extensive fish kills, human respiratory irritation, and illness in shellfish consumers (reviewed by Kirkpatrick et al. 2004).

Despite being an important component to the Gulf of Mexico phytoplankton community, the ecology of *Karenia brevis* has been difficult to describe given that blooms of *K.brevis* generally develop in the oligotrophic waters 18-74 km offshore (Steidinger 1975; Steidinger and Haddad 1981). In the oligotrophic waters of the Gulf of Mexico dissolved inorganic nitrogen (DIN) concentrations approach zero and as a result dissolved organic nitrogen (DON) has been suggested to play an important role in *K.brevis* nutrition (Baden and Mende 1979; Steidinger and Haddad 1981; Walsh and Steidinger 2001; Vargo et al. 2008). Due to its oligotrophic environment the nutrients that support *K.brevis* growth have not yet been identified (Vargo et al. 2008). While correlations have been made between some *K.brevis* blooms and riverine flow, these relationships are not universal and are locally dependant (Dixon and Steidinger 2004); therefore, while riverine nutrients supply some of the nitrogen (N) required to support coastal blooms, it is likely just one of the sources of N supporting offshore blooms. Calculations based on nutrients provided from groundwater, riverine, upwelling and atmospheric inputs together cannot account for the N required to sustain large blooms that can reach concentrations of 1×10^7 cells L^{-1} (Walsh and Steidinger, 2001;

Mulholland et al. 2004; Walsh et al. 2006). Since allochthonous nutrient sources fall short of the total amount of N required for growth, efforts have focused on autochthonous nutrient sources.

Trichodesmium sp. is a diazotroph and the most ecologically important N_2 fixing cyanobacteria in the oceans (Carpenter and Romans 1991; Capone et al. 1997). As *Trichodesmium* sp. fixes N_2 gas from the atmosphere it leaks fixed N into the surrounding water in the form of ammonium (NH_4) and DON (Glibert and Bronk 1994; Mulholland and Capone 2001) where it can then be used as a nutrient source by other organisms. DON represents a significant portion of the total cellular N released by *Trichodesmium* sp. (Gilbert and Bronk 1994) and can increase the background DON concentrations of the Gulf of Mexico by a factor of three or four (15-20μM N, Lenes et al. 2001). The bulk of the DON appears to be released as glutamate and glutamine (Capone et al. 1994). Calculations based on *K.brevis* N demand show that *Trichodesmium* sp. released DON may represent a significant fraction of the N required for growth (Mulholland et al. 2004; Walsh et al. 2006).

It is common for *Trichodesmium* sp. and *Karenia brevis* to co-occur and correlations between *Trichodesmium* sp. presence and *K.brevis* bloom events have been found (Lenes et al. 2001; Walsh and Steidinger 2001; Walsh et al. 2006). Isotopic assessments of natural *K.brevis* populations reveal relatively low $\delta^{15}N$ signatures indicating the use of 'new'/recently fixed N (Havens et al. 2004). Uptake of ^{15}N labeled DON from *Trichodesmium* sp. cultures to a natural non-axenic *K.brevis* population showed that N produced by *Trichodesmium* sp. can be used as a nutrient by *K.brevis* directly or indirectly through bacterial remineralization (Bronk et al. 2004).

Bacterial populations within *Karenia brevis* blooms are important to consider because bacteria serve as a nutrient source and sink as well as potential predators and prey. In an oligotrophic region with low nutrient availability, the balance between nutrient regenerators and competitors may be crucial to bloom initiation. Bacteria liberate nutrients from DOM compounds that are otherwise not available to phytoplankton thus providing a significant source of nutrients under oligotrophic conditions (Pomeroy 1974; Azam et al. 1983). The bacteria also provide a direct source of nutrients through phagotrophic grazing by heterotrophic phytoplankton (Stoecker et al. 2006). Finally algicidal bacteria aid in bloom termination (Doucette et al. 1999).

While we know that bacteria-algal interactions are common in aquatic systems especially among harmful algal species, there is relatively little known about the role bacteria play in *Karenia brevis* growth (Kodama et al. 2006). *K.brevis* has not been successfully cultured axenically, suggesting that bacteria may be important in bloom development. Algicidal bacteria active against *K.brevis* have been identified in culture experiments; however other bacterial species associated with *K.brevis* have been suggested to have antagonistic effects on these algicidal bacteria (Maylai and Doucette 2002; Roth et al. 2008). The investigation by Maylai and Doucette (2002) also suggested a negative correlation between DOM concentration and algicidal affects indicating that nutrient limitation likely plays a key role in bacterial- *K.brevis* interactions. While laboratory experiments provide insight into potential bacterial-algal interactions, they often cannot mimic the microbial dynamics that occur in nature.

Given this, the objectives of this study were to investigate how DON produced by *Trichodesmium* sp. affects the growth of *Karenia brevis*, identify what compounds (as

mass to charge ratios) are bioavailable, and determine how bacterial community composition changes with *K.brevis* growth. To achieve these objectives, DOM from *Trichodesmium* sp. cultures was concentrated and supplied as a nutrient source for a natural *K.brevis* bloom. The changes in the responses of *K.brevis*, bacterial abundance and community composition and changes in N concentrations were monitored.

4.3 Methods

Trichodesmium sp. culture exudates were collected, concentrated and then supplied as a nutrient source for a field population of *Karenia brevis* in a 9 day bioassay experiment to determine its bioavailability. The experiment consisted of four bioassay treatments run in triplicate. The treatments: control, nitrate, urea, and *Trichodesmium* sp. cellular exudates (TCE), varied in the form and total quantity of nitrogen added.

4.3.1Trichodesmium sp. culture concentrates

Trichodesmium IMS101 cultures were grown on low nutrient YBCII media (Chen et al. 1996) in 10L acid washed (12% H_2SO_4 with repeated deionized water (DIW) rinses) polycarbonate carboys. Cultures were maintained at 26^{o}C on a 12:12 light:dark cycle with constant aeration. *Trichodesmium* sp. cultures were harvested by filtering the culture through 0.7μm Whatman GF/F filters to separate *Trichodesmium* sp. cells from the exudates. The TCE was sub-sampled for nutrient analyses [dissolved organic carbon (DOC), total dissolved nitrogen (TDN), dissolved inorganic nitrogen (DIN =NO_3 + NO_2 + NH_4), and phosphate] and immediately frozen in 1L aliquots for salt removal and dissolved organic matter (DOM) concentration.

TCE was concentrated and de-salted using a tandem ultra filtration (UF) and solid phase extraction (SPE) method (Simjouw et al. 2005). Briefly, a 5.3 L sample of TCE filtrate was extracted using a stirred ultra filtration cell (Millipore model 8200) fitted with a 1000 Da Millipore regenerated cellulose ultra filtration membrane, in 100 mL increments. Stirred cell extractions were run under N_2 gas at 25 psi pressure to reduce microbial degradation and increase filtration flow rate.

SPE disks were conditioned with two 10 mL aliquots of methanol (MeOH) and two 10 mL aliquots of DIW. Filtrate from the stirred cell extraction was then acidified with 1.7 mL 6N HCl per 1 L sample and filtered onto a 47 mm 3M Empore C18 SPE disk to extract additional compounds. DOM retained on the C18 SPE disks was eluted from the disks with two 10 mL aliquots of MeOH. The eluent was then heated at 55^{o}C until all MeOH had evaporated. The DOM was then reconstituted with DIW and re-combined with the UF fraction of the DOM to its original proportions based on the concentration factors of each component. With this method DOM is retained while salts and inorganic nutrients past through as filtrate.

4.3.2 Field Sample Collection and Bioassays

A bloom of *Karenia brevis* was identified via satellite and ship board observations near Sanibel Island, FL in the southeastern portion of the Gulf of Mexico at 26^{o} 15.916' N, 82^{o} 01.04'W. Bloom water was collected on October 17, 2007 with a bucket at the surface and was gently filtered through a 64 μm mesh to remove *Trichodesmium spp.* colonies and large zooplankton. Initial cell counts at this site were approximately 10-14 million *K.brevis* cells L^{-1}.

Bloom water was then evenly divided into 12 acid washed (12 % H_2SO_4 followed by DIW rinse) 2.5 L polycarbonate bottles. There were four treatments: (1) control, (2) +nitrate, (3) + urea, (4) +TCE. The control treatment contained no additional nitrogen. Both nitrate and urea were added to a final 10 μM N addition. Urea and nitrate are both known to be bioavailable to *Karenia brevis* and were used as comparative compounds to determine N limitation and the N preference of this field population. The TCE treatment received a 20 μM N addition of the TCE; the concentration was twice that of the nitrate and urea additions because we assumed that not all of the DON produced by *Trichodesmium* sp. would be bioavailable. Phosphate was added to a final addition concentration of 2.5 μM in all treatments to ensure that the bioassays did not become P limited.

The bioassays were incubated at ambient temperature and light in a flow through system shaded by screen to reduce light saturation. Incubations began at sunset. Bioassays were sub-sampled for phytoplankton cell counts, bacterial community composition, nutrient analyses (DOC, TDN, DIN, total dissolved phosphorus (TDP) and phosphate), and chemical characterization at time points 0, 12, 24, 72, 144 and 216 hours. Nutrient samples were immediately frozen and remained frozen until analyzed. Duplicate phytoplankton cell count samples were taken per bioassay bottle; one was preserved in Lugols iodine solution and the other in 2 % gluteraldehyde. Cell counts as well as changes in N concentrations (DIN and DON) were used to assess the bioavailability of N sources to *Karenia brevis* field populations.

4.3.3 Analyses

Karenia spp. cells were enumerated using an inverted microscope on a bright field setting. *Karenia* species were identified based on cell morphology as *Karenia sp*. Growth rates were determined from cell counts by calculating for specific time intervals (days) using the equation :

$$r = \ln (N_t/N_0)/t$$

Division rates were calculated by dividing r by ln 2 and are expressed in cellular divisions day^{-1}.

DOC and TDN concentrations were measured via high temperature combustion using a Shimadzu 5000A TOC analyzer operating in tandem with an Antek 7000B Total Nitrogen Analyzer (Sharp et al. 1993). Ammonium, nitrate + nitrite and phosphate concentrations were analyzed using a Lachat 8500 Nutrient Analyzer (Lachat Instruments). TDP was measured via sector field inductively coupled plasma mass spectrometer (SF-ICP-MS; Field et al. 2007). DON and DOP were determined as the difference between the inorganic and TDN or TDP concentration, respectively. Standard deviations were determined through the propagation of error associated with each subsequent analysis.

4.3.4 Chemical characterization of DOM

DOM from the TCE concentrate, the control time zero and the TCE bioassay (times 0, 12, 24, and 72 hours) were chemically characterized using electrospray ionization mass spectrometry (ESI-MS) with single quadrupole detection (Agilent 1100 Liquid Chromatograph/ Mass Spectrometer with ESI source). Samples were run under the conditions described in Seitzinger et al. (2005) with the exceptions of the mobile phase which was a 50:50 v:v methanol: DIW and the mass range scanned was 50-1000

m/z. Samples were run in the positive ionization mode. Six replicate injections were analyzed per sample to obtain a statistically significant replication for interpretation of *m/z* ion abundance changes. DIW was used for blank correction.

ESI- MS is a soft ionization method which allows non-fragmented compounds to be detected. This method provides molecular weight information represented as a mass to charge ratio (*m/z*). Each unit mass (*m/z*) represents one or more compounds (Kebarle and Ho 1997). In the positive ionization mode singly charged compounds (*m/z's)* represent the molecular weight of the compound +1 $(MW+H)^+$(McEwen and Larsen 1997). Compounds detected in the positive mode contain basic functional groups including N heterocycles, alcohols, amines, and amides (Van Berkel 1997; Seitzinger et al. 2005). ESI-MS allows for the assessment of masses present and tracks changes in each of those masses via changes in the ion abundance. For more information on ESI mass spectrometry and investigating environmental samples see Sipler and Seitzinger (2008).

Raw mass spectra data were recorded on Chemstation (version A.7.01) and then statistically analyzed using a custom program designed in C++ where the average ion abundance (± SD) was determined for each mass within the replicate injections. A t-test with 0.05 confidence level was performed on all *m/z*'s allowing for the retention of all masses with ion abundances significantly different from zero. Instrumental and analytical noise was addressed through blank correction. Ion abundances of *m/z*'s found within the DIW blank were subtracted from that same *m/z* detected in each sample.

TCE concentrate, control and the TCE bioassay samples were chemically characterized and compared statistically using a standard paired t-test with 95 %

confidence based on ion abundance between two samples (i.e. TCE concentrate and control) for each individual *m/z*. The TCE concentrate was diluted with DIW to the same final vol:vol ratio as the TCE bioassay for direct comparison, prior to ESI-MS analysis. To assess temporal changes in the average ion abundance (± 95 % of the SD) of individual masses during the bioassay experiment, the slope of the line connecting each time point in series was determined. By applying a positive, negative or zero slope to each line throughout the time series a total time series trend could be applied to each individual mass.

4.3.5 Bacterial community composition

Changes in bacterial community composition were assessed using 16s rRNA SSU GeneFragment Amplification and Terminal Restriction Fragment Length Polymorphism (TRFLP). A modified phenol chloroform DNA extraction method (Sakano and Kerkhof 1998) was used to extract the total nucleic acids from the microbial biomass samples. The DNA was further purified using a cesium chloride density gradient. Briefly, 0.54 g cesium was added to a total volume of 500 µl which included 100 ng of sample DNA and 300 ng of *Halobacerium salinarium* and 2 µl of 1 % ethidium bromide. *H. salinarium* DNA was used as a marker to visualize the DNA in the cesium gradient under a 312 nm Transilluminator UV light. The gradients were spun for 24 hours at 80,000 rpm in a Beckman Optima TL Ultracentrifuge 120.1 (Beckman Coulter, Fullerton, CA). DNA bands were extracted from the centrifuged cesium via pipette. The cesium was dialyzed from the sample using a Millipore membrane dialysis filter (VSWP 02500) for 45 minutes. Dialyzed DNA was then stored at –80 ºC until further analysis.

Cleaned genomic DNA was used to amplify the 16S rRNA genes via polymerase chain reaction (PCR). For the amplification of 16S rRNA genes from DNA, 50 µl PCR reactions were set up with 10 ng template and 20 pmol of the universal primer 27 Forward (5' AGA GTT TGA TCC TGG CTC AG 3') and the bacterial specific primer 1100 Reverse (5' GGG TTG CGC TCG TTG 3') per reaction. The amplification parameters were as follows: 94 ºC for 5 minutes followed by 25 cycles of 94 ºC for 30 seconds, 57 ºC for 30 seconds, and 72 ºC for 1.1 minutes and a final extension period of 72 ºC for 10 minutes.

For TRFLP profiling, all forward primers were labeled with 6-carboxylfluorescien (6-FAM; Applied Biosystems, Foster City, CA). These fluorescently labeled amplicons were run on a 1 % agarose gel for quantification via image analysis and 15 ng were digested with the endonuclease MnlI (New England Biolab, Beverly, MA) at 37 ºC for 6 hours. The 20 μL digestion reactions were then precipitated using 2.3 μL mixture of 0.75 M sodium acetate, 5 μg of glycogen, and 37 μl of 9 5% ethanol. The reactions were then dried briefly and resuspended in 19.7 μL of deionized formamide and 0.3 μL of ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 minutes and denatured for 94ºC for 2 minutes before analysis. TRFLP profiling was carried out using Genescan software and an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). TRFLP peak detection was set at 50 arbitrary fluorescent units. All detected peaks were downloaded into custom made spreadsheets and those peaks representing <0.5 % of the total peak area were discarded. The remaining peaks were parsed and normalized to a uniform total profile area to adjust for small loading differences between samples.

The Sorensen similarity index and Simpson's index of diversity were calculated to compare chemical characterization samples. The Sorensen similarity index was used to assess the relationship between two samples based on masses present. It is calculated as twice the number of masses (species) shared between two samples divided by the sum of the total number of masses detected in each sample. The Simpson's index of diversity differs from the Sorensen similarity index in that it takes into account not only the number of species but the relative abundance of each species. Both tests have the same interpretational range of 0-1 with 1 indicating the greatest degree of similarity or diversity, respectively.

4.4 Results

4.4.1 Site characteristics

Trichodesmium spp. was found in dense patches at the surface and dispersed throughout the water column in the area surrounding a *Karenia brevis* bloom. The bloom was dominated by *K.brevis* and *Karenia mikimotoi* which together represented 99.9% of the total phytoplankton community. *In situ* DOC, DIN, DON, PO_4, and DOP concentrations within the bloom were 375 ±18 μM C, 0.2 ± 0.1 μM (NO_3 + NO_2+ NH_4), 13.6 ±1.4 μM N, 0.6 ± 0.2 μM PO_4 and 1.1 ± 0.4 μM P, respectively.

4.4.2 TCE concentrate

The salt removal method does not retain all of the DOM from the initial sample. Within this experiment, 53% of the DON initially present within the *Trichodesmium* sp. culture filtrate was recovered after the salt removal/ DOM concentration process. Similarly, 51% of the initial DOC in the *Trichodesmium* sp. culture filtrate was recovered after the same salt removal process. Final nutrient additions for the TCE bioassay

additions differed in nutrient concentration from the initial culture concentrations (Table 4-1).

4.4.3 Biological and chemical response to nutrient additions

An increase in *Karenia* spp. cell number was observed in all treatments where N was added (Fig.4-1). *Karenia* cell number doubled within the first 24 hours when TCE was added resulting in a division rate of 1.05± 0.15 divisions day^{-1}(μ= 0.73± 0.1). Cell number decreased in the urea treatment after the first 12 hours while the nitrate bioassay achieved an average division rate of 0.28 ± 0.09 divisions day^{-1} (μ= 0.19± 0.06). The urea and nitrate bioassays each achieved short term division rates of 0.84±0.2 (between 0 and 12 hours) and 0.66± 0.03 (between 12 and 24 hours), respectively. There was an initial and continuous decline in cell number within the control treatment. Over the course of this experiment the morphology of the remaining populations shifted from the initial large vegetative cells to smaller vegetative and eventually to reproductive cells by time 6-9 days as cell numbers decreased (data not shown).

The changes in nutrient concentrations were larger than expected. There was no significant (α=0.05) decrease in nitrogen observed in the no nitrogen addition control indicating that background DON concentrations represent compounds that may not be readily bioavailable to this community. An initial decrease in nitrogen (DON or nitrate) was observed in all treatments in which nitrogen was added (nitrate, urea, and TCE; Fig. 4-2A and B). DIN as nitrate was only detected in the nitrate treatment at time zero. The nitrate was quickly consumed to concentrations below our detection limit (<0.1μM) within 12 hours of the addition. DON increased over time in both the nitrate and control bioassays. DON concentrations decreased to background levels (13 ± 0.7μM TDN) by

time 12 hours in both the TCE and urea bioassays indicating complete utilization of DON supplied.

All of the PO_4 supplied (2.5 μM) was consumed in each treatment. Residual PO_4 concentrations did not go below 0.4 ± 0.1 μM (Fig. 4-2C). DOP was only added with the TCE treatment as a component of the DOM. Background DOP concentrations increased by 2.1± 0.5 μM with the TCE addition. Sixty percent of the DOP supplied with the TCE DOM addition was consumed by the *K.brevis* community (Fig. 4-2D). DOC concentrations decreased initially and then subsequently increased after the first 12 hours in all treatments except the TCE treatment (Fig. 4-2E). The DOC concentration in the TCE treatment continued to decrease in concentration through the first 24 hours mirroring the increase in cell number and mimicking the observed decreases in DON, DOP and PO_4.

To further investigate the bioavailability of *Trichodesmium* sp. DOM to *K.brevis*, DOM samples were extracted sequentially and analyzed using ESI-MS. The total recovery of initial DON ranged between 66% and 81% in all TCE time series treatments. The DOC recoveries were similar to the DON recoveries and ranged between 53% and 77%.

The TCE DOM addition was bioavailable at the bulk and compound level and contained unique compounds (*m/z*'s) that were not found within the *Karenia spp.* bloom water (control). Fifty-three percent (174) of the masses detected in the TCE initial (background bloom DOM + TCE) were unique to that treatment and were not detected in the *Karenia spp.* bloom water (control). Of those unique compounds 56% (98 *m/z*'s) significantly decreased in concentration during the first 24hours of the experiment (Fig.

4-3). The remaining masses (107) that significantly (α=0.05) decreased were shared with the *Karenia spp.* bloom water (control) indicating that consumption of ambient DOM did occur. Sixteen percent (17) of the masses that decreased were shared by the control, TCE concentrate and TCE bioassay indicating that some bioavailable masses produced by the *Trichodesmium* culture were also present within the *K.brevis* bloom water. The masses that decreased in ion abundance represent those masses that were bioavailable to the plankton community dominated by *K.brevis*, regardless of their origin. The median mass unique to the TCE treatment was *m/z* 322, however, the median unique mass that significantly decreased in concentration was *m/z* 617, indicating that masses greater than *m/z* 500 appear to be more dynamic than those masses less than *m/z* 500.

4.4.4 Bacterial community composition

The total number of operational taxonomic units (OTUs; one or more bacterial species) was initially stimulated with the TCE DOM addition but quickly decreased after the first 12 hours as nutrient levels decreased and *K.brevis* abundance increased. The number of observed OTUs detected in the TCE bioassay increased from 26 at time zero to 30 by time 12 hours. While the number of species increased, the Simpson's diversity index decreased from 0.23 to 0.1. By time 24 hours the total number of observed OTUs detected had decreased to 16, however, the proportion of the each species had once again become dispersed as indicated by a diversity index of 0.24.

Only 5 OTUs (102, 104, 84, 248 and 179) persisted throughout all three time points (0, 12, and 24hours). Combined, these five OTUs represented between 35% and 65% of the total population (Fig. 4-4). Thirteen OTUs were present at both times zero

and 12 hours but disappeared by time 24 hours. This decrease in OTUs preceded the observed decrease in *Karenia spp*. abundance.

A principal component analysis (PCA) of the areas of observed OTUs, ion abundance of masses detected using ESI-MS and *Karenia* spp. abundance revealed three possible components with component 1 representing 92% of the variance. This component was most strongly correlated to *Karenia spp*. abundance however, the relationship between bacterial community composition and *Karenia spp*. abundance was not clear.

4.5 Discussion

For a red tide to reach concentrations of several million cells L^{-1} a significant source of N is required. DON is the largest pool of fixed N in many aquatic systems (Bronk 2002) and due to extremely low DIN concentrations in the Gulf of Mexico DON has been suggested as a likely form of N used by *K.brevis* for growth (Walsh and Steidinger 2001). Previous estimates indicate that riverine transport may account for 5-20% of the N required to sustain blooms of 3 $x10^5$ cells L^{-1} in certain coastal regions (Vargo et al. 2004). However, bloom concentrations reach up to 50 times greater cell density and N demand. By fixing N_2 *Trichodesmium* sp. provides an additional source of N to the ecosystem. Through *Trichodesmium*, *K.brevis* may not only benefit from an initial pulse of N during bloom initiation when cell numbers are low but more importantly could be provided a somewhat continuous source of regenerated N throughout their growth stage.

Within this study we show that *K.brevis* used complex DON as a nutrient source and achieved a division rate of 1.05± 0.15 division day^{-1} (μ= 0.73± 0.1 day^{-1}) when

supplied with DOM released by *Trichodesmium* sp. The observed division rate in the TCE bioassay is higher than typical field observations of 0.16-0.98 divisions day^{-1} (μ= 0.11-0.68 day^{-1}; examples in VanDolah et al. 2008). However, the maximum achieved division rate during any 12 hour period ranged between 0.2 and 0.94 (μ= 0.14 and 0.65 day^{-1}) have been documented in laboratory studies using several different *K.brevis* clones (Loret et al. 2002). High *K.brevis* division rates are not commonly observed in field populations which has made modeling the growth of *K.brevis* difficult, requiring the development of complex ecological models to explain what conditions might allow this "slow-growing" species to thrive (Bissett et al. 2008). However, traditional sampling techniques poorly resolve dynamics associated with HAB populations (Stolte and Garcés 2006; Schofield et al. 2008). The high growth rates observed with the nutrient additions suggests that *K.brevis* can achieve high growth rates which, simplifies the dynamics required to establish a red tide.

The growth response observed in the nitrate treatment differed from that of either the TCE or urea treatments in that cell number only significantly increased in abundance between times 12 and 24 hours, when light was available for photosynthesis. This delay in growth likely reflects the metabolic cost of reducing the oxidized nitrate and can account for the reduced μ potential for *K.brevis* cultures grow on nitrate. The decrease in overall cell number between 12 and 24 hours in the urea treatment is likely due to N limitation, as the TCE treatment, which received twice the N addition achieved a cell concentration that was twice that of the urea treatment. Based on the initial uptake and growth response of the population to the urea addition, the urea and TCE treatments would likely have had similar division responses if supplied at the same final N

concentration. This is significant as urea is an important component of anthropogenic N and represents greater than 50% of the nitrogenous fertilizer used globally (Glibert et al. 2006). Beyond being a potential source of N to coastal systems, increased urea fertilization correlates with an increase in observed HABs (Anderson et al. 2002). The stimulation of *K.brevis* growth by urea has implications for the effects of agriculturally based eutrophication on coastal *K.brevis* blooms.

The maximum decrease in concentration (uptake) of N and phosphorus (P) were compared to the maximum change in *Karenia spp*. cell number to determine the amount of N and P required to support an increase of one million *Karenia* cells (Table 4-2). This increase in population was found to require 0.44-0.49 µmols P and 1.83-2.25 µmols N per million *Karenia* cells. The observed stoichiometry per million *Karenia spp.* cells is similar to literature values of 0.48 µmols P (Wilson 1966) and 3.0 µmols N (Odum et al. 1955) but much lower than more recent estimates (Vargo et al. 2004). The molar uptake of nutrients per million cells gives a final molar N:P ratio of 4.4-4.7 required for growth. The observed N:P ratio is lower than previous estimates of particulate molar ratios, 9-489 (Heil 1986; Heil et al. 2001; Vargo et al. 2004), however, this difference is small (<1 order of magnitude) compared to the large degree of variability in observations. The calculated molar DOC: N uptake ratios from the TCE treatment is 11.6, falling at the higher end of the observed 6-11 C:N range for *K.brevis* (Heil 1986). Documented N:P and C:N ratios for natural *K.brevis* blooms show a high degree of variability (Heil 1986; Heil et al. 2001) and the differences in molar ratios may be explained by population and resource variability (White et al. 2006; Heil et al. 2001).

Several factors may have contributed to the observed N:P and C:N ratios including the stage of the bloom at the time that it was sampled, nutrient availability and the relative uptake of organic compared to inorganic nutrients. Stoichometric observations of natural *K.brevis* blooms showed that N:P ratios increased significantly from 15 to 51 as the bloom aged and became P limited (Heil et al. 2001). Therefore, it is logical that the N:P ratio in this study is lower than other observations based on the N limitation of this population. This difference in stoichiometry has implications for our current understanding of bloom dynamics and nutrient cycling within the Gulf of Mexico. The observed stoichiometry of nutrient concentrations required to double the population of *K.brevis* was lower than recent estimates (Vargo et al. 2004), indicating a potential inflation of bloom requirements.

DOM produced by *Trichodesmium* sp. is bioavailable to and supports the growth of *K.brevis*. At least 53% of DON produced by *Trichodesmium* sp. was directly or indirectly available to the *K brevis* bloom community. This is based on the recovery of DON during the salt removal process and the uptake of DON in the TCE bioassays. In addition to DON, the DOP and DOC concentrations also decreased within the TCE bioassay.

At the compound (unit mass) level, 56% of the masses unique to the TCE treatment (detected in the TCE treatment but not the control) significantly decreased in ion abundance. These masses represent previously uncharacterized compounds produced by *Trichodesmium* sp. that are bioavailable to the microbial community dominated by *K.brevis*. Now that these bioavailable masses have been identified, chemical composition and structural information could be obtained for each bioavailable mass through the use

of higher resolution analyses such as ESI with Fourier transform ion cyclotron resonance mass spectrometry (ESI FT-ICR MS) and ESI with tandem mass spectrometry (ESI-MS/MS) (Sipler and Seitzinger 2008).

While the nutrients supplied in the bioassay treatments resulted in an increase in *Karenia* spp. abundance, it also supported a bacterial community. Field observations show that bacterial abundance as non-algal particles negatively correlate with *K.brevis* abundance indicating that a negative relationship may exist between *K.brevis* and bacterial populations (Schofield et al. 2006). Although several studies have investigated the bacterial species associated with *K.brevis* cultures the relationship of natural *K.brevis* blooms to their co-occurring bacterial community remains understudied at the community level. A recent but unpublished investigation suggests that alpha Proteobacteria and Bacteroidetes groups, typically associated with non-HA blooms, are dominant during natural *K.brevis* blooms (discussed in Kodama et al. 2007). Based on the data presented within the current investigation, *K.brevis* abundance appears to negatively affect a significant number of the bacterial species present. Some possible ways that *K.brevis* may affect bacteria include competition for nutrients (Caron 1994), negative impacts on the community due to toxin production, or direct phagotrophic grazing of cyanobacterial cells (Procise and Mulholland 2008; Glibert et al 2009). While the total number of bacterial species (OTUs) detected decreased over the first 24 hours of the TCE bioassay, some bacterial species were resilient and maintained dominance within the total bacterial population (Fig. 4-4). These resilient OTUs may represent natural populations of algicidal bacteria and those bacteria unaffected by *K.brevis* neurotoxins.

Karenia brevis is an opportunistic species that appears to have the ability to take full advantage of a wide range of DOM compounds and sources (Bronk et al. 2004; Walsh et al. 2006; Vargo et al. 2008). This study has provided evidence that *K.brevis* affects the natural bacterial community composition and DOM pool through at least 205 *m/z*'s that significantly decreased in ion abundance. Due to the diversity in known compounds used including urea, amino acids (Baden and Mende 1979; Bronk et al. 2004), and the complete utilization of *Trichodesmium* sp. derived DON in this study we suggest that both natural and anthropogenic N sources support and enhance growth within *K.brevis* blooms.

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Table 4-1

Concentration (μM)	*Trichodesmium* sp. culture exudates initial	*Trichodesmium* sp. Culture exudates final addition
DOC	497.9 ± 2.6	137.7± 1.4
$NO_3 + NO_2$	0.4± 0.1	0.0± 0.1
NH_4	2.4± 0.1	0.1± 0.1
DON	64.8± 1.8	18.9± 1.1
PO_4	1.1± 0.1	0.2± 0.1
DOP	N/A	1.0± 0.1

Nutrient concentrations present in the *Trichodesmium* sp. culture exudates (TCE) initial and the final nutrient addition of TCE bioassay treatments.

Treatment	N uptake (µM TDN)	P uptake (µM TDP)	Uptake N:P molar ratio	Change in *Karenia* spp. (10^6cells L^{-1})	µmol P per million *Karenia* spp. cells	µmol N per million *Karenia* spp. cells
Control	-0.1 ± 2.1	0.1 ± 0.9	-0.1	-0.1 ± 1.1	-	-
***Trichodesmium* DOM**	18.7 ± 1.7	4.1 ± 0.5	4.6	10.1 ± 1.4	0.41	1.85
Urea	10.5 ± 0.9	2.4 ± 0.4	4.4	4.9 ± 1.1	0.49	2.14
Nitrate	10.8 ± 0.2	2.3 ± 0.1	4.7	4.8 ± 0.4	0.49	2.25
Cultures			9-489 (Heil 1986, Vargo et al. 2004)		0.48 Wilson 1966	3.0 Odum et al. 1955

Table 4-2 Maximum stoichiometric response of a natural population of *Karenia spp* to three different nitrogen additions during the first 24hours of the incubation. The maximum change in concentration of N, P and *Karenia* spp. cell abundance was used to determine the total possible community response.

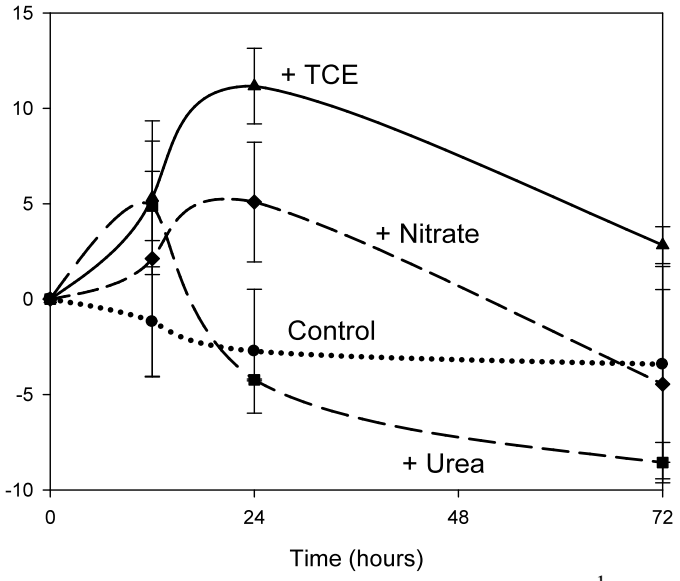


Fig 4-1 Change in millions of *Karenia spp*. cells L^{-1} in bioassay treatments (Control= dotted line, +TCE= solid line, +Urea= short dashed line, + Nitrate= long dashed line). Points are the average difference in concentration ± S.D. of triplicate incubations (positive = increased in cell number, negative = decreased in cell number).

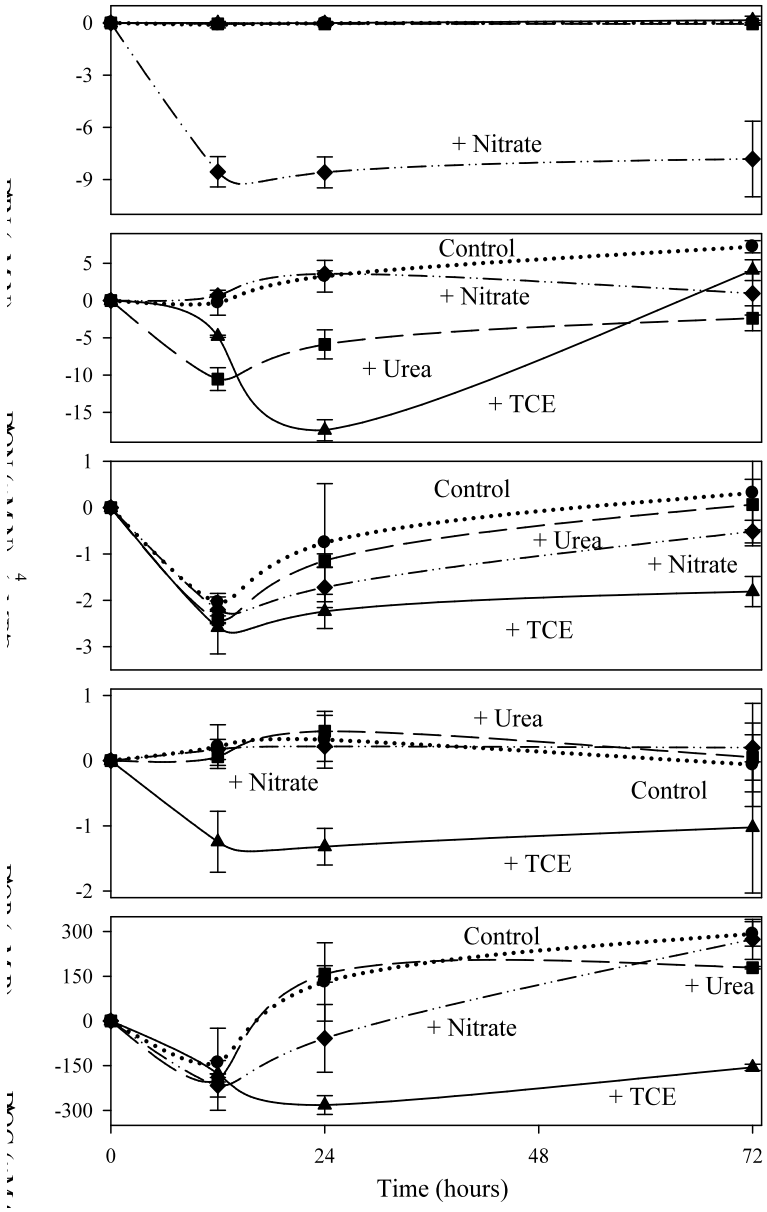


Fig. 4-2 Change in nutrient concentrations in bioassay treatments (Control= dotted line, +TCE= solid line, +Urea= short dashed line, + Nitrate= long dashed line). Points are the average difference in concentration ± S.D. of triplicate incubations (positive = nutrient increased in concentration, negative = nutrient decreased in concentration).

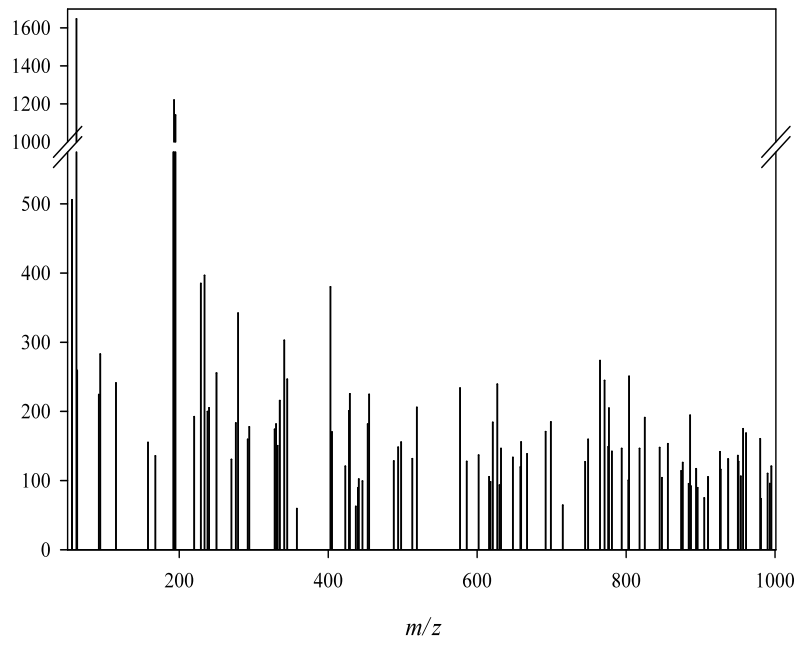


Fig. 4-3. ESI spectra of masses unique to the *Trichodesmium* cellular exudates (TCE) bioassay that significantly (p=0.05) decreased in ion abundance within the first 72 hours.

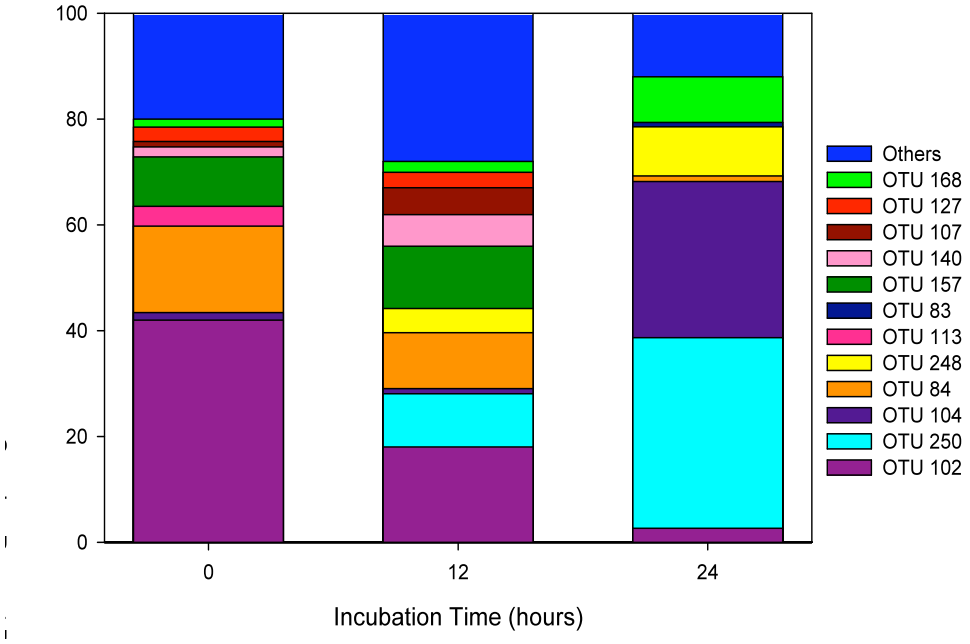


Fig. 4-4. Percent dominance of bacterial species detected using TRFLP at times 0, 12, and 24 hours in the TCE addition bioassays. Species are represented as operational taxonomic units (OTUs)

Chapter 5. Allelopathic effects of brevetoxin on natural microbial community composition

5.1 Abstract

Bacterial community composition is directly affected by brevetoxin, a marine neurotoxin produced by the dinoflagellate *Karenia brevis* and raphidophyte *Chattonella cf. verruculosa*. The effects of brevetoxin on the abundance and community composition of natural microbial communities was investigated by adding synthesized brevetoxin to bacterial communities from three different coastal marine locations with varying historical brevetoxin exposure. Bacterial communities differed in the concentration of brevetoxin required to observe a significant decrease in live bacterial number. Those populations with limited or no previous exposure to brevetoxin were more susceptible to its affects than those populations frequently exposed. The community with no documented exposure to brevetoxin was the most sensitive to brevetoxin with a significant change in live cell number occurring with additions of 25 $\mu g\ L^{-1}$ or more brevetoxin. No significant decrease in live cell number was observed within the community frequently exposed to brevetoxin. Assessments of bacterial community composition revealed that bacterial communities with limited or no previous exposure became more similar with increasing brevetoxin concentrations indicating that brevetoxin may have been selecting for specific species or groups of species and against others. Based on the number of species that disappeared in the 200 $\mu g\ L^{-1}$ brevetoxin treatment after the 48 hours, as much as 37% of the species present in a system could be lost through the introduction of brevetoxin to a previously unexposed community. If the presence of brevetoxin increases in frequency and concentration in systems with limited

or no previous exposure, decreases in bacterial number and changes in community composition would be expected.

5.2 Introduction

Brevetoxins are potent neurotoxins naturally produced by the dinoflagellate *Karenia brevis* (*K.brevis;* Davis) and raphidophyte *Chattonella cf. verruculosa* (*C. cf. verruculosa;* Landsberg 2002). Blooms of these species and their subsequent toxins have been linked to fish, shellfish, marine mammal, marine invertebrate and sea bird mortalities as well as negative human health affects including neurotoxin shellfish poisoning (NSP) and respiratory distress (Landsberg 2002; Kirkpatick et al. 2004). Chemically, brevetoxins are a suite of hydrophobic, polycyclic ether compounds (Fig.5-1; Baden 1989; Nicolaou et al. 1998; Kirkpatrick et al. 2004; Vilotijevic and Jamison 2007). There are 2 structural types and at least 9 forms of brevetoxin with brevetoxin 2 being the most abundant (Baden 1989; Pierce et al. 2002). These compounds are known to negatively affect organisms by activating and thus opening the voltage gated sodium channels allowing excess amounts of sodium to enter the cell leading to depolarization and alteration of the cell membrane (Baden, 1983; Purkerson et al. 1999; Kirkpatrick et al. 2004).

Toxic *K.brevis* blooms frequently disrupt the marine communities along the west Florida shelf and occasionally along the south Atlantic Bight with a range between Jacksonville, FL to Beaufort, NC (Gessey and Tester 1993; Buck and Pierce 1989; Tester and Steidinger 1997, Landsberg 2002). In 2000, brevetoxin production associated with a *C. cf. verruculosa* bloom appeared in Rehoboth Bay, Delaware and its tributaries (Bourdelais et al. 2002). These two brevetoxin producing species exist in very different

aquatic niches. *K.brevis* blooms nearly annually in the tropical ologotrophic waters of the Gulf of Mexico and *C. cf. verruculosa* is often observed in the shallow eutrophic canals and bays of the mid Atlantic (Delaware). This wide range of environmental conditions suggests that brevetoxin may pose a risk to a diverse and thus large number of estuarine and coastal regions and communities. While extensive research has been done on the effects of brevetoxin on species ranging from zooplankton to marine mammals and even humans (Landsberg 2002), there are relatively few studies on the effects of brevetoxin on co-occurring phytoplankton and to our knowledge no studies of the direct affect of brevetoxin on bacterial community composition.

The relationship between bacteria and phytoplankton is complex including known symbiotic, antagonistic, predatory and competitive associations (Cole 1982). The role that many marine toxins, including brevetoxin, play in microbial community structure is still an open question. Although algicidal bacteria active against *K.brevis* have been identified in culture (Doucette et al. 1999; Mayali and Doucette 2002; Roth et al. 2008), it is not understood how these relationships translate to natural microbial assemblages. Field observations have thus far provided contrasting evidence of the relationship between bacteria and the toxic species *K.brevis*. There is evidence that bacterial biomass as non-algal particles decrease with increasing *K.brevis* biomass, indicating that *K.brevis* may negatively affect natural bacterial populations (Cannizzaro 2004; Schofield et al. 2006). Thus it has been suggested that decreased bacterial abundance during *K.brevis* blooms may be linked to brevetoxin production (Schofield et al. 2006). However, there are also examples of increased bacterial abundance and productivity within *K.brevis* blooms compared to surrounding waters (Evans 1973; Heil et al. 2004) and yet other

studies suggest that there is no relationship between bacterial abundance and *K.brevis* abundance (Buck and Pierce 1989). These very different assessments suggest a complex relationship exists between bacteria and *K.brevis*.

Beyond the effects on the total number of bacteria present, little is known about the effects that brevetoxins may have on microbial community composition. Field observations of saxitoxin producing *Alexandrium spp*. blooms indicate shifts in bacterial species dominance may occur when toxin levels are high (Pomati et al. 2003; Jasti et al. 2005). This indicates that some species may be more or less tolerant to saxitoxin than others. Better understanding of the mechanisms, including toxin effects, that control competition and community composition are crucial to understanding bloom dynamics. *K.brevis* does not grow axenically, so better understanding of the relationship between *K.brevis* and the bacteria associated with it will yield further insight into the dynamic nature of these monospecific blooms.

Why species produce these complex toxins is still unknown. Under nutrient deplete conditions some species of harmful algae may be more likely to produce allelopathic chemicals/ toxins than in nutrient replete conditions (Granéli 2006). This strategy is believed to aid in the reduction of nutrient stress by not only eliminating competitors but also gaining bound nutrients through their remineralization (Granéli 2006) as bacterial cell lysis can be a significant source of DOM (Middelboe and Lyck 2002; Kawasaki and Benner 2006). It is also possible that toxins play a role in changing the microbial community composition, controlling competition and nutrient regulation not simply deterring grazing (Granéli and Turner 2006; Granéli 2006). The ability to take full advantage of nutrient resources may give organisms like *K.brevis* a competitive

edge over other bloom forming phytoplankton (Liu et al. 2001; Bronk et al. 2004; Chapter 4).

This study investigated the effects of brevetoxin on the bacterial abundance and community composition of natural bacterial populations from sites with varying levels of previous brevetoxin exposure ranging from unexposed to frequently exposed. To do this synthesized brevetoxin was supplied to three different microbial communities - one in each, Great Bay, New Jersey, Rehoboth Bay, Delaware, and Sarasota Bay, Florida. Each of these sites represents regions of different previous exposure. Change in bacterial abundance was evaluated through live/ dead bacterial cell counts and terminal restriction length polymorphism (TRFLP) was used to investigate the effects of brevetoxin on bacterial community composition. The community composition after brevetoxin addition for each of the three sites was then compared to the bacterial community composition of a natural *K.brevis* bloom and the bacteria associated with two *K.brevis* cultures of varying historical brevetoxin production.

5.3 Methods

Brevetoxin was added to three different coastal microbial populations to investigate its affect on the bacterial community abundance and composition. Locations were chosen based on available data of brevetoxin exposure. The three locations [Great Bay, NJ ($39^{o}32'22"N$, $74^{o}23'13"W$), Rehoboth Bay, DE ($38^{o}39'45"N$, $75^{o}07'53"W$) and Sarasota Bay, FL ($27^{o}19'54"N$, $82^{o}34'34"W$)] represent communities with no documented exposure to brevetoxin, occasional brevetoxin exposure (Bourdelais et al. 2002) and frequent exposure to bevetoxin (Fire et al. 2007; Pierce and Henry 2008; Pierce et al. 2008), respectively (Fig. 5-2). This experiment was designed to determine if

any or all of these communities were positively or negatively affected by brevetoxin and what concentrations of brevetoxin elicit the greatest change in the community composition and abundance. Bacterial community composition samples were compared to the bacterial communities associated with cultures of two different *K.brevis* clones, Wilson strain and Wilson low toxin strain maintained at Mote Marine Laboratories and a natural *K.brevis* bloom.

5.3.1 Field sample collection and preparation- Brevetoxin additions

Bay water samples were collected via bucket at the surface and were gently filtered through a 3µm polycarbonate inline filter to remove phytoplankton and zooplankton. The bay water was then divided into 32 mL aliquots and pipetted into 16 acid washed (12% H_2SO_4) and combusted (500^oC) 50 mL glass culture tubes fitted with acid washed and autoclaved (121^oC) caps. The bay water samples were spiked with 0, 2.5, 5, 10, 25, 50, 100, and 200 µg L^{-1} brevetoxin 2 (PbTx-2) standard reference material (Calbiochem, San Diego, CA, USA).

Due to brevetoxin's hydrophobic nature, 1 mL of 99.9% pure methanol (MeOH) was used to bring 100µg of the solid brevetoxin reference material into solution. The brevetoxin solution was then diluted with 10 mL deionized water (DIW). All samples were normalized with a 10:1 DIW:MeOH to ensure that all contained the same amount of methanol. The final MeOH addition per sample was 0.002% v/v (MeOH: DIW+ field sample). The control treatment (0 µg L^{-1} brevetoxin) contained no brevetoxin but an equal amount of methanol required to bring brevetoxin into solution. All treatments were run in duplicate. No nutrients were added. Ambient temperatures at the time of collection ranged between 25.5^oC and 31.5^oC at all 3 locations. Samples were incubated

at the appropriate corresponding temperature. Each treatment was sampled at time 0, 24, and 48 hours for bacterial abundance and community composition. Bacterial community composition was analyzed for the 0 μg L^{-1}, 25 μg L^{-1} and 200 μg L^{-1} brevetoxin treatments at all three bay locations.

5.3.2 Field sample collection of K.brevis bloom transect

K.brevis cell counts and bacterial community composition samples were taken from a three point transect through a natural *K.brevis* bloom in the Gulf of Mexico on October 23, 2007. The *K.brevis* bloom was identified through ship board observations near Sanibel Island, FL in the southeastern portion of the Gulf of Mexico. The bloom transect was based on *K.brevis* cell counts and samples were collected at 26^{o} 25.9335' N, 82^{o} 11.520'W, 26^{o} 23.268' N, 82^{o} 10.864'W, and 26^{o} 26.708' N, 82^{o} 12.718'W (Fig.5-2). Bloom water was collected with a bucket at the surface. Duplicate phytoplankton cell count samples were taken per transect site and were preserved in Lugols iodine solution.

5.3.3 Sample collection from K.brevis cultures

Bacteria communities associated with two different *K.brevis* strains maintained at Mote Marine Laboratory (Sarasota, FL) were compared to the bay and bloom communities. The two strains differ on the amount of toxin produced, one producing lower levels of toxin than the other (Pierce, personal comms). For this study the two strains will be designated Wilson high toxin and Wilson low toxin.

5.3.4 Analyses

Direct bacterial cell counts were performed using a Live/ Dead BacLight Bacterial Viability stains (Invitrogen L-7012) to monitor the bacterial population through cell membrane viability. The SYTO 9 nucleic acid stain stains live cells while cells with

compromised membranes that are dead or dying are stained by propidium iodide (Leuko et al. 2004). This method allowed us to determine what proportion of the bacterial population was compromised but had not yet lysed (Fig. 5-2). Stains were added simultaneously (3 µL each) to duplicate 1mL bioassay subsamples in 2.5 mL microcentrifuge tubes. Samples were vortexed and incubated in the dark for 15 minutes. After their incubation, samples were filtered at 5 psi onto 0.2 µm 25 mm Whatman black polycarbonate filters. Once the sample had been filtered, a 2 mL 2% gluteraldehyde wash was applied to the filter to preserve the sample. The filters were then mounted on microscope slides and counted using an epifluorescent microscope (Hobbie et al. 1977). *Karenia spp*. cells were enumerated using an inverted microscope on a bright field setting. *Karenia* species were identified based on cell morphology as *Karenia sp.* (Steidinger et al. 2008).

5.3.5 Bacterial community composition

Bacterial composition samples were gently filtered (5 psi) onto 0.2 µm Supor-200 membrane filters (Pall Corporation Ann Arbor, MI). The membrane filters were placed in sterile 2 mL low temperature freezer vials and immediately frozen in liquid nitrogen, on dry ice or placed in a -80^{o}C freezer. All samples remained frozen until analysis.

Changes in bacterial community composition were assessed using 16s rRNA SSU GeneFragment Amplification and Terminal Restriction Fragment Length Polymorphism (TRFLP). A modified phenol chloroform DNA extraction method (Sakano and Kerkhof 1998) was used to extract the total nucleic acids from the microbial biomass samples. The DNA was further purified using a cesium chloride density gradient. Briefly, 0.54 g cesium was added to a total volume of 500 µl which included 100 ng of sample DNA and

300 ng of *Halobacerium salinarium* and 2 µl of 1% ethidium bromide. *Halobacterium* DNA was used as a marker in order to visualize the DNA in the cesium gradient under a 312 nm Transilluminator UV light. The gradients were spun for 24 hours at 80,000 rpm in a Beckman Optima TL Ultracentrifuge 120.1 (Beckman Coulter, Fullerton, CA). DNA bands were extracted from the centrifuged cesium via pipette. The cesium was dialyzed from the sample using a Millipore membrane dialysis filter (VSWP 02500) for 45 minutes. The dialyzed DNA was then stored at –80ºC until further analysis.

The cleaned genomic DNA was used to amplify the 16S rRNA genes via PCR (polymerase chain reaction). For the 16S rRNA genes from DNA, 50 µl PCR reactions were set up with 10 ng template and 20 pmol of the universal primer 27 Forward (5' AGA GTT TGA TCC TGG CTC AG 3') and the bacterial specific primer 1100 Reverse (5' GGG TTG CGC TCG TTG 3') per reaction. The amplification parameters were as follows: 94ºC for 5 minutes followed by 25 cycles of 94ºC for 30seconds, 57ºC for 30 seconds, and 72ºC for 1.1 minutes and a final extension period of 72ºC for 10 minutes.

For TRFLP profiling, all forward primers were labeled with 6-carboxylfluorescien (6-FAM; Applied Biosystems, Foster City, CA). These fluorescently labeled amplicons were run on a 1% agarose gel for quantification via image analysis and 15 ng were digested with the endonuclease MnlI (New England Biolab, Beverly, MA) at 37ºC for 6 hours. The 20 µL digestion reactions were then precipitated using 2.3 µL mixture of 0.75 M sodium acetate, 5 µg of glycogen, and 37 µl of 95% ethanol. The reactions were then dried briefly and resuspended in 19.7 µL of deionized formamide and 0.3 µL of ROX 500 size standard (Applied Biosystems, Foster City, CA) for 15 minutes and denatured for 94ºC for 2 minutes before analysis. TRFLP profiling was carried out using Genescan

software and an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). TRFLP peak detection was set at 50 arbitrary fluorescent units. All detected peaks were downloaded into spreadsheets and those peaks representing <0.1% of the total peak area were discarded. The remaining peaks were parsed and normalized to a uniform total profile area to adjust for small loading differences between samples. Peaks represent operational taxonomic units (OTUs) which in turn represent at least one bacterial species.

5.3.5 Statistical analyses

A standard paired t-test with 95% confidence level was used to assess significant difference between replicate bacterial count samples, each brevetoxin treatment (2.5, 5, 10, 25, 50, 100, and 200 µg L^{-1} brevetoxin) and the control (0 µg L^{-1} brevetoxin) treatment and the peak areas of different TRFLP samples. The results of this analysis were reported as p values. Those comparisons with p values < 0.05 were considered to be significantly different. The Sorensen similarity index was used to assess the relationship between two samples based on species (OTUs) detected. It is calculated as twice the number of masses shared between two samples divided by the sum of the total number of masses detected in each sample. The Sorensen similarity index has an interpretational range of 0-1 with 1 indicating the greatest degree of similarity.

5.4 Results

5.4.1 Brevetoxin addition experiment

All brevetoxin addition samples were collected between June 17, 2008, and August 25, 2008. The salinities and temperatures were 28.5 and 25.5^{o}C at the NJ site, 25.5 and 29.7^{o}C at the DE site, and 31.0 and 31.5^{o}C at the FL site. Initial live bacterial cell numbers were 1.5 x 10^5 ± 0.86 x 10^5 cells L^{-1at} the NJ site, 3.4 x 10^5 ± 1.5 x 10^5 cells

L^{-1} at the DE site and $1.3 \times 10^5 \pm 0.37 \times 10^5$ cells L^{-1} at the FL site. There were 32, 38, and 48 OTUs (one or more species) detected in the initial NJ, DE and FL samples, respectively. Although NJ and FL were relatively similar in bacteria abundance, the FL site had 33% more OTUs than the NJ site. The community composition of the initial bay water samples from NJ and DE were more similar to each other with a shared similarity index of 0.76 than to FL (0.54 and 0.50, respectively).

While differences in live cell number were observed after 24 hours this difference became more pronounced after 48 hours. A dose response was observed in both the NJ and DE communities with a larger decrease in live cell number occurring after 48 hours in the 200 μg L^{-1} brevetoxin addition compared to the 25 μg L^{-1} brevetoxin or 0 μg L^{-1} brevetoxin (control) addition (Fig.5-3). Bacterial communities differed in the concentration required to observe a significant decreases in live bacterial number compared to the control (0 μg L^{-1} brevetoxin; Table 5-1). The NJ community showed a significant change in cell number with additions of 25 μg L^{-1} or more brevetoxin. The DE community was slightly more tolerant, with significant changes occurring with brevetoxin additions of 50 μg L^{-1} or more. There was no significant decrease in bacterial cell number within the FL community at any concentration.

The number of cells with compromised membranes was determined through the use of propidium iodide cellular stain (Fig. 5-3). There was no significant difference in the number of cells with compromised membranes (dead/ dying cells) in any of the brevetoxin additions within the NJ treatments. Within the DE community, significantly more ($p<0.001$) compromised cells were observed with brevetoxin additions of 50 μg L^{-1} and 100 μg L^{-1} (data not shown) compared to the control. The 200 μg L^{-1} addition was

not significantly different from the control based on number of cells with compromised membranes, however the total number of cells (live and compromised) was significantly ($p<0.05$) different from the control. Although there was no difference in the number of live bacterial cells observed between any of the brevetoxin additions in the FL community, there was a significant difference in the number of compromised cells. Significantly more compromised cells were observed in the 50 μg L^{-1} and 200 μg L^{-1} treatments compared to the control.

The observed bacterial community composition represents all of the bacterial cells present at the time of sampling including both the live and compromised but not degraded cells. Changes in bacterial community composition were observed in all treatments (Fig. 5-4). The differences between the control (0 μg L^{-1}) and brevetoxin additions based on those OTUs detected were only significant in the 200 μg L^{-1} brevetoxin treatment for the NJ ($p= 0.004$) and DE ($p= 0.04$) communities based on OTUs detected. Those species detected in the FL 200 μg L^{-1} brevetoxin treatment were not significantly different ($p=0.1$) from those detected in the control.

The bacterial communities of the 0 μg L^{-1} and 25 μg L^{-1} treatments in the NJ site shared a similarity index of 0.71. In comparison, the DE bacterial communities of the 0 μg L^{-1} and 25 μg L^{-1} treatments shared a similarity index of 0.93. This difference in composition between the 0 μg L^{-1} and 25 μg L^{-1} likely reflects the sensitivity of these communities to the brevetoxin. The NJ site was found to be more sensitive to these effects showing a significant decrease in live cells within the 25 μg L^{-1} treatment compared to the insignificant decrease in live cell number observed at the same brevetoxin addition within the DE community.

The similarity indices of the NJ and DE bacterial communities increased with increasing brevetoxin concentrations. Similarity index values of the NJ and DE initial population, 0, 25 and 200 μg L^{-1} brevetoxin additions were 0.57, 0.63, 0.64 and 0.75, respectively. The initial FL community was only 50% and 54% similar to DE and NJ communities, respectively. Similarities between the FL and DE or NJ sites did not change with brevetoxin additions.

Thirteen of the species present in the NJ community were more dominant in the treatment containing 200 μg L^{-1} brevetoxin than in 0 μg L^{-1} brevetoxin treatment. Sixteen of the species present in the DE community were more dominant in the 200 μg L^{-1} treatment than the 0 μg L^{-1} treatment. While these species appear to be resistant to brevetoxin, 26 OTUs in NJ and 20 OTUs in DE decreased in dominance. Eleven of the OTUs that decreased in NJ community and 14 of those DE OTUs that decreased in dominance fell to concentrations below the detection limit in the 200 μg L^{-1} treatments.

5.4.2 K.brevis bloom microbial communities

A transect of a natural *K.brevis* bloom revealed the bacterial communities associated with 3.8± 0.3 x 10^6, 9.7± 0.7x 10^5, and 4.1± 0.2 x 10^4 *K.brevis* cells L^{-1}. Brevetoxin concentrations were not assessed; however, dead fish were observed at the surface at the site with 3.8 x 10^6 *K.brevis* cells L^{-1}. The *K.brevis* bloom of 3.8 x 10^6cells L^{-1} had a total of 40 associated OTUs, the *K.brevis* bloom of 1 x 10^6cells L^{-1} had a total of 38 associated OTUs and a bloom of 4 x 10^4 cells L^{-1} had a total of 44 associated OTUs. Twenty of the observed OTUs were common among all bloom samples. Thirty OTUs were present in both the 1 x 10^6 and 4 x 10^4 *K.brevis* cells L^{-1} samples. Twenty-three OTUs were common among the 4 x 10^6 and 1 x 10^6 *K.brevis* cells L^{-1} samples.

Based on OTUs detected the 1 x 10^6 and 4 x 10^4 *K.brevis* cells L^{-1} samples had a similarity index of 0.73. These samples were less similar to the 4x 10^6 *K.brevis* cells L^{-1} bloom site with similarity index values of 0.52 and 0.53, respectively.

5.4.3 Bacterial communities of K.brevis cultures

A total of 16 OTUs were detected associated with the Wilson high toxin strain and 25 OTUs were detected associated with the Wilson low toxin strain. Fourteen of the OTUs were shared by both the high and low toxin *K.brevis* cultures. These shared OTUs represent between 97% and 99% of the total bacterial community. Only 1 of the unique OTUs represented more than 1% of the population, OTU 279 represented 1.6% of the community in the low toxin Wilson strain. The bacterial community associated with these two cultures shared a similarity index of 0.63 based on OTUs detected.

5.4.4 Comparison of bacterial community composition

Thirteen of the OTUs detected at all 3 brevetoxin addition bay sites were also detected in the natural *K.brevis* bloom. Six of these shared OTUs (102, 107, 127, 131, 248, and 250) were within the 12 most dominant OTUs in the brevetoxin treatments. The OTUs displaying an increase in dominance with increasing brevetoxin in the 3 bay communities also increased in dominance with increasing *K.brevis* concentration within the natural bloom (Fig. 5.5). OTUs 107, 131, 248, and 250 were not only consistently detected in the brevetoxin addition treatments and blooms but were also detected in both the low and high *K.brevis* cultures. The OTUs unique to the low toxin *K.brevis* culture (OTU 279 and 461) were detected in all 3 bay communities; however, they represented less than 1% of the community in any give sample and thus no definite trends could be

determined. All TRFLP signatures from replicate bottle incubations were found to be between 82% and 92% similar in OTUs detected and their relative abundances.

5.5 Discussion

Brevetoxin affects both bacterial abundance and bacterial community composition. Bacterial communities differed in the concentration of brevetoxin required to observe a significant decreases in live bacterial number based on previous exposures. Those populations with limited or no exposure were more susceptible to the effects of brevetoxins than those populations frequently exposed to brevetoxin. The NJ community which has no documented exposure to brevetoxin was the most sensitive to brevetoxin with a significant change in cell number occurring between 10 $\mu g\ L^{-1}$ and 25 $\mu g\ L^{-1}$ brevetoxin. The DE community was slightly more tolerant with significant changes in cell number occurring between 25 $\mu g\ L^{-1}$ and 50 $\mu g\ L^{-1}$ brevetoxin. There was no significant decrease in bacterial cell number within the FL community at any concentration, however, the change in bacterial number associated with the 200 $\mu g\ L^{-1}$ brevetoxin addition fell just below the 95% confidence range ($p = 0.051$).

An investigation of bacterial populations associated with *K.brevis* blooms showed that agar plate-based bacterial counts did not differ between bloom and non-bloom years near Sarasota, FL (Buck and Pierce 1989). However, bacterial counts taken during a rare bloom near Beaufort, NC showed that bacterial abundance was reduced during the *K.brevis* bloom compared to non-bloom seasons (Buck and Pierce 1989). This supports the current investigation in that decreased bacterial abundance was only observed at the site with limited previous exposure to brevetoxin.

Previous studies of the allelopathic capabilities of *K.brevis* on phytoplankton cultures have minimized the role of brevetoxin as an allelopathic compound (Kubanek et al. 2005; Prince et al. 2008). In one of these studies, brevetoxin purified from *K.brevis* cultures was added at 8.8 μg L^{-1} final addition to cultures of different phytoplankton species (Kubanek et al. 2005). No significant change in the abundance of the tested phytoplankton cultures was observed with that addition. According to the findings herein based on natural bacterial communities, the 8.8 μg L^{-1} addition would have been too low to observe a significant change in the microbial population compared to the control. The concentration of brevetoxin may be more significant than simply its presence, and phytoplankton, like the bacteria in this study, may be affected at greater concentrations than those tested. Therefore, the effect of brevetoxin on microbial populations is not only linked to the presence of the toxin but also the level of exposure (concentration) and the frequency of exposure.

Natural concentrations of brevetoxin during *K.brevis* blooms range from ~2.5 to nearly 100 μg L^{-1} with typical concentrations reaching ~ 10-30 μg L^{-1} (Landsberg, 2002). Concentrations of 2.5 x 10^6 *K.brevis* cells L^{-1} have been reported as a typical concentration observed to cause fish mortality (Quick and Henderson 1974); however longer exposure times at lower brevetoxin concentrations can also result in mortality (Landsberg 2002). Bloom conditions of approximately 2 million *K.brevis* cells L^{-1} are required to reach waterborne brevetoxin concentrations of 20 μg L^{-1} (Tester et al. 2008). Based on the concentration assessed in this study for brevetoxin to play a role in altering microbial communities during bloom initiation when *K.brevis* cellular concentrations are low, bacteria would need to be in close proximity to *K.brevis* cells. It has been suggested

that direct contact between *K.brevis* and other phytoplankton cells may be required for allopathic affects to be observed (Kubanek et al. 2005). At lower cell concentrations, brevetoxin production may be a mechanism by which *K.brevis* eliminates cell surface bacteria and those bacteria and phytoplankton in close proximity. If the toxin is too dilute then no significant response in adjacent specie number would be observed. This suggests that waterborne brevetoxin concentrations must be high in well mixed conditions or *K.brevis* cells must be in close proximity to competitors (bacteria or phytoplankton) at time of release under stratified/ stable conditions.

By negatively affecting bacterial populations brevetoxin may also play a role in nutrient acquisition. Bacteria are thought to indirectly promote phytoplankton growth through the remineralization of DOM (Azam 1998). When nutrient concentrations are low, bacteria compete with phytoplankton for the limiting available nutrients (Caron 1994). Brevetoxin may be the vector by which toxin producing species control their environment and nutrient supply. Since *K.brevis* has the ability to use a variety of organic nutrients including DON (Baden and Mende 1979; Bronk et al. 2004; Chapter 4), eliminating competitors (bacteria) during low nutrient conditions would be a beneficial growth strategy. If one can not only eliminate competition but also benefit through the release of nutrients via their elimination, cell lysis, then producing toxin may be crucial to the survival of these organisms (Granéli 2006).

Although this investigation suggests a clear trend showing a decrease in live bacterial cell number with increasing brevetoxin concentration, the mechanism by which brevetoxin attacks bacterial cells is unknown. In eukaryotic cells, brevetoxin has been shown to act as a sodium channel activator causing depolarization and compromising the

membranes of affected cells (Kirkpatrick et al. 2004). To further investigate the likelihood of brevetoxin affecting bacterial cell membranes, compromised cell number was assessed. Only 4 treatments showed a significant increase in the number of compromised cells relative to the control, the DE 50 μg L^{-1} and 100 μg L^{-1} and the FL 50 μg L^{-1} and 200 μg L^{-1} brevetoxin additions. None of the brevetoxin concentrations elicited an increase in the number of compromised cells within the NJ community. It is possible that an increase in compromised cells was not detected because with less tolerance to the toxin, they lysed at a faster rate. The DE samples showing a significant increase in compromised cells were also samples showing a significant decrease in live cells. This supports that bacterial cell membranes may be compromised by brevetoxin leading to the observed decrease in bacterial abundance and changes in community composition.

In an investigation of changes in bacterial community composition associated with the addition of DOM from *Trichodesmium* supplied to a natural population of *K.brevis,* OTU 250 was found to increase in dominance as *K.brevis* concentrations increased (chapter 4 of this dissertation). This is in agreement with this investigation in that OTU 250 was persistent through out all brevetoxin additions, was found in both high and low toxin *K.brevis* cultures and increased in dominance in the natural bloom with increasing concentrations of *K.brevis*. Because the response of the microbial populations from different aquatic systems varied based on previous exposure and several of the resilient species within these samples also displayed the same dynamics in a natural *K.brevis* bloom, brevetoxin production has, through this study, been directly linked to changes in bacterial community composition.

Brevetoxin negatively affects bacterial abundance and significantly alters the bacterial community composition. Due to the diverse ecological niches that sustain blooms of *K.brevis* and *C. cf. verruculosa* understanding brevetoxin's effect on previously unexposed populations is critical to understanding bloom dynamics and community composition of affected areas. Both of the communities with limited (DE) or no (NJ) previous exposures to brevetoxin showed a significant decrease in bacterial abundance with increasing brevetoxin concentrations. The NJ and DE bacterial communities became more similar with increasing brevetoxin concentrations indicating that brevetoxin was selecting for specific species or groups of species and against others. Based on the number of OTUs that decreased in dominance to below detection limits compared to the control as much as 37% of the species present in a system could be lost through the introduction of brevetoxin to a previously unexposed community. Therefore, if the presence of brevetoxin increases in frequency and concentration in systems similar to Rehoboth Bay, DE and Great Bay, NJ, decreases in bacterial number and changes in community composition would be expected.

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Brevetoxin concentration ($\mu g\ L^{-1}$)	Response of NJ bacterial population	Response of DE bacterial population	Response of FL bacterial population
2.5	0.60	ND	0.90
5	0.082	0.38	ND
10	0.074	0.55	0.95
25	0.016 *	0.45	0.32
50	0.0011 *	0.0004 *	0.066
100	0.0033 *	$p<0.0001$ *	0.083
200	$p<0.0001$ *	$p<0.0001$ *	0.051

* = significant difference ($p<0.05$) between control and brevetoxin addition treatment after a 48hour incubation, ND= not determined

Table 5-1. Results of a standard paired t-test, represented as p-values, to evaluate brevetoxin concentrations that elicited a significant difference in the number of live bacterial cells between the control (no brevetoxin added) and brevetoxin addition experiments after a 48 hour incubation.

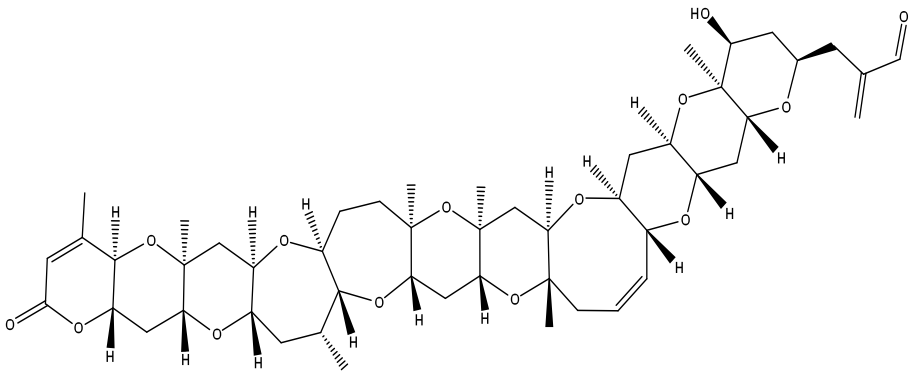


Fig. 5-1. Chemical structure of brevetxoin 2 (PbTx-2), the most abundant form found in nature and the form used within this investigation

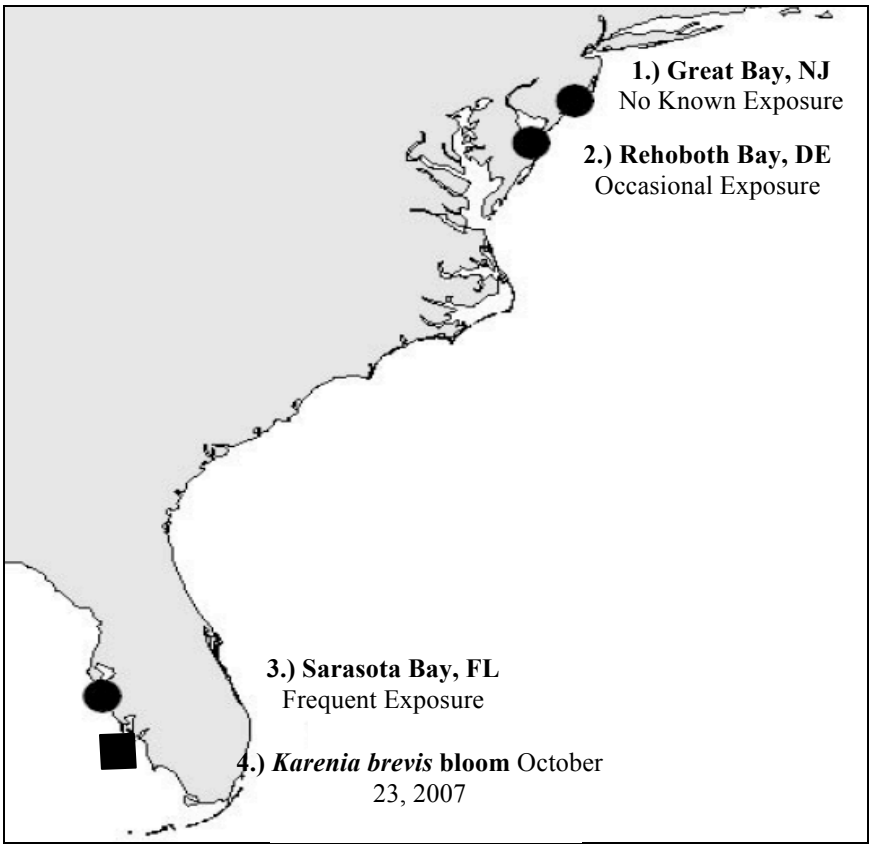


Fig. 5-2. Map of sample sites. Site 1 is Great Bay, NJ (NJ); site 2 is Rehoboth Bay, DE (DE); site 3 is Sarasota Bay, FL (FL); site 4 is the location of the October 2007 *Karenia brevis* bloom.

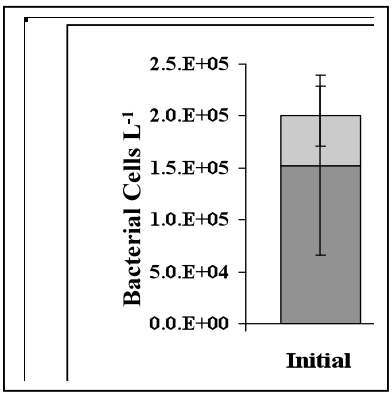


Fig. 5-3. Live (dark gray) and dead (light gray) ± S.D. of bacterial cell number remaining after a 48 hour incubation at 0 µg L^{-1}, 25 µg L^{-1} and 200 µg L^{-1} brevetoxin 2

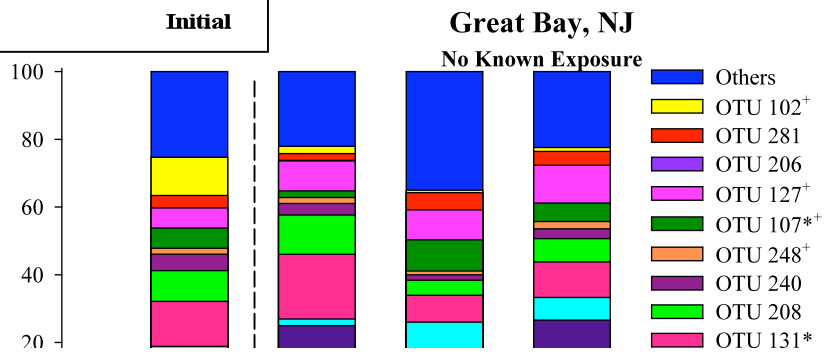


Fig. 5-4. Changes in bacterial community composition as percent dominance associated with changes in cell number in three treatments 0 µg L^{-1}, 25 µg L^{-1} and 200 µg L^{-1} brevetoxin within a 48 hour incubation. * = OTU detected in both *K.brevis* Wilson clone cultures, $^{+}$= OTU detected in a natural *K.brevis* bloom

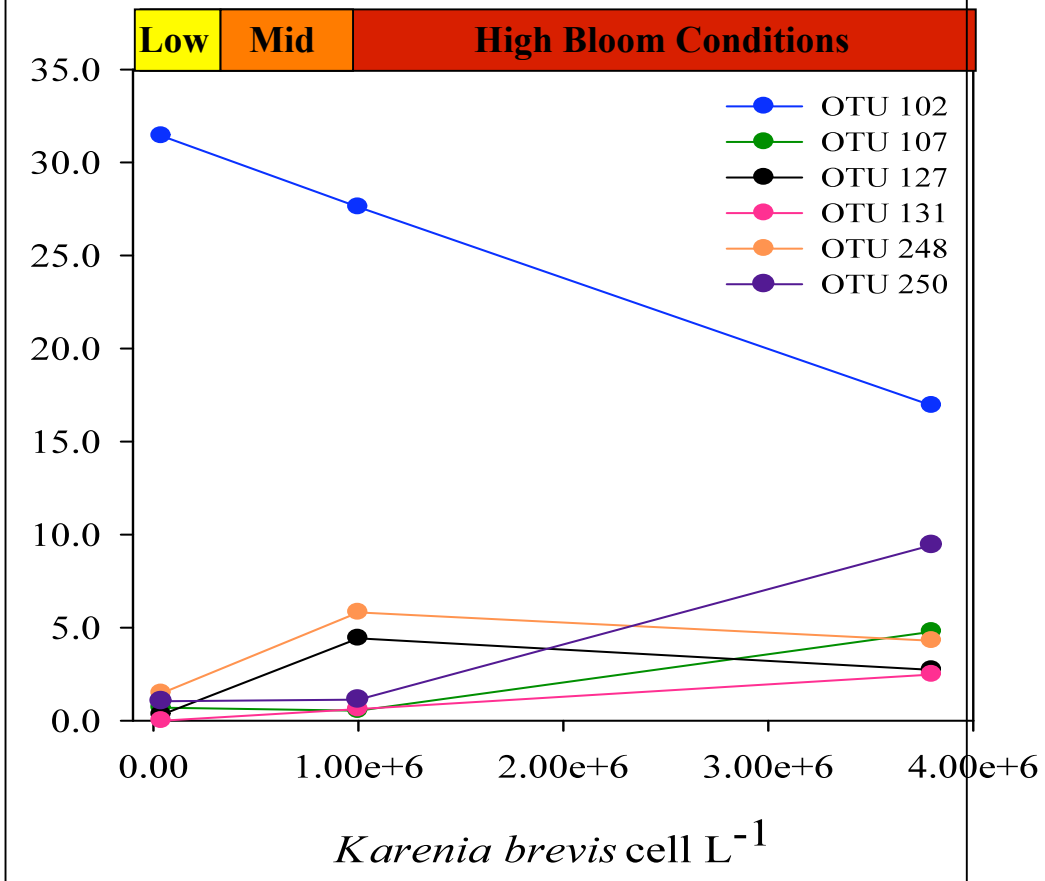


Fig. 5-5. Percent species dominance of species present in gradient of *K.brevis* cells L^{-1}

Appendix A: Supplemental Information for Electrospray Ionization Mass Spectrometry Analyses

Appendix A1
Masses Detected in Positive Mode ESI-MS Analyses

Masses Detected Within Three Natural Phytoplankton Blooms, a Cyanobacteria Culture, Taylor Slough and Shark River

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
50						
51						
52	X		X			
53	X		X			
54	X		X			
55	X	X	X	X		X
56	X	X	X			
57	X	X	X			
58	X	X	X			
59	X					
60	X	X	X			
61	X	X	X			
62	X	X	X			
63			X	X	X	X
64	X	X	X			
65			X			
66		X	X			
67					X	
68	X	X	X			
69	X	X	X			
70	X	X	X			
71	X	X	X			
72	X	X	X		X	
73			X			
74	X	X	X			
75	X		X			
76	X	X	X			
77	X		X	X	X	X
78	X		X			
79			X			
80	X					
81	X	X	X			

82						
83	**X**	**X**	**X**			
84	**X**				**X**	
85	**X**	**X**	**X**			
86	**X**	**X**	**X**			
87	**X**	**X**	**X**			

m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
88	**X**	**X**	**X**			
89	**X**	**X**	**X**			
90	**X**					
91	**X**	**X**	**X**			
92	**X**	**X**	**X**			**X**
93	**X**	**X**	**X**			
94	**X**					**X**
95	**X**	**X**	**X**	**X**		**X**
96	**X**					
97	**X**		**X**			
98	**X**				**X**	
99	**X**	**X**		**X**	**X**	**X**
100						
101	**X**					
102	**X**					
103	**X**			**X**		**X**
104	**X**					
105	**X**					
106	**X**					
107	**X**				**X**	
108	**X**					
109	**X**	**X**	**X**			**X**
110	**X**					
111			**X**			
112	**X**			**X**		
113	**X**			**X**	**X**	**X**
114	**X**					
115	**X**		**X**			
116	**X**					
117	**X**			**X**		
118	**X**					
119	**X**	**X**				
120	**X**	**X**	**X**			
121	**X**			**X**	**X**	**X**
122	**X**				**X**	
123	**X**	**X**	**X**	**X**	**X**	
124	**X**	**X**	**X**			
125	**X**	**X**	**X**			
126	**X**	**X**	**X**			
127	**X**	**X**	**X**	**X**	**X**	
128	**X**	**X**		**X**	**X**	
129			**X**	**X**	**X**	**X**

130	X					
131	X					
132	X					
133	X					
134	X					
135	X			X	X	
m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
136	X	X				
137	X			X		
138	X			X		X
139	X	X	X			
140	X					
141	X	X	X			
142	X					
143	X		X		X	
144			X			
145	X		X		X	
146	X		X			
147	X			X		
148	X					
149	X	X	X			
150	X	X				
151	X	X	X			
152	X	X				X
153	X	X	X			X
154	X		X		X	X
155	X	X	X	X	X	X
156	X		X			
157	X		X	X	X	
158	X				X	
159	X					
160	X				X	
161	X					
162	X					
163	X			X	X	
164						
165	X	X	X			
166	X					
167	X		X	X	X	X
168	X					
169	X			X	X	X
170						
171	X			X	X	X
172						
173	X					
174	X				X	
175	X	X	X			X
176	X		X			
177	X	X	X			
178	X		X			X
179	X	X				

180	**X**				**X**	
181	**X**	**X**	**X**			
182	**X**				**X**	
183	**X**	**X**				

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
184	**X**	**X**			**X**	**X**
185	**X**					
186	**X**					
187	**X**				**X**	
188	**X**				**X**	
189	**X**					
190	**X**			**X**	**X**	
191	**X**	**X**	**X**			
192	**X**		**X**		**X**	
193	**X**	**X**	**X**			
194						
195	**X**			**X**	**X**	**X**
196	**X**					
197	**X**	**X**	**X**			
198	**X**					
199	**X**	**X**	**X**			
200	**X**					
201	**X**					
202	**X**					
203	**X**					**X**
204	**X**					
205	**X**					
206	**X**					
207	**X**	**X**	**X**	**X**	**X**	
208	**X**					
209	**X**		**X**	**X**	**X**	**X**
210	**X**					
211	**X**			**X**	**X**	**X**
212	**X**					
213	**X**			**X**	**X**	
214	**X**			**X**	**X**	
215	**X**			**X**	**X**	
216	**X**					
217	**X**				**X**	
218	**X**					
219	**X**	**X**				
220	**X**				**X**	
221	**X**			**X**		**X**
222	**X**					
223	**X**		**X**			
224	**X**					
225	**X**		**X**	**X**	**X**	**X**
226	**X**					**X**
227	**X**				**X**	**X**

228	**X**					
229	**X**				**X**	**X**
230	**X**					**X**
231	**X**			**X**	**X**	

m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
232	**X**					
233	**X**	**X**	**X**		**X**	**X**
234	**X**					
235	**X**	**X**	**X**		**X**	
236	**X**		**X**			
237	**X**	**X**	**X**			
238	**X**					
239	**X**					**X**
240	**X**					
241	**X**			**X**	**X**	**X**
242	**X**				**X**	**X**
243	**X**	**X**		**X**	**X**	**X**
244	**X**					**X**
245	**X**					
246	**X**			**X**	**X**	**X**
247	**X**					
248	**X**				**X**	
249	**X**	**X**	**X**			
250	**X**					
251	**X**		**X**			
252	**X**		**X**			
253	**X**			**X**		**X**
254	**X**					
255	**X**	**X**	**X**			
256	**X**				**X**	**X**
257	**X**	**X**	**X**			
258	**X**			**X**		
259	**X**	**X**	**X**			
260	**X**					
261	**X**	**X**	**X**	**X**	**X**	
262	**X**					
263	**X**					
264	**X**					
265	**X**		**X**	**X**	**X**	**X**
266	**X**					
267	**X**			**X**		**X**
268	**X**					
269	**X**				**X**	**X**
270	**X**			**X**		
271	**X**	**X**				
272	**X**				**X**	
273	**X**				**X**	**X**
274	**X**				**X**	
275	**X**	**X**	**X**			

276	**X**					
277	**X**	**X**	**X**	**X**	**X**	**X**
278	**X**					
279	**X**		**X**		**X**	**X**

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
280	**X**					**X**
281	**X**		**X**		**X**	
282	**X**					
283	**X**	**X**				
284	**X**					
285	**X**			**X**	**X**	
286	**X**					
287	**X**			**X**		
288	**X**					
289	**X**					**X**
290	**X**					**X**
291	**X**	**X**	**X**	**X**	**X**	
292	**X**				**X**	
293	**X**	**X**	**X**			
294	**X**			**X**		**X**
295	**X**	**X**	**X**			**X**
296	**X**	**X**				
297	**X**					
298	**X**					
299	**X**			**X**		**X**
300	**X**					
301	**X**					
302	**X**					
303	**X**		**X**		**X**	**X**
304	**X**		**X**	**X**		
305	**X**		**X**	**X**		
306	**X**					
307	**X**		**X**		**X**	**X**
308						
309	**X**					
310	**X**					
311	**X**					**X**
312	**X**			**X**		
313	**X**	**X**	**X**		**X**	
314	**X**	**X**				
315	**X**		**X**			
316	**X**					
317	**X**	**X**	**X**		**X**	**X**
318	**X**				**X**	**X**
319	**X**	**X**	**X**	**X**	**X**	**X**
320	**X**					
321	**X**		**X**	**X**	**X**	**X**
322	**X**					
323	**X**			**X**	**X**	**X**

324	**X**					
325	**X**			**X**	**X**	**X**
326	**X**					
327	**X**		**X**			

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
328	**X**				**X**	
329	**X**	**X**	**X**			
330	**X**				**X**	
331	**X**	**X**	**X**	**X**	**X**	**X**
332	**X**					
333	**X**		**X**			
334	**X**			**X**		
335	**X**					
336	**X**					
337	**X**					
338	**X**				**X**	
339	**X**					
340	**X**					
341	**X**					
342	**X**					
343	**X**	**X**	**X**			
344	**X**			**X**		
345	**X**		**X**		**X**	
346	**X**		**X**			
347	**X**				**X**	
348	**X**					
349	**X**		**X**			
350	**X**					
351	**X**		**X**		**X**	
352	**X**					**X**
353	**X**		**X**			
354	**X**					
355	**X**	**X**				
356	**X**					
357	**X**					**X**
358	**X**					
359	**X**			**X**	**X**	**X**
360	**X**					
361	**X**			**X**	**X**	**X**
362	**X**					
363	**X**				**X**	
364	**X**				**X**	
365	**X**					**X**
366	**X**					
367	**X**		**X**			
368	**X**					
369	**X**					
370	**X**					
371	**X**	**X**				

372	**X**					
373	**X**		**X**			
374	**X**					
375	**X**		**X**			

m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
376	**X**					
377	**X**		**X**	**X**		
378	**X**			**X**		
379	**X**					
380	**X**			**X**		
381	**X**					
382	**X**					
383	**X**					**X**
384	**X**					
385	**X**		**X**		**X**	
386	**X**					
387	**X**		**X**			
388	**X**		**X**			
389	**X**	**X**	**X**			
390	**X**		**X**			
391	**X**					
392	**X**					
393	**X**					
394						
395	**X**		**X**	**X**		**X**
396	**X**					
397	**X**		**X**			
398	**X**					
399	**X**					**X**
400	**X**	**X**				
401	**X**		**X**			
402	**X**					**X**
403	**X**		**X**			
404	**X**					**X**
405	**X**		**X**			
406	**X**				**X**	
407	**X**					
408	**X**			**X**		**X**
409	**X**					
410	**X**		**X**			
411	**X**		**X**		**X**	
412	**X**					
413	**X**					
414	**X**					
415	**X**			**X**	**X**	**X**
416	**X**				**X**	
417	**X**					
418	**X**					
419	**X**					

420	**X**					
421	**X**			**X**	**X**	
422	**X**			**X**		
423	**X**			**X**	**X**	**X**

m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
424	**X**			**X**		**X**
425	**X**					
426	**X**		**X**			**X**
427	**X**			**X**		
428	**X**					
429	**X**	**X**	**X**			
430	**X**					
431	**X**		**X**			
432	**X**					
433	**X**					
434	**X**					
435	**X**		**X**			
436	**X**					
437	**X**					
438	**X**					
439	**X**					
440	**X**					
441	**X**					
442	**X**			**X**		
443	**X**		**X**	**X**	**X**	**X**
444	**X**					
445	**X**		**X**			**X**
446	**X**					
447	**X**				**X**	
448	**X**					
449	**X**		**X**			
450	**X**				**X**	
451	**X**					
452	**X**					
453	**X**		**X**			
454	**X**					
455	**X**					**X**
456	**X**					
457	**X**				**X**	
458	**X**					
459	**X**		**X**		**X**	
460	**X**		**X**		**X**	
461	**X**		**X**			
462	**X**					
463	**X**					
464	**X**				**X**	
465	**X**		**X**		**X**	
466	**X**				**X**	
467	**X**		**X**			

468	**X**					
469	**X**					
470	**X**				**X**	
471	**X**		**X**			

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
472	**X**					
473	**X**	**X**				
474	**X**			**X**		
475	**X**					**X**
476	**X**					
477	**X**			**X**		
478	**X**					
479	**X**					
480	**X**		**X**			**X**
481	**X**					
482	**X**				**X**	**X**
483	**X**		**X**			**X**
484	**X**					**X**
485	**X**		**X**			
486	**X**		**X**		**X**	
487	**X**		**X**			
488	**X**					**X**
489	**X**					
490	**X**					
491	**X**		**X**			**X**
492						
493	**X**		**X**			
494	**X**				**X**	
495	**X**		**X**		**X**	**X**
496	**X**					
497	**X**					**X**
498	**X**			**X**		
499	**X**					**X**
500	**X**			**X**		
501	**X**					
502	**X**					
503	**X**				**X**	
504	**X**					
505	**X**				**X**	
506	**X**					**X**
507	**X**		**X**	**X**		
508	**X**					
509	**X**					
510	**X**					
511	**X**				**X**	**X**
512	**X**					
513	**X**			**X**		**X**
514	**X**				**X**	
515	**X**					**X**

516	**X**					
517	**X**		**X**			
518	**X**		**X**	**X**		
519	**X**					

m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
520						
521	**X**			**X**	**X**	
522	**X**			**X**		
523	**X**					**X**
524	**X**					**X**
525	**X**					
526	**X**		**X**			
527	**X**		**X**			
528	**X**					
529	**X**		**X**	**X**		**X**
530	**X**		**X**			**X**
531	**X**		**X**			**X**
532	**X**				**X**	**X**
533	**X**	**X**				
534	**X**				**X**	
535	**X**					
536	**X**				**X**	
537	**X**			**X**		
538	**X**					**X**
539	**X**		**X**			**X**
540	**X**				**X**	
541	**X**		**X**			
542	**X**					
543	**X**					
544	**X**				**X**	**X**
545	**X**			**X**		
546	**X**		**X**			
547	**X**					
548	**X**					
549	**X**				**X**	
550	**X**					
551	**X**					
552	**X**		**X**		**X**	
553	**X**	**X**	**X**	**X**		
554	**X**					
555	**X**		**X**		**X**	
556	**X**					
557	**X**					
558	**X**					**X**
559	**X**					**X**
560	**X**		**X**			
561	**X**				**X**	
562	**X**		**X**			

563	**X**			**X**		**X**
564	**X**		**X**	**X**		
565	**X**					
566	**X**					
567	**X**		**X**	**X**		

m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
568	**X**			**X**		
569	**X**					
570	**X**					
571	**X**					
572	**X**		**X**		**X**	
573	**X**					**X**
574	**X**					
575	**X**		**X**			
576	**X**					**X**
577	**X**			**X**		
578	**X**		**X**			
579	**X**					
580	**X**					**X**
581	**X**					
582	**X**				**X**	
583	**X**		**X**			**X**
584	**X**					
585	**X**			**X**		**X**
586	**X**					
587	**X**		**X**			**X**
588	**X**					
589	**X**		**X**			
590	**X**					
591	**X**		**X**			
592	**X**					
593	**X**		**X**		**X**	
594	**X**					
595	**X**	**X**			**X**	
596	**X**					
597	**X**					**X**
598	**X**					**X**
599	**X**					
600	**X**					
601	**X**					
602	**X**		**X**			**X**
603	**X**				**X**	
604	**X**					
605	**X**		**X**			**X**
606	**X**		**X**			
607	**X**					
608	**X**		**X**			
609	**X**					**X**
610	**X**		**X**			

611	X			X	X	
612	X		X			
613	X	X				
614	X					
615	X					
m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
616	X					
617	X					
618	X					
619	X					
620	X		X			X
621	X					X
622	X					
623	X					X
624	X					X
625	X					
626	X					
627	X		X			X
628	X					
629	X		X			
630	X					
631	X					
632	X					
633	X					
634	X					X
635	X				X	
636	X					
637	X				X	
638	X					
639	X					
640	X					
641	X					
642	X		X	X		
643	X					
644	X					
645	X	X				
646	X				X	
647	X					
648	X					X
649	X					
650	X	X				
651	X					
652	X		X			
653	X					X
654	X		X			
655	X					
656	X			X		
657	X			X		
658	X		X			
659	X				X	
660	X					

661	**X**	**X**				
662	**X**		**X**	**X**		
663	**X**					

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
664	**X**		**X**			
665	**X**					
666	**X**		**X**			**X**
667	**X**		**X**			
668	**X**		**X**		**X**	
669	**X**					
670	**X**		**X**		**X**	
671	**X**					
672	**X**		**X**	**X**		
673	**X**		**X**			
674	**X**					
675	**X**					
676	**X**					**X**
677	**X**			**X**		**X**
678	**X**					**X**
679	**X**					
680	**X**		**X**			
681	**X**	**X**				
682	**X**				**X**	
683	**X**		**X**			
684	**X**	**X**				**X**
685	**X**					
686	**X**					
687	**X**					
688	**X**			**X**		
689	**X**					
690	**X**					
691	**X**		**X**			
692	**X**					
693	**X**					
694	**X**		**X**			
695	**X**		**X**			**X**
696		**X**				**X**
697	**X**	**X**				
698	**X**				**X**	
699	**X**	**X**				
700	**X**		**X**			
701	**X**					
702	**X**		**X**			
703	**X**		**X**			**X**
704	**X**		**X**			
705	**X**					**X**
706	**X**		**X**			**X**
707	**X**					
708	**X**		**X**		**X**	**X**

709	**X**		**X**			
710	**X**					
711	**X**					

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
712						
713	**X**					
714	**X**					
715	**X**		**X**	**X**	**X**	
716				**X**		
717	**X**		**X**			
718	**X**	**X**				
719	**X**		**X**	**X**		
720	**X**				**X**	
721	**X**			**X**		
722	**X**		**X**			**X**
723	**X**					
724						
725	**X**			**X**		
726	**X**					
727	**X**					
728	**X**					**X**
729	**X**		**X**			
730	**X**	**X**	**X**			
731	**X**			**X**		
732	**X**					
733	**X**	**X**			**X**	
734	**X**					
735	**X**	**X**				**X**
736	**X**					**X**
737	**X**					
738	**X**		**X**			
739	**X**			**X**		**X**
740	**X**			**X**		
741	**X**	**X**	**X**			**X**
742	**X**		**X**			
743	**X**					**X**
744	**X**					
745	**X**				**X**	**X**
746	**X**					
747	**X**					
748						
749	**X**					
750	**X**					
751	**X**		**X**			
752	**X**	**X**				
753	**X**	**X**				
754	**X**					
755	**X**					
756	**X**		**X**			**X**

757	**X**		**X**			**X**
758	**X**				**X**	
759	**X**					

m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
760	**X**		**X**			
761	**X**		**X**			
762	**X**					
763	**X**					
764	**X**					
765	**X**				**X**	
766	**X**					
767	**X**					
768	**X**		**X**			
769	**X**				**X**	**X**
770	**X**					
771	**X**		**X**			**X**
772	**X**					
773	**X**				**X**	
774	**X**					
775	**X**		**X**			**X**
776	**X**					**X**
777	**X**	**X**	**X**			
778	**X**					
779	**X**			**X**	**X**	**X**
780	**X**					
781	**X**		**X**			
782	**X**					
783	**X**					
784	**X**	**X**	**X**			
785	**X**		**X**	**X**		**X**
786	**X**	**X**				
787	**X**				**X**	**X**
788	**X**					
789	**X**				**X**	
790	**X**		**X**			
791	**X**		**X**			
792	**X**					
793	**X**				**X**	
794	**X**		**X**			
795	**X**		**X**	**X**		**X**
796	**X**					
797	**X**					**X**
798	**X**					
799	**X**			**X**		
800	**X**		**X**	**X**		
801	**X**	**X**	**X**		**X**	**X**
802	**X**					**X**
803	**X**		**X**			
804	**X**		**X**			**X**

805	**X**					
806	**X**					
807	**X**			**X**		

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
808			**X**			
809	**X**				**X**	
810	**X**		**X**			
811	**X**					
812	**X**		**X**			
813	**X**		**X**			**X**
814	**X**	**X**				
815	**X**			**X**	**X**	**X**
816	**X**		**X**			
817	**X**		**X**	**X**		
818	**X**			**X**	**X**	**X**
819	**X**	**X**	**X**			**X**
820	**X**					
821	**X**					
822	**X**		**X**			
823	**X**		**X**			
824	**X**					
825	**X**			**X**		**X**
826	**X**					
827	**X**	**X**	**X**			**X**
828	**X**			**X**		
829	**X**			**X**		**X**
830	**X**					
831	**X**			**X**		
832	**X**					
833	**X**	**X**		**X**		
834	**X**	**X**	**X**			
835	**X**					
836	**X**					
837	**X**					
838	**X**					
839	**X**		**X**			
840	**X**		**X**	**X**	**X**	
841	**X**					
842	**X**		**X**		**X**	
843	**X**		**X**			**X**
844	**X**			**X**	**X**	
845	**X**					
846	**X**		**X**		**X**	**X**
847	**X**		**X**	**X**		**X**
848	**X**					
849	**X**			**X**		
850	**X**				**X**	
851	**X**					**X**

852	X		X			
853	X		X			
854	X					X
855	X					X
m/z	***Chattonella cf. verruculosa* Bloom**	***Synechococcus* spp. Bloom**	***Karenia brevis* Bloom**	***Trichodesmium* sp. Culture**	**Taylor Slough**	**Shark River**
856	X	X	X			
857	X				X	
858	X		X			X
859	X		X			
860	X			X	X	
861	X					
862	X					
863	X		X		X	
864	X				X	
865	X			X		
866	X				X	X
867	X					X
868	X					X
869			X			
870	X					
871	X					
872	X		X			
873	X					X
874	X		X			
875						
876	X					X
877	X					
878	X	X			X	
879	X		X			
880	X		X			
881	X					
882	X		X	X	X	
883	X	X				
884	X				X	
885	X					
886	X		X	X		
887	X					
888	X					
889	X				X	X
890	X		X			X
891	X	X	X	X		
892	X		X			X
893		X		X	X	X
894	X	X				X
895	X					
896	X		X			
897	X					X
898	X					X
899	X					
900	X				X	
901	X					X

902	**X**					
903	**X**					**X**

m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
904						
905						
906	**X**		**X**	**X**		
907	**X**		**X**		**X**	
908	**X**	**X**		**X**		**X**
909	**X**		**X**		**X**	
910	**X**					
911	**X**					
912	**X**	**X**	**X**	**X**		**X**
913	**X**					
914						**X**
915	**X**					**X**
916	**X**					**X**
917	**X**			**X**		**X**
918	**X**		**X**			
919	**X**					**X**
920	**X**					
921	**X**			**X**		
922	**X**					
923	**X**					
924		**X**		**X**		**X**
925	**X**					
926	**X**	**X**	**X**			
927	**X**					**X**
928	**X**	**X**				
929	**X**					
930	**X**				**X**	**X**
931	**X**			**X**		
932	**X**		**X**			
933	**X**		**X**	**X**		**X**
934	**X**				**X**	**X**
935	**X**					
936	**X**				**X**	
937	**X**					
938		**X**	**X**		**X**	**X**
939	**X**					
940	**X**	**X**		**X**		
941	**X**					
942	**X**					
943	**X**	**X**				**X**
944	**X**					
945	**X**					
946	**X**	**X**		**X**		
947	**X**					
948	**X**					

949	X					
950	X					
951	X			X		
m/z	*Chattonella cf. verruculosa* Bloom	*Synechococcus* spp. Bloom	*Karenia brevis* Bloom	*Trichodesmium* sp. Culture	Taylor Slough	Shark River
952	X	X				
953	X		X			X
954	X				X	
955	X				X	
956	X			X		
957	X			X		X
958	X	X	X			
959	X				X	
960	X		X			
961		X				
962	X	X	X			X
963	X		X		X	X
964	X					
965	X	X				
966	X				X	
967	X	X				
968	X					
969	X					
970	X		X			
971	X		X			
972	X		X			
973	X	X				
974	X	X				
975	X					
976	X		X			
977		X				
978	X	X				
979	X				X	
980	X				X	
981	X	X		X	X	
982	X		X			
983	X					
984	X	X				
985	X					
986	X					
987	X					
988	X		X	X		
989	X					X
990				X		X
991	X		X			X
992	X			X		
993	X					
994	X				X	
995			X			
996	X					X
997						
998	X		X		X	

999	X	X				

Appendix A2
Temporal Changes of All *m/z*'s Measured in Florida Bay Bioassay Experiments

Time series trends of individual *m/z*'s detected within the Florida Bay (control), Taylor Slough DOM addition, and Shark River DOM addition treatments. Trends are identified as those that significantly ($p<0.05$) decreased (D), increased (I), increased and then decreased (ID), increased, decreased and then increased (were variable; V), and those that did not change (NC) in ion abundance.

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
54	ID	I	V
55	NC	NC	D
56	V	I	I
57	ID	ID	D
58	NC	NC	ID
60	ID	V	ID
61	D	NC	ID
62	D	V	V
64	ID	ID	D
65	V		
66	ID	NC	ID
67		ID	
68	NC	I	ID
69	ID	V	ID
70	ID	ID	NC
71	I	ID	D
72	ID	I	ID
73	V	V	
74	ID	V	ID
75	V	I	ID
76	ID	V	D
77	ID	ID	D
78	V	V	ID
81	ID	ID	ID
83	ID	ID	ID
84		D	
85	ID	V	D
86	NC	I	I
87	ID	V	ID
88	ID	V	NC
89	ID	V	ID
91	ID	ID	ID
92	D	ID	I
93	ID	I	NC
95	NC	NC	ID
97	V	ID	D
99	D		D

104	**ID**		

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
109	**ID**	**V**	**D**
111	**ID**	**I**	**D**
112	**V**	**ID**	**ID**
113		**I**	
119	**D**	**D**	
120	**NC**	**I**	**I**
123	**ID**	**ID**	**ID**
124	**ID**	**ID**	**ID**
125	**ID**	**ID**	**ID**
126	**I**	**I**	**ID**
127	**NC**	**ID**	**D**
128	**ID**	**ID**	**ID**
136	**ID**	**D**	**ID**
137	**ID**	**ID**	**ID**
139	**ID**	**ID**	**ID**
141	**ID**	**ID**	**D**
143	**ID**		**ID**
149	**ID**	**ID**	**ID**
150	**D**	**ID**	
151	**ID**	**ID**	**ID**
152	**NC**	**V**	**ID**
153	**NC**	**NC**	**NC**
155	**ID**	**I**	**NC**
156	**ID**	**I**	**D**
157	**ID**		
165	**ID**	**I**	**NC**
168		**ID**	**I**
175	**ID**	**ID**	**ID**
176	**ID**	**D**	**V**
177	**ID**	**ID**	**NC**
178	**ID**		**ID**
179	**D**	**NC**	**V**
181	**D**	**ID**	**I**
183	**D**		
184	**D**		**ID**
191	**I**	**I**	**ID**
193	**I**	**NC**	**D**
197	**I**	**ID**	**NC**
199	**ID**	**ID**	**NC**
203		**ID**	**ID**
207	**ID**	**I**	**ID**
208	**ID**	**ID**	**ID**
209	**ID**	**ID**	**D**
217	**I**	**NC**	**I**
218		**I**	**ID**
219	**ID**	**NC**	**I**
230			**I**
233	**ID**	**ID**	**NC**
234	**I**	**D**	**D**

235	ID	NC	NC
236	ID	ID	NC
Measured m/z	**Florida Bay Water (Control)**	**Taylor Slough**	**Shark River**
237	I	ID	NC
238	ID		
243	D	V	ID
245	ID	V	
246		ID	
249	ID	I	ID
255	I	I	I
257	NC	ID	V
259	ID	ID	I
260	I	ID	ID
261	ID	I	I
262	V	I	
269		D	ID
271	D	ID	NC
273	I	I	D
275	NC	I	ID
276		ID	ID
277	D	ID	NC
278	I		
279	ID	D	D
283	V	I	V
285	ID	V	ID
287	ID	I	V
288		I	
290			D
291	ID	ID	NC
292	ID	V	
293	ID	NC	NC
294	ID		I
295	I	NC	ID
296	D	D	I
297	I	D	D
301	I	V	D
302			D
303	I	NC	I
304	ID		V
313	D	NC	V
314	D		D
315	I	ID	V
317	NC	NC	I
318		D	ID
319	ID	I	D
320		ID	
328	I	I	ID
329	ID	ID	NC
330	ID	D	
331	ID	I	NC
332		ID	ID
236	ID	ID	NC

237	**I**	**ID**	**NC**
334			**ID**

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
343	**D**	**ID**	**NC**
344	**ID**		**I**
345	**ID**	**D**	**I**
346	**I**	**ID**	
347		**ID**	**ID**
348		**ID**	
350			**ID**
351			**D**
353	**ID**	**ID**	**I**
355	**ID**	**NC**	**D**
356		**I**	
361		**D**	
362		**I**	
367	**ID**	**I**	**I**
369		**ID**	**D**
370			**ID**
371	**V**	**NC**	**ID**
372			**D**
373		**V**	**V**
374			**ID**
375	**V**	**I**	**ID**
377	**ID**		**ID**
382			**D**
384			**ID**
385	**ID**	**I**	**I**
386		**ID**	
387	**ID**	**ID**	**ID**
388		**ID**	**D**
389	**ID**	**ID**	**ID**
390	**ID**	**I**	
391	**V**	**I**	**ID**
392			**ID**
396			**D**
397	**ID**	**I**	**I**
398		**I**	
400	**D**		
401		**ID**	
403			**ID**
404			**I**
406			**D**
411		**I**	
413		**D**	**D**
415	**ID**	**NC**	**ID**
416		**ID**	**ID**
426		**I**	
428		**ID**	**D**
429	**ID**	**D**	**ID**
430	**ID**	**ID**	

431		**ID**	**ID**
432		**I**	

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
436			**I**
437	**I**	**ID**	**D**
438	**ID**		
445			**D**
446		**I**	**D**
447	**ID**	**ID**	**I**
455		**V**	**D**
456		**D**	
457	**ID**	**V**	**ID**
459	**ID**	**ID**	**I**
465		**ID**	**ID**
469			**D**
470		**V**	**D**
471	**ID**	**D**	**V**
472		**ID**	
473	**D**	**I**	**ID**
474		**D**	
481	**I**	**I**	**D**
485	**ID**	**V**	**ID**
486			**ID**
490	**ID**		
492	**I**	**I**	**ID**
494		**ID**	
495	**ID**	**I**	
496			**ID**
497		**ID**	
502			**I**
503	**I**		
504			**D**
508	**I**		
510		**ID**	
511		**D**	**D**
515			**I**
517			**D**
522			**ID**
524		**D**	**D**
526		**ID**	
527	**ID**	**D**	**NC**
528		**D**	
529	**ID**	**I**	**I**
530		**ID**	
531	**ID**	**V**	**I**
532	**ID**		
533	**D**	**ID**	
537			**I**
538		**D**	
540		**D**	
541	**ID**		**I**

542		**ID**	
545		**ID**	**ID**

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
546		**ID**	**I**
547		**ID**	
548			**ID**
549	**ID**	**ID**	**D**
550			**I**
553	**D**	**ID**	**I**
555	**ID**		**ID**
556	**ID**	**D**	
558			**I**
560		**ID**	**ID**
561		**ID**	
562	**ID**		**ID**
563		**D**	
564		**D**	**ID**
566		**I**	
568			**I**
570		**D**	**ID**
571			**D**
574		**D**	
575			**D**
577			**ID**
578			**V**
579		**ID**	**ID**
580			**ID**
582			**ID**
583		**I**	
584			**ID**
585	**ID**		**I**
586		**ID**	**I**
587			**ID**
588		**ID**	**ID**
589	**ID**	**I**	**V**
590	**ID**		**ID**
591		**ID**	**I**
592		**ID**	
594		**ID**	**I**
595	**D**	**ID**	
597	**I**	**ID**	**ID**
598		**ID**	**D**
599			**I**
600	**ID**		
601		**I**	
602		**ID**	
603	**I**	**D**	**D**
604			**ID**
605		**ID**	**I**
606		**ID**	**ID**
607	**ID**	**D**	**ID**

608	ID	ID	D
610		ID	

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
611	ID	D	ID
612			ID
613	D		D
614	ID	I	
615	I	I	
616	I		I
617		ID	I
618			V
619	ID		
620		V	D
621		ID	ID
623	I	ID	V
624			ID
625			ID
626		V	ID
627		D	
628		ID	
629		ID	ID
631		D	D
632		I	D
633		ID	ID
634			D
636			ID
637		ID	
639	ID	ID	D
640		I	I
641		D	V
642	ID		ID
643		D	I
644			I
645	D	V	ID
646			D
647	ID	V	V
648	ID		D
649	I		
650	D	D	
651			D
652		ID	
653		I	D
654	ID	D	I
656			V
657		D	D
658			ID
659		D	
660	I	V	V
661	D		I
662	I		ID
663	ID	ID	I

664			ID
665	ID	D	I
Measured m/z	**Florida Bay Water (Control)**	**Taylor Slough**	**Shark River**
666			V
667	I		V
668		ID	ID
669		D	
670	I	ID	ID
671		ID	ID
672	ID		I
673		ID	ID
674		I	
676		ID	V
677	I	D	
679			D
680		ID	D
681	D	ID	D
682			D
684	ID		V
685	I	V	ID
686	ID	D	I
687		I	I
688		ID	D
689		D	ID
690	ID	D	
691	ID		ID
693	ID	ID	I
694	ID	ID	ID
695		I	
696	ID		D
697	D		ID
698	ID	ID	ID
699	D		I
700	ID	ID	D
701	ID		ID
702		I	D
704	V	V	ID
705		I	D
706		I	I
707	I	D	D
708		ID	D
709		D	D
710	ID		ID
711			D
713	ID		ID
714			D
716	I		ID
718	D	I	ID
719		ID	
720	I	ID	
721			V
722		D	D

723			**ID**
724	**V**	**ID**	**D**

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
725	**I**	**ID**	**ID**
726	**I**	**ID**	**I**
728		**D**	**D**
729	**ID**	**V**	**ID**
730	**D**	**I**	
731		**V**	**I**
732		**ID**	**D**
733	**ID**	**ID**	**I**
734	**ID**	**D**	**D**
735	**D**		**ID**
736			**D**
737		**ID**	
738		**ID**	**ID**
739	**ID**		
740	**I**		**D**
741	**D**		**D**
742	**ID**	**ID**	**D**
743		**D**	**I**
744		**ID**	
745	**ID**		**D**
746	**I**		**I**
747	**I**	**ID**	
748		**I**	**ID**
749	**I**		**I**
750	**I**	**ID**	**ID**
751	**ID**	**ID**	**D**
752	**D**	**ID**	
753	**D**	**D**	
754		**I**	**ID**
755		**I**	**ID**
756	**ID**	**V**	**ID**
757		**ID**	
758	**ID**	**ID**	**V**
759			**ID**
761		**ID**	**ID**
762	**I**	**V**	
763		**ID**	**ID**
764	**ID**	**V**	**D**
765	**ID**	**ID**	
766		**V**	**V**
767	**ID**	**ID**	
768		**I**	**D**
769	**I**		**ID**
770	**ID**	**I**	**I**
771	**I**	**D**	
772	**ID**	**I**	**D**
774	**V**	**V**	**ID**
775	**ID**	**ID**	**ID**

776	ID		I
777	D		I

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
778	ID	ID	ID
779	ID		ID
780			ID
781			V
782	I	I	D
783	I	D	V
784	D	I	I
785	ID	D	
786	D	ID	I
787		ID	ID
788	ID	I	I
789	ID		
790	ID	ID	
791		ID	I
792		ID	D
793		I	
794	I	ID	D
795			I
796		D	ID
797		V	I
798		I	D
800		ID	D
801	D	ID	I
802	V	I	ID
804		ID	ID
805	ID	ID	ID
806	ID		
807		ID	ID
808		ID	ID
809		I	D
810	ID	I	ID
811	ID	I	ID
812	I		
814	ID	D	ID
815		ID	
816	I		I
818	V	D	ID
819	D		
820	I	D	NC
821		I	I
822		D	D
823		V	ID
824	ID	V	I
826	ID		ID
827	D		
828	ID	NC	D
830	ID	ID	ID
831		ID	

832		ID	D

Measured m/z	Florida Bay Water (Control)	Taylor Slough	Shark River
833	D		
834	D	V	I
835	ID	ID	ID
836		V	V
838			ID
840		V	ID
841			I
842		ID	D
843	I	ID	ID
844	ID	ID	
845		ID	D
846	ID	I	ID
847		ID	ID
848			ID
849		ID	ID
850	I	ID	ID
851		ID	
852		V	ID
853	ID	ID	D
854	ID	ID	ID
856	D	D	ID
857			V
858	ID	ID	
859		D	I
860		ID	D
861			ID
862			V
863		D	ID
864	I	ID	V
865	I	ID	
866	ID	D	
867			D
868	ID	D	
869			D
870	I		I
871			D
872	ID	D	
873		I	D
874		V	
875	ID	ID	I
876	ID		D
877	ID	I	ID
878	ID	ID	
879	ID	ID	
880	I	I	D
881	I		I
882	ID	ID	ID
883	ID		ID
884	ID	ID	

885			I
Measured m/z	**Florida Bay Water (Control)**	**Taylor Slough**	**Shark River**
886	ID	ID	
887			ID
888		I	ID
890			ID
891	ID		D
893	D		D
894	D		D
895	ID	I	
896	ID	ID	ID
897		ID	ID
898	V	D	I
899		ID	I
900	ID	ID	
901		D	I
902	ID	I	ID
903	I	V	ID
904			ID
905		D	D
906		D	I
907		I	V
908	D	D	D
909			I
910		D	D
911	ID	ID	
912	D	ID	ID
913	ID		ID
914	ID		ID
915		ID	I
916	I	ID	ID
917	ID		
918	ID	ID	D
919		I	
920	I	ID	I
921	V	ID	I
922	ID	ID	D
923		D	V
924	D	D	D
926	D	V	ID
927			D
928	ID	V	ID
929	ID	ID	
930	I	ID	
931		D	
932	ID	I	V
933	ID	I	ID
934		D	ID
935		ID	
936	ID		I
937			D
938	D	ID	

939		ID	
Measured m/z	**Florida Bay Water (Control)**	**Taylor Slough**	**Shark River**
940	ID	ID	I
941		ID	D
942		I	D
943	D	I	ID
944	I	I	I
945	V	I	ID
946	D	V	V
947			D
948			I
949		ID	ID
950			I
951		D	
952	D	I	D
953	I		ID
954		ID	I
955		V	D
956	ID	ID	D
958	D		D
959		ID	V
960	ID	D	ID
961	D		ID
962	D	D	NC
963		D	D
964	I	I	ID
965	ID		
966	ID	ID	D
967	D	ID	I
968	ID	V	ID
970	ID		ID
971	ID	ID	
972	ID	D	D
973	D	ID	ID
974	D	ID	ID
975	ID	V	ID
976	ID	I	D
977	D	I	D
978	D	V	D
979		D	ID
980	ID	ID	V
981	D		
982	ID	D	ID
983	ID	ID	ID
984	D	ID	D
985		D	ID
986	V	D	D
987	ID		V
988		I	V
990	ID	D	ID
991	I	ID	ID
992	ID		I

993	I	ID	D
Measured m/z	**Florida Bay Water (Control)**	**Taylor Slough**	**Shark River**
994		ID	V
995	I	ID	ID
996	I	D	D
997	ID	D	ID
998	ID	I	ID
999	ID	D	D

Appendix B: Supplemental Information for Terminal Restriction Fragment Length Polymorphism (TRFLP) Analyses

Appendix B1
Operational Taxonomic Units Detected in Terminal Restriction Fragment Length Polymorphism Analyses

All operational taxonomic units (OTUs) representing >0.1% of the total peak area detected within three coastal bays, a natural *Karenia brevis* bloom and two *Karenia brevis* cultures of varying historical brevetoxin production.

OTU	Great Bay New Jersey	Rehoboth Bay Delaware	Sarasota Bay Florida	*Karenia brevis* Bloom	High Toxin *Karenia brevis* Culture	Low Toxin *Karenia brevis* Culture
59	X	X	X			
84	X	X	X	X		
86				X		
88	X	X	X			X
90			X			
95	X	X	X			
97				X		
102	X	X	X	X		
104	X		X	X		
105					X	X
107	X	X	X	X	X	X
109			X			
111			X	X		
113			X	X		
117			X			
121			X		X	
122			X	X	X	X
125				X	X	X
127	X	X	X	X		
131	X	X	X	X	X	X
137			X			
139			X	X		
140	X	X	X	X		
142				X		
146				X		
148					X	X
152				X		
166	X	X	X	X		
167	X	X	X	X		
168				X		
173			X			
174	X	X	X	X		
175					X	
177			X	X		
178	X		X	X	X	X
179	X	X	X	X		
182	X					

OTU	Great Bay New Jersey	Rehoboth Bay Delaware	Sarasota Bay Florida	*Karenia brevis* Bloom	Low Toxin *Karenia brevis* Culture	High Toxin *Karenia brevis* Culture
185	X	X			X	X
186	X					
187	X	X	X		X	X
188			X			
192			X	X		
198		X	X			
203				X		
204		X		X		
206		X	X	X		
208	X	X	X		X	
212				X		
213			X	X		
217	X				X	
219					X	X
222				X		
231			X			
232	X	X	X	X		X
234			X			
235		X		X	X	X
236			X			
237			X	X		
239	X	X		X		
240	X	X	X		X	
242			X			
243			X	X		
245			X			
246				X		
248	X	X	X	X	X	X
250	X	X	X	X	X	X
253				X		
254		X	X			
255		X				
259					X	
261					X	
264				X		
268			X			
275			X	X		
277	X	X	X	X	X	X
279	X	X			X	
281	X	X	X			
282					X	
284			X			
285	X	X	X	X		
286		X				
296			X			
297			X			
299	X	X	X	X		
301	X	X			X	

OTU	Great Bay New Jersey	Rehoboth Bay Delaware	Sarasota Bay Florida	*Karenia brevis* Bloom	Low Toxin *Karenia brevis* Culture	High Toxin *Karenia brevis* Culture
308				**X**		
327				**X**		
330		**X**	**X**	**X**		
333		**X**	**X**			
336			**X**			
339			**X**			
461		**X**	**X**		**X**	
463		**X**				

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Sipler, R. E. and S. P. Seitzinger. 2008. Use of electrospray ionization (ESI) mass spectrometry to investigate complex dissolved organic matter (DOM) and its potential applications in phytoplankton research. Harmful Algae. 8:182-187.