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# Production of Bioactive Secondary Metabolites by Florida Harmful Bloom Dinoflagellates Karenia brevis and Pyrodinium bahamense

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# Production of Bioactive Secondary Metabolites by Florida Harmful Algal Bloom Dinoflagellates *Karenia brevis* and *Pyrodinium bahamense*

by

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## Dedication

A few people influenced me to pursue science. I would like to thank Jeff Schwarz, Jonathan Harris, Mike Raney, Pat Levin, Lalita Calabria, and Ken Dunton. I would also like to thank Cindy, Terry, and Cobra, and Minx Bears for their support.

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**Abstract**

Despite the critical role algae serve as primary producers, increases or accumulation of certain algae may result in Harmful Algal Blooms (HABs). Algal toxins from these blooms contribute significantly to incidences of food borne illness, and evidence suggests HABs are expanding in frequency and distribution. Mitigation of these HABs without knowledge of the ecological purpose and biochemical regulation of their toxins is highly unlikely. The production, function, and potential of secondary metabolites produced by the dinoflagellates *Karenia brevis* and *Pyrodinium bahamense*, were investigated.

Brevetoxins were demonstrated by two different methods to localize within the cytosol of *Karenia brevis*. Differential and density-dependent centrifugation followed by Enzyme Linked Immunosorbant Assays (ELISAs) indicated that brevetoxin was not contained by any cellular organelles. Light microscopy of brevetoxin immunolabeled preserved cells visually confirmed these results, showing stain to be distributed throughout the cytosol and notably absent from the nucleus. These results have implications for brevetoxin synthesis and function. The complex cyclization process of brevetoxin therefore likely occurs in the cytosol after export of a polyketide precursor from the chloroplast. Functionally, this cellular location suggests use of brevetoxin in cytosolic functions such as signaling and chelation.

Culture experiments of *Pyrodinium. bahamense* var. *bahamense* were undertaken to determine the effects of nutrients and environmental conditions on growth requirements and toxin production. HPLC analysis was employed to separate and quantify the saxitoxins. As eutrophication is a concern where this species is most problematic, in the Indian River Lagoon area of Florida, utilization of urea and ammonium were explored and compared to nitrate. While all nitrogen conditions yielded similar growth curves in *P. bahamense,* the cultures using urea contained a substantially lesser amount of the potent STX congener. This difference implies the urease enzyme utilized by *P. bahamense* is inefficient and urea based fertilizers are unlikely to create blooms with greater toxicity. Cyst production in *P. bahamense* was found to depend on nutrient limitation. Cultures utilizing ammonium displayed a smaller proportion of cysts, presumably attributable to the bioavailablility of ammonium. The total toxin content of *P. bahamense* was found to vary inversely with growth rate, although mole percents of the saxitoxins were largely unchanged over a suite of environmental parameters including temperature, salinity, and pH. Possible reasons for the reported increase in HABs include global warming, dumping of ballast water, and nutrient influx. These studies outline controls on toxin synthesis and production and conditions needed for growth and will aid in predicting environmental and human health effects pending these global changes.

Extracts of *K. brevis* and *P. bahamense* cultures were assayed against various pathogenic agents. Growth of *K. pneumoniae* was inhibited by extracts of both *K. brevis* and *P. bahamense*. An extract of *K. brevis* additionally inhibited MRSA, while a *P. bahamense* extract additionally inhibited both *S. aureus* and MRSA as well as the most

common protozoan vector of malaria, *P. falciparum*. The activity of a dinoflagellate against an Apicomplexan (*P. falciparum*) found in this study is especially interesting as the phyla are closely phylogenetically related. Differences in activity of extracts against *P. falciparum* between a clonal culture on *P. bahamense* from the Indian River Lagoon and a 2011 bloom sampled from Tampa Bay were observed. Drugs are losing their effectiveness against these infectious agents, making pursuit of new drugs an important field. These results suggest that HAB dinoflagellates hold promise in drug discovery similar to other phytoplankton.

# Chapter One: Introduction

## Harmful Algal Blooms

Algae are essential primary producers providing fixed carbon to consumers and contributing a significant portion of Earth's oxygen supply as a by-product. They contributed a central evolutionary role as the first photosynthetic organisms, changing the primordial atmosphere to one conducive to aerobic respiration. Despite their critical ecosystem functions, rapid increases or accumulation of certain algae may result in harm to other organisms or the environment. These events are dubbed Harmful Algal Blooms (HABs). Many HABs reach a concentration that alters the color of the water, resulting in the pseudonym, Red Tides. Although some HABs are classified as harmful due to the subsequent microbial depletion of oxygen upon the bloom's demise, approximately 2% of phytoplankton species produce toxic secondary metabolites (Smayda, 1997) that can result in impacts to public health, wildlife, livestock, and the economy (Anderson et al., 2000). Occurring worldwide, they have been documented throughout history:

"all the waters that were in the river turned to blood, and the fish that were in the river died…" (Exodus 7: 20-21).

Cabeza de Vaca in 1534 noted that the Avavares Indians characterized one seasonal change as:

"the times when the fruit comes to ripen and the fish die" (Adorno and Pautz, 1999).

Approximately 20% of foodborne illness in the U.S. result from consumption of seafood, and half of these are attributable to algal toxins (Ahmed, 1991). Worldwide, there are 60,000 intoxication events yearly by marine algal toxins, resulting in a mortality rate of 1.5% (Van Dolah, 2000). Some blooms can cause other health effects such as contact dermatitis and respiratory irritation. Fish kills are a common result of HABs, and many other taxa that feed on contaminated fish are also affected. Chronic exposure of wildlife to these toxins is difficult to investigate, but effects may include promotion of tumors (Landsberg, 1996; Landsberg et al., 1999) and a decrease in survival in larvae (Edmunds et al., 1999).

A global increase in occurrence, duration, and geographic spread of HABs has been reported (Smayda and White, 1990; Hallegraeff, 1993; Van Dolah, 2000). Although increased awareness and scientific sampling may contribute to the inflation of recent numbers, in some cases the expansion of blooms has been well documented. Anthropogenic alterations to the environment including climate change, deposition of species via ballast water, and nutrient run-off are conjectured to be eliciting this increase. Although anecdotal accounts of some widespread blooms were reported prior to significant anthropogenic nutrient inputs (Kirkpatrick et al., 2004), there is strong evidence that nutrient runoff and subsequent eutrophication of coastal waters in particular increases the incidence of HABs (Smayda and White, 1990; Hallegraeff, 1993; Burkholder, 1998; Paerl and Whitall, 1999). Mining of phosphorus and production of urea has increased exponentially over the past few decades (Nixon, 1995). An 8-fold increase in occurrence of HABs in Hong Kong from 1976-1986 correlated with a nutrient increase of 2.5 times, concurrent with a 6-fold population increase. Negative

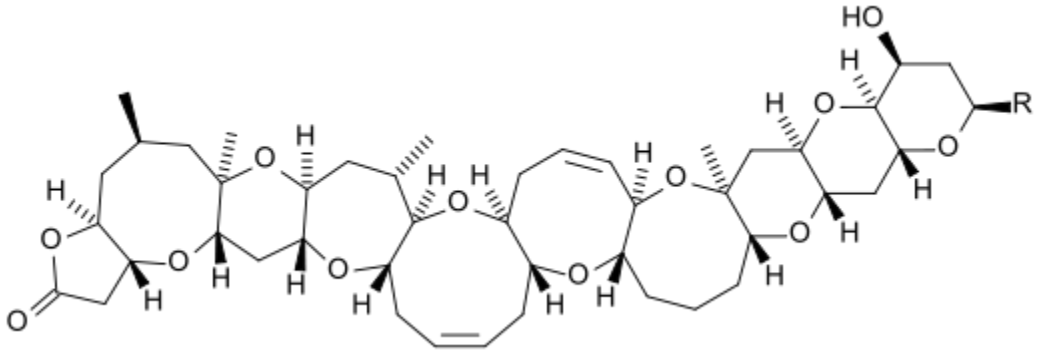
environmental effects and costs associated with HABs make it important to investigate their toxin production and environmental stimuli.

*Karenia brevis* and Brevetoxins

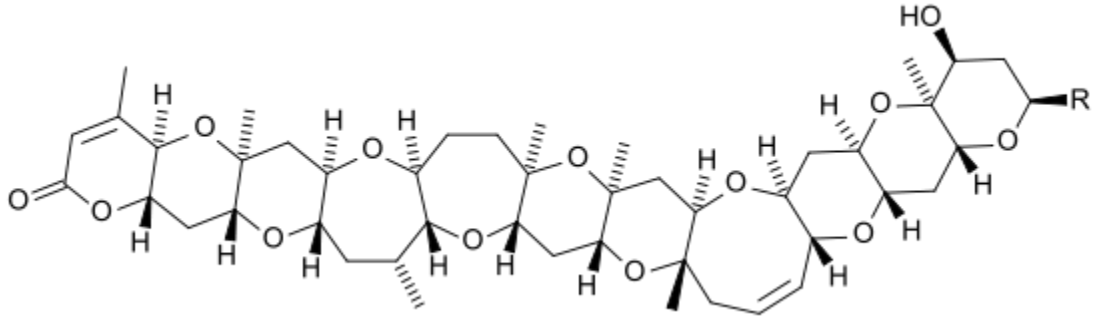
The most prevalent HABs along the Florida gulf coast are caused by *Karenia brevis* , an unarmoured gymnodinoid dinoflagellate (Davis, 1948). *Karenia brevis* (formerly *Ptychodiscus brevis* and *Gymnodinium breve*) naturally occurs in the Gulf of Mexico, the Caribbean, and New Zealand. Blooms frequent Texas and Florida coasts and the Loop Current and Gulf Stream occasionally transport blooms up the East coast of the US (Tester and Steidinger, 1997). *Karenia brevis* cells are positively phototactic, and concentrate in the upper water column when sunlit (Steidinger, 1975; Heil, 1986). Blooms originate offshore and are transported inshore by tides and currents, while thermal and salinity fronts act as both barriers and transport mechanisms concentrating the cells (Vargo et al., 2008).

The brevetoxins (Figure 1.1) produced by *K. brevis* are neurotoxins that depolarize voltage gated sodium channels and inhibit repolarization (Baden et al., 1984). They are large lipid-soluble molecules characterized by a rigid cyclic polyether ladder structure (Lin et al., 1981). There are more than 20 compounds in the brevetoxin family which fall into two distinct backbone structures; A-type backbone and B-type backbone (Figure 1.1) (Abraham et al., 2006). Though PbTx-1 (A-type) is the most toxic brevetoxin, PbTx-2 (B-type) is the most common in bloom water. Environmental impacts of these *K. brevis* blooms include fish kills, marine mammal, sea turtle, and sea

bird mortalities and effects on benthic communities including sea grasses and corals (Steidinger, 1973; Bossart et al., 1998; Trainer and Baden, 1999; Flewelling et al., 2005)




**Brevetoxin A**




**Brevetoxin B**

Figure 1.1. Backbones of brevetoxin.

In fact, *K. brevis* was first identified after a devastating kill of an estimated 50 million fish from a 1946-1947 bloom (Gunter et al., 1948). Populations of species in ecologically precarious positions can be intensely affected; in 1996 at least 149 endangered manatees died during a persistent red tide (Bossart et al., 1998).

Human consumption of brevetoxin-contaminated shellfish causes neurotoxic shellfish poisoning (NSP), which is characterized by gastrointestinal and neurological symptoms. *Karenia brevis* cells are fragile, and when exposed to shear forces and wave action, break, releasing brevetoxins into the water and air. Respiratory irritation similar to asthma may occur after inhalation of aerosolized brevetoxins (Fleming et al., 2005; Pierce et al., 2005). No human fatalities have been reported, but hospitalizations have occurred.

The economy of Florida is closely associated with its coasts and is greatly affected by costs and lost income attributable to *K. brevis* blooms. From 1987-1992 the economic loss from blooms on the West Florida shelf were estimated to total approximately 49 million dollars (Anderson et al., 2000).

*Pyrodinium bahamense* and Saxitoxins

*Pyrodinium bahamense* is a thecate, chain-forming dinoflagellate, with var. *bahamense* found in the Atlantic, Caribbean, and Gulf of Mexico. Like its Pacific variety, var. *compressum*, it produces saxitoxins (Landsberg et al., 2006). Several other genera of dinoflagellates (*Alexandrium* and *Gymnodinium*) and cyanobacteria also produce saxitoxin (Oshima et al., 1987; Oshima et al., 1993; Cembella, 1998).

Saxitoxins are a group of hydrophilic neurotoxic purine alkaloids (Figure 1.2). More than 50 forms exist and are classified by their sidechains: carbamate, sulfate, hydroxyl, hydroxylbenzoate, and acetate (Wiese et al., 2010). The guanidinum groups on saxitoxins potently interact with the carboxyl groups at site one of voltage gated sodium channels, reversibly blocking the sodium influx that would create action potentials along

nerve cells (Catterall, 1985; Ahmed, 1991). They also have been demonstrated to block calcium channels and prolong gating of potassium channels (Wang et al., 2003; Su et al., 2004). Each different form of saxitoxin possesses characteristic toxicity to these receptors.

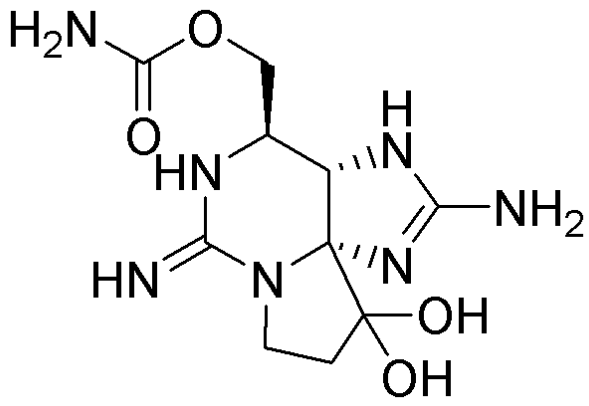



Figure 1.2. General form of saxitoxin.

While filter feeding, shellfish can bioaccumulate saxitoxins that may cause paralytic shellfish poisoning (PSP) if consumed (Sommer and Meyer, 1937; Gainey Jr and Shumway, 1988; Shumway, 1995). Symptoms include gastrointestinal effects, parasthesias, ataxia, slurred speech, and death by respiratory paralysis. Approximately 2,000 cases of PSP occur each year with a mortality rate of 15% (Hallegraeff, 1993). Most occurrences of PSP fatalities worldwide have ultimately been attributed to saxitoxins derived from *P.bahamense* var. *compressum* (Usup and Azanza, 1998). In the United States, PSP incidents have occurred in New England and the Pacific Northwest by exposure to *Alexandrium* species exclusively (Gessner, 2000).

Saxitoxin became a concern in Florida after *P.bahamense* var. *bahamense* specifically was implicated in 28 cases from 2002 to 2004 consumption of puffer fish

caught in the Indian River Lagoon (Landsberg et al., 2006; Deeds et al., 2008). Poisonings in this region prior to this date were assumed to by tetrodotoxin related, as pufferfish have modified sodium channel proteins and have long been known as vectors of tetrodotoxin. Because saxitoxin is a guanidine alkaloid structurally and functionally similar to tetrodotoxin (Figure 1.3), it is bioaccumulated by pufferfish in a similar fashion; however, it is distributed to a greater extent in the muscle, making human consumption increasingly dangerous (Abbott et al., 2009). A moratorium on pufferfish harvesting in the IRL has since been practiced. The terms pufferfish poisoning (PFP) and saxitoxin pufferfish poisoning (SPFP) denote tetrodotoxin and saxitoxin respectively as the causative toxins (Landsberg et al., 2006).

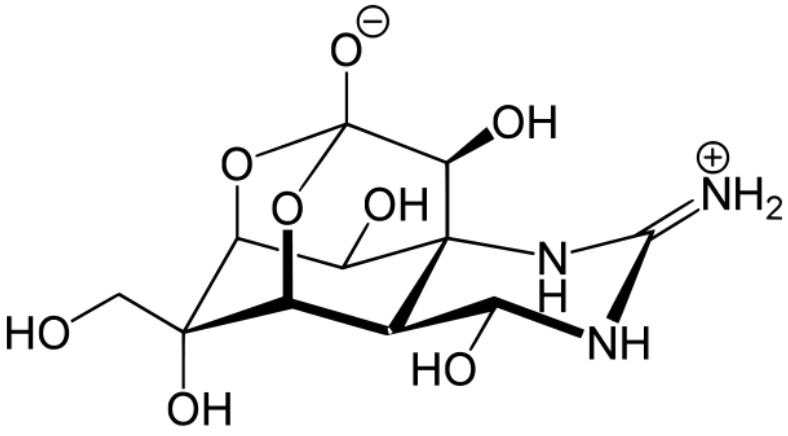



Figure 1.3. Tetrodotoxin.

**Research Objectives**

The two most devastating HAB dinoflagellates in Florida are *K. brevis* and *P.bahamense*. It is not fully known how toxin production in these dinoflagellates changes under different environmental parameters, where the toxins are stored, or whether other bioactive compounds are produced. These studies are essential to

understand the biology of these organisms and the dynamics of the ecosystems in which they occur. Understanding the production of algal toxins may aid in prediction of bloom toxicity and potentially bloom mitigation. Further, enhancing knowledge of biosynthetic regulation and chemical diversity can aid in comprehending the role of specific compounds in chemical ecology. Production of secondary metabolites by Florida harmful algal bloom species was investigated by a variety of methods.

Specific objectives of the current research were the following:

- To determine the cellular localization of brevetoxins in *K. brevis* (Chapter 2).
- To determine the effect of nitrogen source on growth, toxin production, and protein content in *P. bahamense* (Chapter 3).
- To screen for specific environmental factors controlling toxin production in *P. bahamense* (Chapter 4).
- To investigate activity of *K. brevis* and *P.bahamense* against infectious agents (Chapter 5).

## Chapter Two: Cellular Localization of Brevetoxin in *Karenia brevis*

### Introduction

Brevetoxins are large and complex polyether molecules produced by *K. brevis* upwards of daltons. The cellular expense to produce such a molecule constitutively would suggest its import; yet the existence of hearty non-brevetoxin producing *Karenia* species, including *K. mikimotoi*, implies that it is dispensable. Brevetoxin immunochemistry has been used primarily to identify target tissues in animals affected by the toxins. However, understanding where brevetoxins are stored within the organism producing them, *K. brevis*, may aid in understanding its biosynthesis and role within the cell and the ecosystem. Unpublished density centrifugation (Baden, UNCW) and immunocytochemistry data (Naar, UNCW) have not been sufficient to indicate a point source of brevetoxin in the cell (Baden et al., 2005).

The ecological function of the brevetoxins remains elusive. Two theories accounting for the toxin production by dinoflagellates include allelopathy and grazing deterrence, both of which have been demonstrated with karlotoxins (Adolf et al., 2007; Sheng et al., 2010). But while many other toxin-producing dinoflagellates display allelopathy, this effect is seldom linked to the toxins (Prince et al., 2010). For example, saxitoxins produced by *Alexandrium* species do not account for the allelopathic effects of filtrates toward phytoplankton competitors (Tillman, 2002; Fistarol et al., 2004). *Karenia brevis* was also found to produce allelopathic compounds (Kubanek et al., 2005; Prince et al., 2010), however, no inhibition was found in *Asterionellopsis glacialis* when exposed to the brevetoxins specifically (Prince et al., 2010). The massive fish kills that

occur due to brevetoxins have been conjectured to play a role in grazer deterrence, but this may be an indirect consequence of toxin production (Turner et al., 1998) A recent study points to salinity changes as an initiator of brevetoxin production (Errera and Campbell, 2011), but further studies are needed to confirm this function.

Brevetoxins are thought to be synthesized by type I polyketide synthases (PKS) within the chloroplast and then cyclized by an epoxide cascade (Shimizu, 1990; Leadlay et al., 2001; Shimizu, 2003). A PKS gene with similarities to both type I and II PKSs was recently sequenced in *K. brevis* (Monroe and Van Dolah, 2008), but the recent discovery of the far simpler brevetoxin relative, brevisamide, suggests use of a nonribosomal peptide synthetase polyketide synthase complex (NRPS-PKS) (Satake et al., 2008). This type of complex had previously been found in bacteria and cyanobacteria, and was recently found in *K. brevis* ((López-Legentil et al., 2010). Unlike microcystins, which remain in the thylakoid region (Young et al., 2005), brevetoxins are not found in the chloroplast fraction (personal correspondence with Dr. Fran Van Dolah via Dr. Karen Steidinger). They therefore must be exported to another cellular location.

**Objectives**

The present study was undertaken to examine the location of brevetoxin accumulation in *K. brevis* . Two classic methods of studying cellular components are cellular fractionation and microscopy, and both were employed. Cellular fractionation by differential centrifugation was followed by Enzyme Linked Immunosorbant Assays (ELISAs) to determine cellular localization. Fixed cells were also immunolabeled for

brevetoxin and examined by light microscope. Immunolabeling in *Karenia mikimotoi*, a non-toxin producing member of the same genus, was used as a control.

## Materials and Methods

### Cells and Culture Conditions

*K. brevis* cells were clone "Jacksonville C4" isolated by the FWC Fish and Wildlife Research Institute (FWRI) from Neptune Beach (30.3083 N, 81.3958 W) on October 14th, 1999. *Karenia mikimotoi* cells were clone "TAMU C22" isolated by Texas A&M University. Both clones were grown at 25°C on a 12/12 light cycle in GP/2 medium since their isolation.

### Cell Fractionation

*K. brevis* culture was concentrated in a 50 mL centrifuge tube by centrifugation at 500 x g for ten minutes. Differential centrifugation was then carried out by the following generally accepted methods. The supernatant was discarded and the pellet was disrupted with 0.25 M sucrose buffer. The cells were allowed to lyse for ten minutes in the cold buffer, then further broken in a cooled Dounce homogenizer with approximately 80 passes. The lysate was examined under a microscope to ensure sufficient lysis, and then centrifuged at 3,500 x g for ten minutes at 4°C, producing a nuclear pellet. The supernatant was then centrifuged at 100,000 x g for one hour at 15°C to yield a pellet of most remaining cellular components. Finally, the supernatant was mixed with a 0.78 M sucrose buffer and fractionated in a buoyancy gradient as previously described (Yu et al.,

1998). A 0.27 M sucrose buffer, 0.13 M sucrose buffer, and "Top" buffer containing no sucrose were layered on top of the supernatant and centrifuged at 192,000 x g for two hours at 15°C. All sucrose buffers contained 25 mM Tris-HCl to maintain a pH of approximately 8.1, 2.5 mM EDTA to chelate protease cofactors, and 0.25 M sucrose to keep organelle membranes intact. All steps were kept cold to avoid degradation and enzymatic activity.

To determine the location of lipid bodies, a subsample of each layer of the buoyancy gradient experiment was preserved in 2.5% gluteraldehyde. A 1% stock solution of Oil red O was prepared in isopropanol (Bartz et al., 2007). This solution was diluted 3:2 with deionized water to create a working solution and added dropwise to preserved samples to visualize lipid droplets under light microscope.

ELISA

Enzyme Linked Immunosorbant Assays were used to determine cell fractions rich in brevetoxins (ELISAs) with a procedure modified from (Naar et al., 2002). The protocol will be briefly outlined here. Flat-bottomed 96 well plates were coated with PbTx3-Bovine Serum Albumin (BSA) conjugate followed by blocking buffer and rinsed with phosphate buffered saline (PBS). Samples and standards were next added in serial dilutions down the plate. Reference wells and a quality control well were plated on the last row. Anti-brevetoxin primary antibody was added to all wells and allowed to competitively bind with brevetoxin in the sample, standard, or bound to the plate. The plate was rinsed and horse radish peroxidase (HRP) linked secondary antibody was then allowed to attach to the primary antibody bound to the plate. The plate was again rinsed,

and tetramethylbenzidine (3,3,5,5 TMB) was added to react with the HRP, forming a blue colored product. To stop the reaction, sulfuric acid was added and plates were quickly read at 450 nm (yellow). Due to the competition of toxin in samples with that bound to the plate, wells of samples and standards containing toxin contain less colored product. This ELISA mainly detects brevetoxins with the B type backbone, which are the most prevalent in most *K. brevis* environmental samples and clones including the clone used in this study. The antibodies used also possess a low affinity for A-type brevetoxins.

Fixing, Brevetoxin Labeling, and Light Microscopy

*K.brevis* and *K. mikimotoi* cultures were centrifuged at 500 x g for 10 minutes. The supernatant was discarded, and saline ethanol was added to disrupt the pellet and allowed to preserve the cells for 30 minutes. The cells in suspension were centrifuged for 2 minutes at 3500 x g and the supernatant was discarded. Phosphate buffered saline (PBS) was added to break up the pellet, let sit two minutes, and centrifuged. The supernatant was discarded. This PBS wash step was repeated an additional time. Blocking buffer (2.5% BSA in PBS with 0.1% Triton X) was added to cover and break up the pellet and allowed to incubate 30 minutes. The centrifugation and PBS wash procedure in the previous section was used after this step and each of the following labeling steps. Monoclonal mouse PbTx primary antibody (NeoClone) was diluted in PBS with 0.1% Triton X, added to cover and break up the pellet, and incubated at 4°C overnight. The secondary anti-mouse antibody conjugated to a HRP multimer (Ventana) was added and incubated for 30 minutes at room temperature. Finally, metal enhanced diaminobenzidine (DAB) substrate (Thermo Scientific) was added and allowed to

incubate 15 minutes. After this step, tap water was used to wash the pellet instead of PBS, as use of PBS at this step will rinse the DAB stain away. A *K. brevis* control was produced by following this protocol, but excluding the primary antibody. Cells were viewed on an Olympus IX71 inverted microscope and visualized with an Olympus DP70 camera.

Electron Microscopy Preparation

Cells were transferred to a 2 mL centrifuge tube and centrifuged at 1000 x g five minutes. The supernatant was discarded and 1% osmium tetroxide was added for six hours. The osmicated suspension was centrifuged, and osmium was pipetted off. Warmed histogel was pipetted into the microcentrifuge tube, keeping the pellet intact. The gel was centrifuged for five minutes while it congealed and then removed. Gel pieces were cut to approximately 1mm cubes and dehydrated in increasing ethanol concentrations. The 100% dehydration step was repeated three times for 15 minutes to ensure dehydration. Spurr's resin was prepared and diluted 1:1 with ethanol. The gel pieces were incubated in this mixture for 30 minutes, twice in Spurr's resin for an hour, and finally embedded in Spurr's resin and baked at 65°C for 8 hours.

Embedded samples were sectioned in 90nm sections by ultramicrotome using a diamond knife. The sections were flattened with xylene fumes and captured onto 75x300 copper grids. No additional staining was needed. The grids were viewed by transmission electron microscope.

**Results**

Differential centrifugation was first used to fractionate cellular components in *K. brevis* (Figure 2.1). In theory, centrifugation of the homogenized cells at 3,500 x g produced a pellet rich in nuclei, chloroplasts, and any unlysed cells. The pellet produced by centrifuging the supernatant at 100,000 x g contained most of the smaller cell components such as mitochondria and microsomes. The final pellet produced by centrifugation at 192,000 x g should contain predominately ribosomes. Brevetoxin was retained in the supernatant after increasing differential centrifugations including 192,000 x g for two hours.

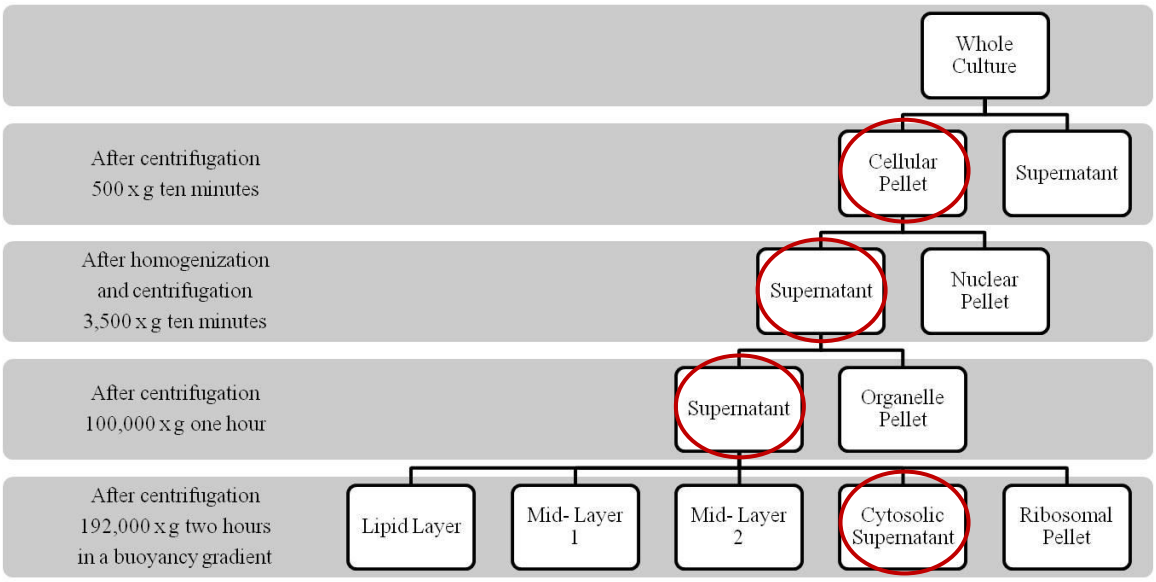



Figure 2.1. Cell fractionation scheme. Circled fractions are those that were found to contain brevetoxins.

Density dependent fractionation was used to further separate the final supernatant. This fractionation separated based on buoyancy, where the lipid components migrated upward during centrifugation. Brevetoxins were found predominantly in the original

supernatant fraction, while lipid droplets had migrated to the top fraction. Upper layers and the ribosomal pellet did not contain appreciable brevetoxin.

DAB immunostaining of *K. brevis* also localized brevetoxin to the cytoplasm (Figure 2.2), characterized by the dark brown coloration. The *K. brevis* control experiment excluded primary antibody and did not show brevetoxin immunolabel. The *K. mikimotoi* control also remained unstained. The nucleus in *K. brevis* was noticeably not stained. Electron microscopy did not indicate point localizations within the cell (not pictured).

**Discussion**

Based upon both fractionation of cellular components and microscopy, brevetoxins appear to be localized to the cytosol in *K. brevis*. Cellular fractionation by centrifugation at a rate of 192,000 x g for two hours revealed that brevetoxins remain in the cytosol. *Karenia brevis* is known to contain lipid droplets which seem likely sinks for the lipophilic brevetoxins, however buoyancy fractionation revealed that the lipid droplets distribute independently of brevetoxin concentration, ruling them out as brevetoxin storage locations. The presence of a lipophilic compound in the cytoplasm seems rather counterintuitive, however, ovatoxin from *Ostreopsis* was also found localized to the cytoplasm by confocal microscopy (Honsell et al., 2011).

Light microscopy supported these findings, showing the brown stain of DAB localized to the cytosolic vesicles and being noticeably absent from nuclei. Both controls

for nonspecific staining were not appreciably stained by DAB. The corroboration of these two techniques suggests that the cytosolic location of brevetoxin found by differential centrifugation is not likely due to rupture of organelles.

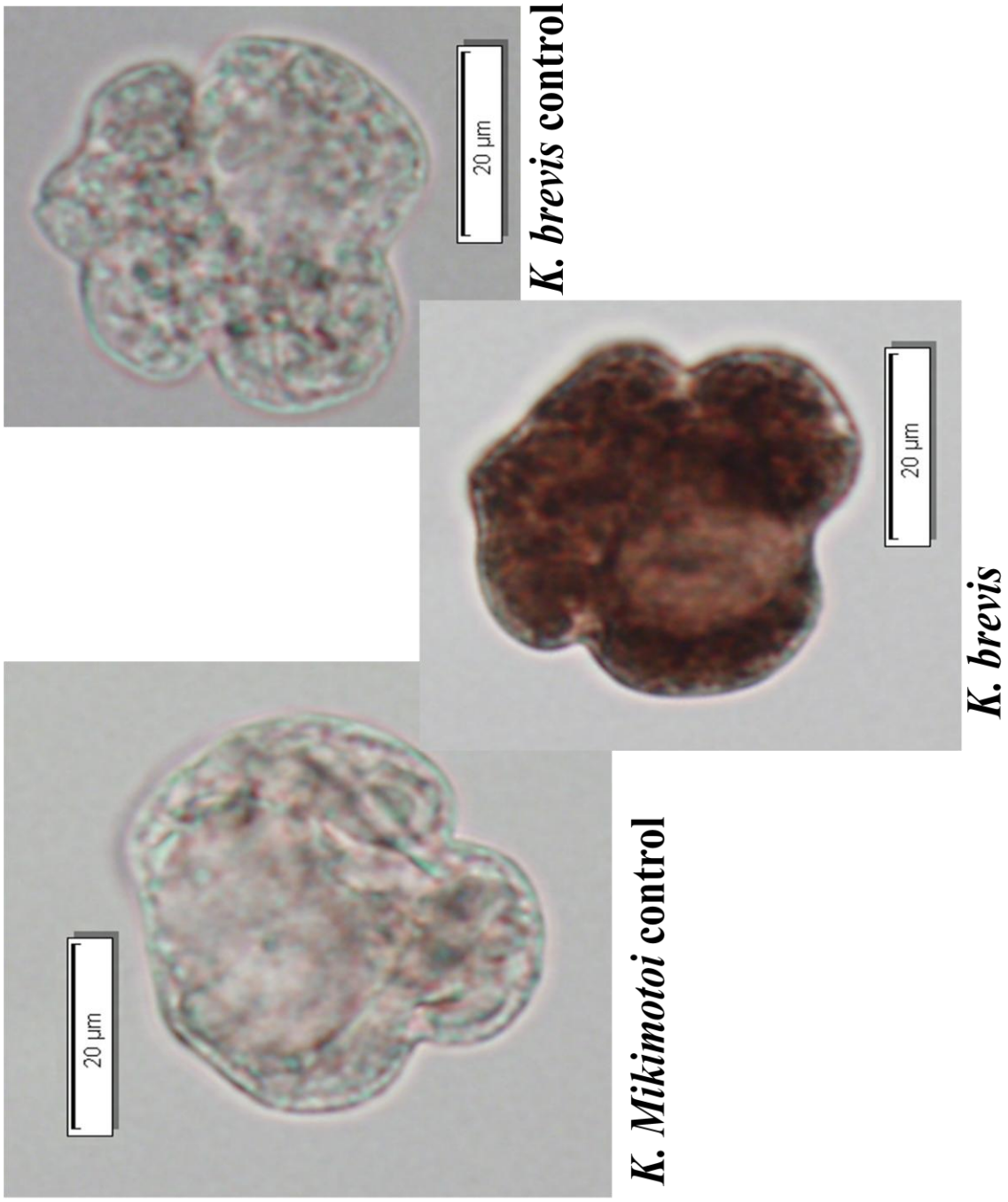



Figure 2.2. Light micrographs of brevetoxin immunolabeling experiment. Brevetoxin accumulation is shown in dark brown. No brevetoxins are apparent in the *K. brevis* control or in *K. mikimotoi*.

Furthermore, gentle homogenization, sucrose solutions common in cell fractionation, and cold temperatures were utilized to minimize rupture and degradation. Electron microscopy was employed, but the lack of staining by this method made this portion of the experiment minimally conclusive. More microscopy will be needed to further resolve this picture. The brevetoxins may be localizing within or to the membranes of cytoplasmic vesicles, or to portions of the cytoskeleton.

Because synthesis of polyketides occurs in the chloroplasts of dinoflagellates, the lack of brevetoxin in this location indicates that brevetoxin is exported to the cytosol after construction. The mechanism of brevetoxin synthesis is thought to involve a cascade of condensations from a polyunsaturated all-Entgegen intermediate that is epoxidized (Dewick, 2001). This configuration could easily be obtained by the final NADPH induced reduction of α,β-unsaturated acyl-ACP in polyketide synthesis. The epoxidation process was reported to occur spontaneously in polar solvent with a hydroxyltetrapyranyl ester intermediate (Vilotijevic and Jamison, 2007). This suggests that the first ether ring formed requires catalysis, and the remaining form spontaneously (Satake et al., 2008). Therefore it is possible that brevetoxin intermediates are synthesized in the chloroplast and exported to the cytoplasm for cyclization. Chloroplasts, therefore, may be a likely location to peruse for long-chain unsaturated brevetoxin precursors. Brevisamide is a recently discovered compound in *K. brevis* that contains a solitary ether ring that is formed by a cyclization proceeding in an opposing direction to polyketide chain assembly (Satake et al., 2008). The method of cyclization itself is useful to synthetic chemists, and determining the cellular location of brevisamide may also be helpful in determining if the

presumed catalysis to produce the first cyclization in brevetoxin synthesis occurs in the chloroplast or in the cytosol.

The cytosolic location and the rigidity of the molecule suggest a role in signaling or chelation. A group of polyketides closely structurally related to the brevetoxins are the polyketide ionophores. These molecules are produced predominantly by *Streptomyces* species, and broadly inhibit bacteria and parasites. Monensin is one of these polyether antibiotics thought to have an analogous biosynthetic pathway to brevetoxin (Dewick, 2001). It causes an influx of sodium ions in coccidae, fatally increasing osmotic pressure (Dewick, 2001). Because brevetoxins are structurally related, they may exhibit similar action: as ionophores in the cytosol to inhibit endosymbionts, in osmoregulation of their own internal environment, or in chelation. Most PKS genes in *K. brevis* were found to be more related to cyanobacterial than other eukaryotic PKS sequences (López-Legentil et al., 2010), which possibly links brevetoxin production even more closely with the bacterial production of polyether ionophores. Brevetoxins, however, are far more rigid than these ionophores, and may not act in the same capacity.

Reflecting the recent findings that brevetoxin production is associated with salinity change (from 35 to 27), it is of note that other osmoregulatory molecules such as trehalose are found in the cytosol. *K. mikimotoi*, however, does not produce brevetoxins. Although the lack of brevetoxins does not preclude the existence of functionally related compounds in *K. mikimotoi*, brevetoxin itself seems non-essential to the genera.

This study identifies potential routes for further experimentation including screening for action of brevetoxin against marine cocci, chelation experiments, and routes to search for brevetoxin precursors. While light microscopy was key in confirming the

cell fractionation results, fluorescence detection by confocal microscope would be ideal to visualize these results with greater resolution. Also, the antibody used both the immunolabeling and centrifugation experiments has a much higher affinity to brevetoxins having the B-type backbone (Figure 1.1). Though not probable, it is possible that the A-backbone brevetoxins are distributed independently. This should be further investigated.

# Chapter Three: Growth and Saxitoxin Production of *Pyrodinium bahamense* in Varied Nitrogen Sources

## Introduction

A global increase in HAB occurrence has been reported (Van Dolah, 2000), with one possible cause being anthropogenic nutrient flux (Smayda, 1990; Hallegraeff, 1993; Nixon, 1995). Population growth and development are contributing to widespread eutrophication (Glibert and Burkholder, 2006). In fact, mobilization of fixed nitrogen into the environment may be the most drastic effect of human population growth and development to date (Vitousek et al., 1997).

The Indian River Lagoon (Florida) is an area of eutrophication concern due to its high rate of human development and the restricted nature of the lagoon (Phlips et al., 2004). The barrier island in Brevard and Indian River counties contains 19 wastewater treatment plants, 3000 on-site sewage diposal systems and 300 septic tanks (Barile, 2004). Permitted total nitrogen from the wastewater facilities alone is 1.94 metric tonnes per day (Barile, 2004), and this accounts for only 6% of the total nitrogen entering the system (Sigua and Tweedale, 2003). Approximately 79% of total nitrogen is attributable to agricultural and urban runoff (Sigua and Tweedale, 2003). While physical parameters may determine distribution of HAB species, nutrient availability regulates growth rate, biomass, and the duration of a bloom (Vargo et al., 2008). Studies indicate that algal blooms of saxitoxin-producing *Alexandrium* species may be bolstered by anthropogenic nutrient sources (Hattenrath et al., 2010).

Nitrogen is a crucial limiting nutrient for most marine primary producers (Dugdale, 1967; Ryther and Dunstan, 1971; Eppley and Peterson, 1979; D'Elia et al., 1986; Nixon et al., 1986; Paerl and Whitall, 1999). The prevalent form of nitrogen in seawater is nitrate and often urea is the dominant form of dissolved organic nitrogen (DON) (Jackson and Williams, 1985; Antia et al., 1991). Oceanic concentrations of urea are nanomolar, but can increase up to 12 uM in coastal regions (Glibert et al., 2006). Though urea is naturally excreted by organisms in the environment, it is now widely synthesized and used as a fertilizer. Worldwide use of urea has been bolstered 100 fold in the last four decades and by anthropogenic enrichment to coastal regions may be associated with increased incidence of some HABs (Glibert et al., 2006). Blooms of *Aureococcus anophagefferens* in Long Island coastal bays occur predominately during low nitrate and ammonium conditions suggesting use of organic nitrogen sources (Keller and Rice, 1989). Changes in DON may alter phytoplankton succession (Paerl, 1988; Berg et al., 1997; Glibert and Terlizzi, 1999).

Urea is an important source of regenerated nitrogen that phytoplankton have been shown to have the capability to utilize (Carpenter et al., 1972; Antia et al., 1991; Collier et al., 1999; Palinska et al., 2000; Berg et al., 2002). The ability to employ diverse nitrogen sources enhances the ability of cells to survive under nutrient deplete conditions. Diatoms are fast-growing and associated with nitrate fluxes (Collos et al., 1992; Collos et al., 1997; Landry et al., 1997); slower-growing dinoflagellates, therefore, may benefit from utilization of organic nitrogen sources. Abundance of dinoflagellates, as well as cyanobacteria and picoplankton have been associated with reduced forms of nitrogen (Paerl, 1988; Berg et al., 1997; Carlsson et al., 1998). In fact, the chrysophyte,

*Aureococcus anophagefferens* and the dinoflagellate, *Lingulodinium polyedra*, were found to take up urea at a higher rate than nitrate (Berg et al., 1997; Kudela and Cochlan, 2000). Increased dinoflagellate counts in particular were associated with increased urea concentrations in aquaculture facilities (Glibert and Terlizzi, 1999). Urea was also shown to support a large amount of the nitrogen demand in *A. catenella* (Collos et al., 2004).

To form the vital precursor molecules glutamate and glutamine, nitrogen-containing molecules must be reduced to a bioavailable form. Ammonia and its positively charged ion, ammonium, are characterized by the -3 oxidation state of the nitrogen atom and are readily used to form these products, however enzymes are required for other nitrogen forms. The highly conserved enzyme nitrate reductase must be employed to transform nitrate. In marine phytoplankton two enzymes are known to break down urea: ATP urea amidolase and a nickel-dependent urease. Chlorophytes are the only marine phytoplankton that have been shown to use ATP urea amidolase (Bekheet and Syrett, 1977). Some dinoflagellates including the saxitoxin-producers *Alexandrium fundyense* and *Alexandrium catenella* require nickel when grown on urea; indicating their use of urease (Antia et al., 1991; Dyhrman and Anderson, 2003). Because *Pyrodinium* is closely related to *Alexandrium* species (Leaw et al., 2005), it too is likely to use urease.

Saxitoxin production has been exhaustively studied in *Alexandrium* species. Toxin content varies with many variables including growth stage, salinity, temperature, light, and nutrients (Ogata et al., 1987; Anderson et al., 1990; Flynn et al., 1994; John and Flynn, 2000; Wang, 2002). Saxitoxins are nitrogen-rich and it has accordingly been reported that high nitrogen availability increases toxin content and nitrogen deprived cells contain lower toxin content (Anderson et al., 1990; Flynn et al., 1994). Toxin content has

been shown to be greater in *Alexandrium sp.* supplied with ammonium rather than nitrate (Levasseur et al., 1993; John and Flynn, 2000), although high levels of ammonium inhibits growth in dinoflagellates (Hamasaki et al., 2001). Suboptimal conditions may engender enhanced toxin content per cell due to the reduced growth rate (Anderson et al., 1990). Therefore, growth rate and toxin content have generally been found to be inversely related (Proctor et al., 1975). However, PSP toxin producers have been shown to contain increased amounts of toxin in exponential phase and decreased amounts in stationary phase (Anderson et al., 1990; Cembella, 1998; Murata et al., 2006; Gedaria et al., 2007). The observed decrease in toxin content during stationary phase may reflect cell lysis and leakage, waning production, increased turnover, or dilution of toxin though partitioning into daughter cells (Cembella, 1998). The toxin content per cell in *P. bahamense* var. *compressum* was reported to be highest in early exponential phase and rapidly decrease at the onset of stationary phase (Usup et al., 1994). No work has been carried out on *P.bahamense* var. *bahamense* to investigate these effects.

In other species, toxin composition has been reported to be invariant and may be a conservative characteristic in each strain (Cembella and Taylor, 1985; Boyer et al., 1987; Cembella et al., 1987; Ogata et al., 1987; Oshima et al., 1993; Anderson et al., 1994; Flynn et al., 1994). For instance, the toxin profile of *A. tamarense* is conserved through changes in light, salinity, and nitrate concentration (Parkhill and Cembella, 1999). However, toxin profiles have been found to vary by others (Boczar et al., 1988; Anderson et al., 1990).

The biosynthetic gene cluster (stx) for saxitoxins in cyanobacteria was recently discovered, enhancing understanding of the biosynthetic route used by these organisms

(Kellmann et al., 2008). The biosynthesis genes for dinoflagellates have yet to be found (Wiese et al., 2010), and although there continues to be debate over the source of saxitoxins in these organisms (Prol et al., 2009), it is probable that dinoflagellates and cyanobacteria share the same biosynthetic route to produce saxitoxin (Shimizu, 1993). However, dinoflagellate stx genes may differ from those in cyanobacteria more than would be expected from a recent gene transfer (Yang et al., 2010).

Saxitoxin is primarily composed of composed of L-arginine, as is evidenced by its guanidine character; its structure is additionally composed of acetate and a C1 unit from methionine (Shimizu, 1990; Dewick, 2001). Arginine is biosynthesized from glutamate. Arginine and toxin content were thus shown to be inversely related, though arginine production continued when toxin production was inhibited (Anderson et al., 1990). However, in *A. minutum*, toxin content has been associated with an increase in arginine and other amino acids, making free arginine alone not the controlling factor in saxitoxin synthesis (Flynn et al., 1994). Protein is a major pool of cellular nitrogen. Both saxitoxin and protein utilize amino acids for their construction, and therefore a direct relationship between the two has been identified (Murata et al., 2006). Nontoxic *Alexandrium sp.* have lower protein content than highly toxic strains (Anderson et al. 1990).

Pyrodinium and Alexandrium species are capable of producing resting cysts when experiencing non-optimal conditions. Cyst dynamics have been studied in *Alexandrium* species (Watras et al., 1982; Anderson et al., 1983; Blanco, 1995; Jensen and Moestrup, 1997; Garces et al., 2002; Band Schmidt et al., 2003) because of their propensity to form destructive blooms (Turner et al., 1998). Dinoflagellate cyst production in culture is typically achieved by nutrient depletion in culture (Pfiester, 1975; Turpin et al., 1978;

Anderson et al., 1984). Temperature increase may also lead to encystment due to higher nutrient demand (Anderson et al., 1983).

## Objectives

Nutrient studies of *P.bahamense* are essential, as saxitoxin and its derivatives are alkaloids and a supply of nitrogen must be allocated for their production. Therefore nitrogen bioavailability and anthropogenic nutrient influx may impact growth and toxicity of blooms. Ability to utilize urea and the presence of reduced forms of nitrogen in seawater may lead to changes in growth, toxin production, and protein content in *P.bahamense*.

In order to evaluate the effects of nitrogen source on toxin production, cultures of *P. bahamense* var. *bahamense* grown in nitrate, ammonium, and urea were monitored for cell counts and toxin content. HPLC analysis was employed to separate and quantify the saxitoxins. Proteins were analyzed using the Bradford (1976) method.

## Materials and Methods

### Culture Conditions

A non-axenic clonal culture of *Pyrodinium bahamense* var. *bahamense* (CCFWC# 394) was isolated from the South Rockledge area of the Indian River Lagoon (IRL), Florida on October 15$^{th}$, 2002 and kept in culture at the FWC Fish and Wildlife Research Institute (FWRI; St. Petersburg FL). It was grown under full spectrum lights on a 12/12 light cycle at 25 °C in ESDK medium since its isolation. Deionized water and

seawater from offshore Florida were filtered and autoclaved to yield media with a salinity of 21±1.

For this experiment, cultures were grown with GP media modified to contain a nutrient profile similar to that of the IRL; the mean nutrient values reported by Phlips et al. (2006) for the Titusville portion of the IRL were used to make all media with a 60 nitrogen: 1 phosphorus ratio. Media containing either nitrate, ammonium, or urea as the nitrogen source were prepared. Target nitrogen content of each media type was approximately 67.5 umol/L and each was tested at FWRI by autoanalyzer (nitrate and ammonium) and spectroscopically (urea) to ensure similar nitrogen concentrations; the three media types were found to vary less than 2% from each other in nitrogen equivalent.

Three weeks prior to the experiment, the culture was seeded into three carboys, each containing a different nitrogen condition (nitrate, ammonium, or urea). These cultures were allowed to acclimate to their respective nitrogen conditions for the following month.

In a follow-up experiment, *P. bahamense* was not acclimated to the new nitrogen conditions. Cells were transferred after being starved two months to either ammonium, urea, or nitrate conditions. There were five replicate flasks of each nitrogen condition.

Sampling and Extraction

After this acclimation period each nitrogen media condition was seeded into 3L flasks at 250,000 cells/L in duplicate. The nine flasks were sampled at seven time points over two months, with more frequent sampling during exponential phase. A subsample

was preserved in Lugol's iodine for counting. Triplicate samples of 25 to 50 mL depending on cell concentration were taken from each flask at each time point and filtered onto a GFD filter (Appendix A).

Saxitoxin Extraction and Analysis

Duplicate GFD filters were ground in one milliliter of 1% acetic acid in a glass Dounce grinder to break the rigid theca of the *P.bahamense*. This extract was poured into a centrifuge tube. The grinder was rinsed with an additional milliliter of 1% acetic acid and combined with the extract in the centrifuge tube. The extract was then centrifuged to remove the filter residue and solids, and the supernatant was poured into a plastic vial for storage at -20°C.

Saxitoxins are not UV active, and they must be oxidized for detection by High Performance Liquid Chromatography (HPLC) as previously described (Lawrence et al., 2005). A Shimadzu HPLC with a Phenomenex 150 x 4.6 mm, 5µm C-18 column was utilized for the separation of saxitoxins. The solvent sytem used was ammonium formate in water (A) and ammonium formate in 5% acetonitrile (B). At t=0 B=0%, t=1min. B=5%, t=5 min. B=70%, and t=7.2 to 10min B=100% (Figure 3.1). Both the periodate and peroxide oxidations were tested on initial samples of the *P. bahamense* clone, however only un-hydroxylated saxitoxin forms were found in this clone and thus only the peroxide oxidation was used for later samples. Decarbamoyl saxitoxin (dcSTX), sulfocarbamoyl saxitoxin (B1), and Saxitoxin (STX) were the only saxitoxins detected in this species eluting at approximately 6.0, 8.1, and 8.7 minutes respectively. DcSTX has a characteristic second peak (Figure 3.1 at approximately 6.5 minutes) that was not utilized

in the quantification of this toxin. Each sample was also injected on the HPLC non-oxidized to help ensure that the UV detection was saxitoxin, and not a UV active cell product. Every ten samples were bracketed by standards of STX, dcSTX, and B1. Standards were plotted using the EZstart chromatography software and used to transform the sample peak areas to toxin quantities (Appendix B).

Protein Extraction and Analysis

A comparative study of the total proteins in each culture condition was carried out for four timepoints as described previously (Dortch et al., 1984; Berges et al., 1993). One GFD filter was ground with a glass Dounce grinder with 0.5 mL of cold 3% trichloroacetic acid (TCA) and poured into a 1.5 mL centrifuge tube. The grinder was rinsed with another aliquot of TCA and the washings were added to the centrifuge tube. Ground samples were centrifuged at 14,500 x g for five minutes to collect the precipitated proteins and the supernatant containing free amino acids was discarded. The pellets were resuspended in 1 mL of 0.1 M sodium hydroxide and incubated at 80°C for 15 minutes. The incubated samples were again centrifuged at 14,500 x g for five minutes, and the supernatant containing solubilized proteins was collected for analysis.

The (Bradford, 1976) method was used for the analysis of total protein. Stock reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 mL methanol, adding 100 mL 85% $H_3PO_4$, and bringing this solution up to 200mL with nanopure water. Stock reagent was stored in a dark bottle at 4°C. Working solution was prepared immediately before analysis by diluting the stock solution 1:5 in nanopure

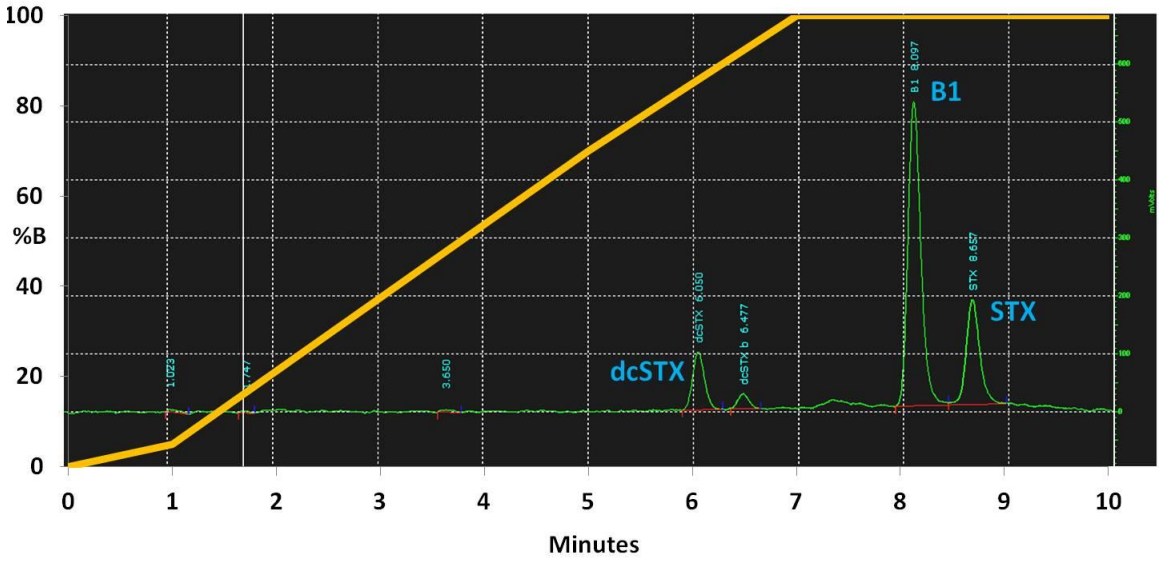



Figure 3.1. Solvent gradient and saxitoxin elution times for HPLC experiments.

water. Bovine serum albumin (BSA) standards were prepared ranging from 80µg/mL to 1.25 µg/mL in 0.1 M sodium hydroxide. Standards, blanks, and samples (100 µL) were pipetted in duplicate onto a 96 well plate. Working Coomassie Blue reagent (100 µL) was pipetted quickly stepwise into these wells and allowed to incubate at room temperature for five minutes. Wells were read by a µquant plate spectrophotometer at 595 and 450 nm. Wells must be read before one hour has elapsed, as proteins begin to precipitate.

All duplicate readings at the 595 nm wavelength were averaged, blank subtracted and divided by their respective 450 nm average measurement to linearize the data (Zor and Selinger, 1996). Graphpad software was utilized to construct the linear regression and transform the sample absorbances to protein values.

It is important to note that the results of this protein assay are meant to be comparative within this study and are only comparable to other results obtained by

similar methods. Algal proteins were found to react similarly to BSA in the Bradford method (Berges et al., 1993), however, other protein analysis methods (Lowry and Smith assays) function differently and therefore will not reproduce these results. The Bradford method generally yields lower results (Berges et al., 1993).

### Calculations

Results from duplicate filters were averaged. For STX equivalents, 51% dcSTX and 6% B1 were added to the STX results to approximate toxicity according to the Oshima Toxicity Equivalence Factors (Oshima, 1995). Statistical analyses were calculated in Graphpad software. Because three replicates were analyzed in most cases, normality could not be checked, and a non-parametric test was used. Toxin mole percents and STX equivalent data were analyzed using the Friedman Test. Cyst count data during the second experiment contained five replicates and were checked for normality using the KS method, then analyzed by two-way ANOVA.

## Results

All nitrogen conditions provided similar growth curves (Figure 3.2). Neither the averaged cell counts nor cyst counts for the conditions within each time point were significantly different. However, it was notable that nearly zero cysts were detected in the culture containing ammonia until the onset of stationary phase, and this was further explored in a follow-up experiment. Total toxin concentration, calculated by summing the three forms of saxitoxin detected without consideration of relative toxicity, ranged between approximately 50 fmol per cell and 350 fmol per cell (Figure 3.3).

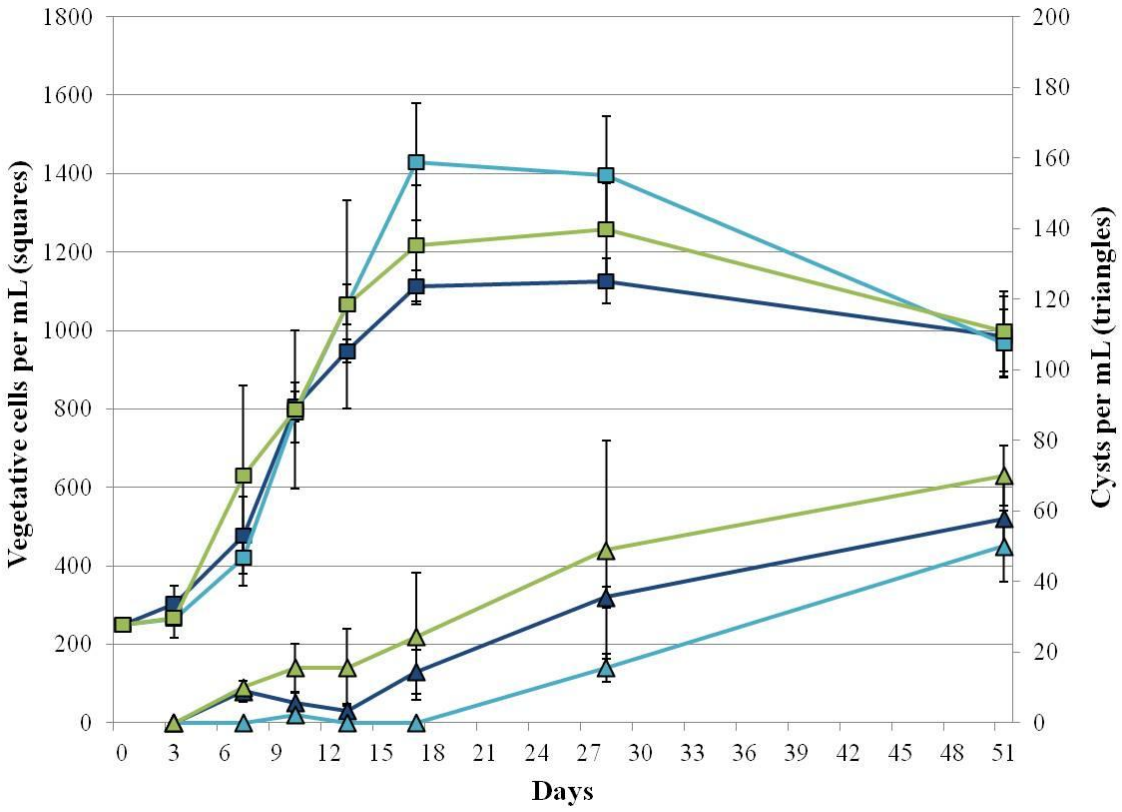



Figure 3.2. *P.bahamense* averaged cell and cyst counts. Lines indicate nitrate (navy), ammonium (teal), and urea (green). Triangles indicate cyst counts, and square markers indicate cell counts.

Toxin concentrations in each culture condition generally reached their minimum at day seven or ten, and maximum on the last day of sampling (Figures 3.3 and 3.4). Nitrate tended to stimulate the highest total toxin production, followed by ammonium, and urea.

The toxin ratio of the culture grown on urea showed less than 20% STX while in both other conditions STX remained higher than 20% (Figure 3.5); this toxin mole ratio analyzed across all sampling days was found to be statistically significant (P=0.0003).

STX equivalent values normalized to per cell values showed significant differences at four time points (day 10,13,17,and 28; P=0.028), and when the STX

equivalent averages were analyzed for each nitrogen condition across all time points (P=0.0003) (Figure 3.6). Cell counts varied between replicates in the early log and late stationary points, leading to larger deviations of cellular toxin concentrations. Protein concentrations were found to range between 0.5 and 1.5 ng per cell (Figure 3.7). No difference in protein production was noted between culture conditions. A follow-up experiment with five replicates was conducted to confirm the lower production of cysts in cultures with ammonium as the nitrogen source (Figure 3.7); at the last two time points significantly differing (P= 0.04 and P= 0.01 respectively) cyst concentrations were counted. Growth rate was plotted to show onset of stationary phase (Figure 3.8).

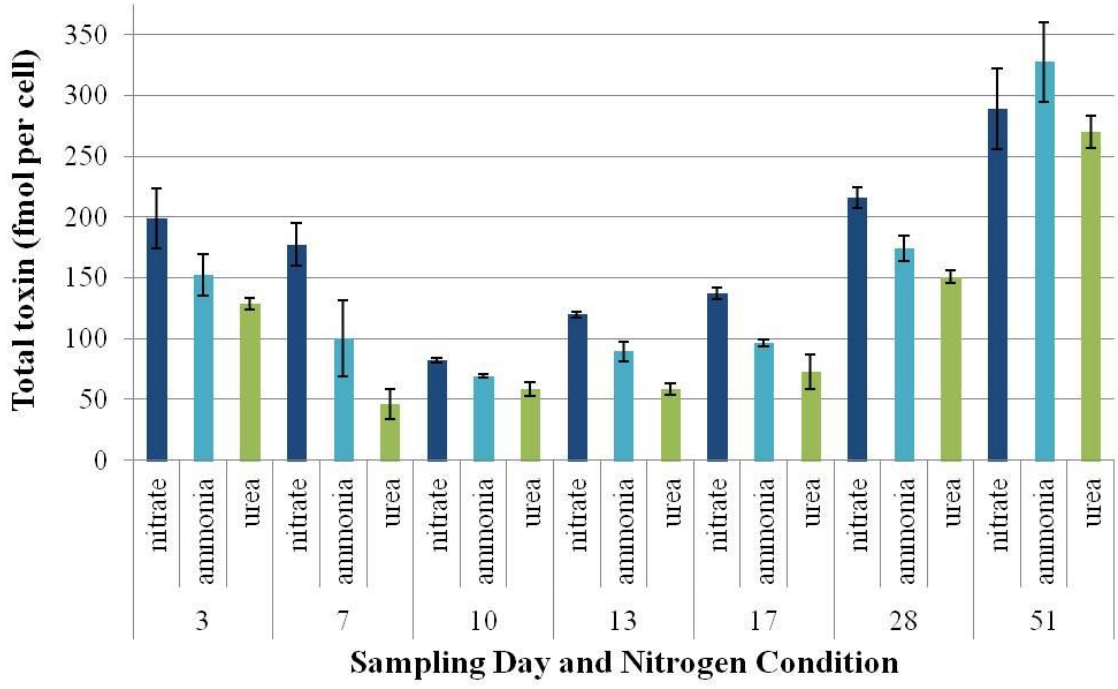



Figure 3.3. Total toxin on each sampling day in each nitrogen condition. Bars indicate standard error.

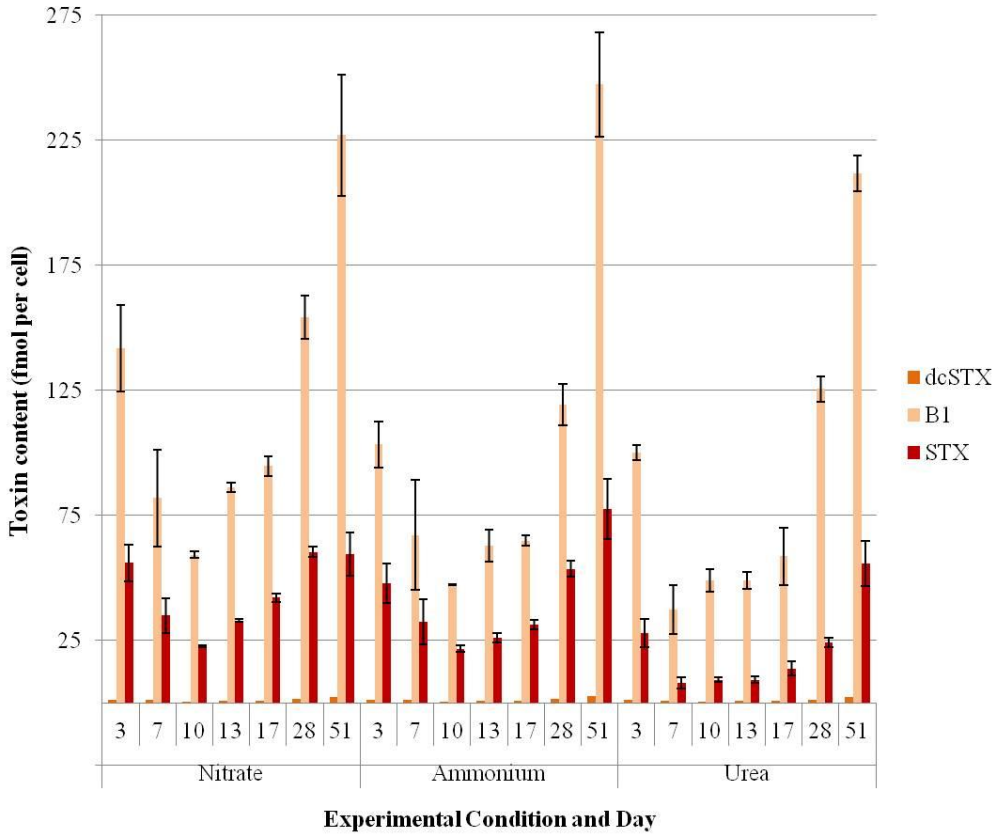



Figure 3.4. Toxin levels in each culture condition on each sampling day. Bars display standard error.

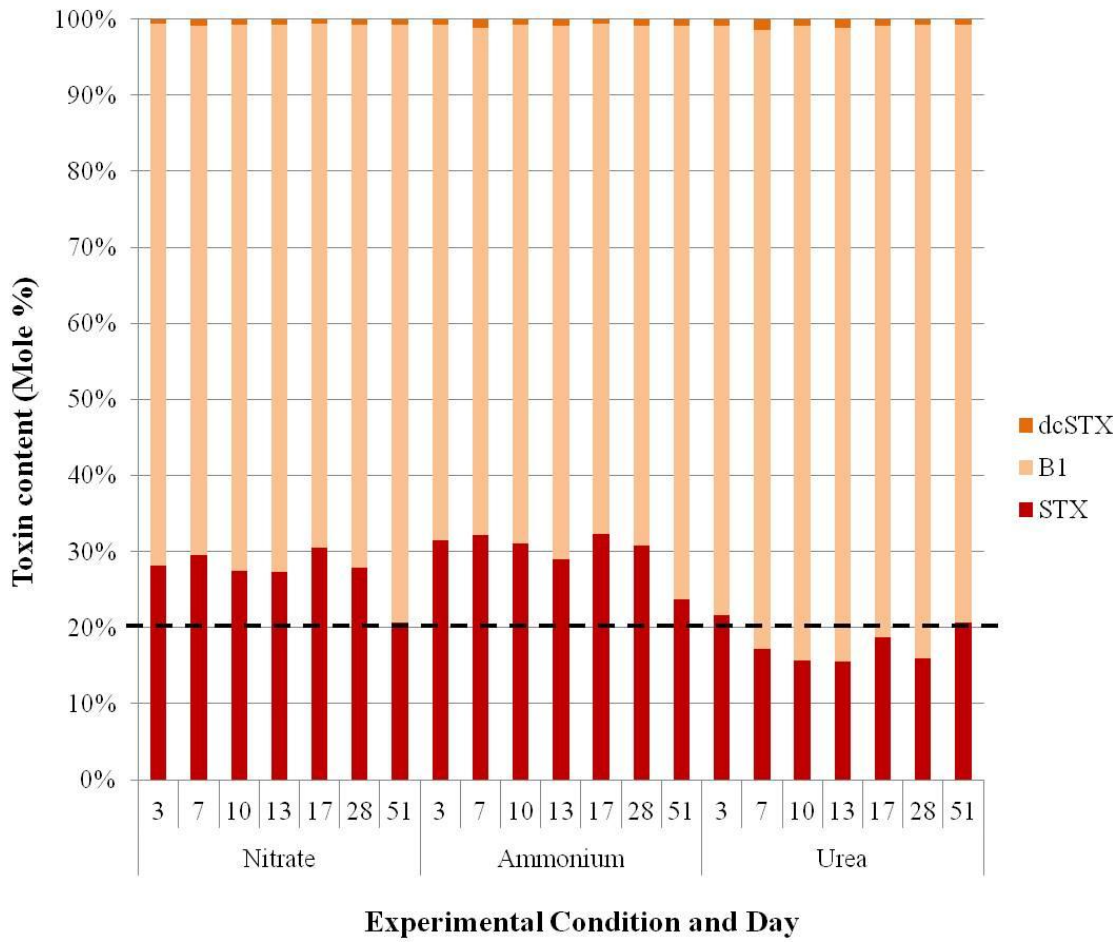



Figure 3.5. Mole percent of the toxins in each nitrogen condition on each sampling day. The dashed bar delineates 20% STX.

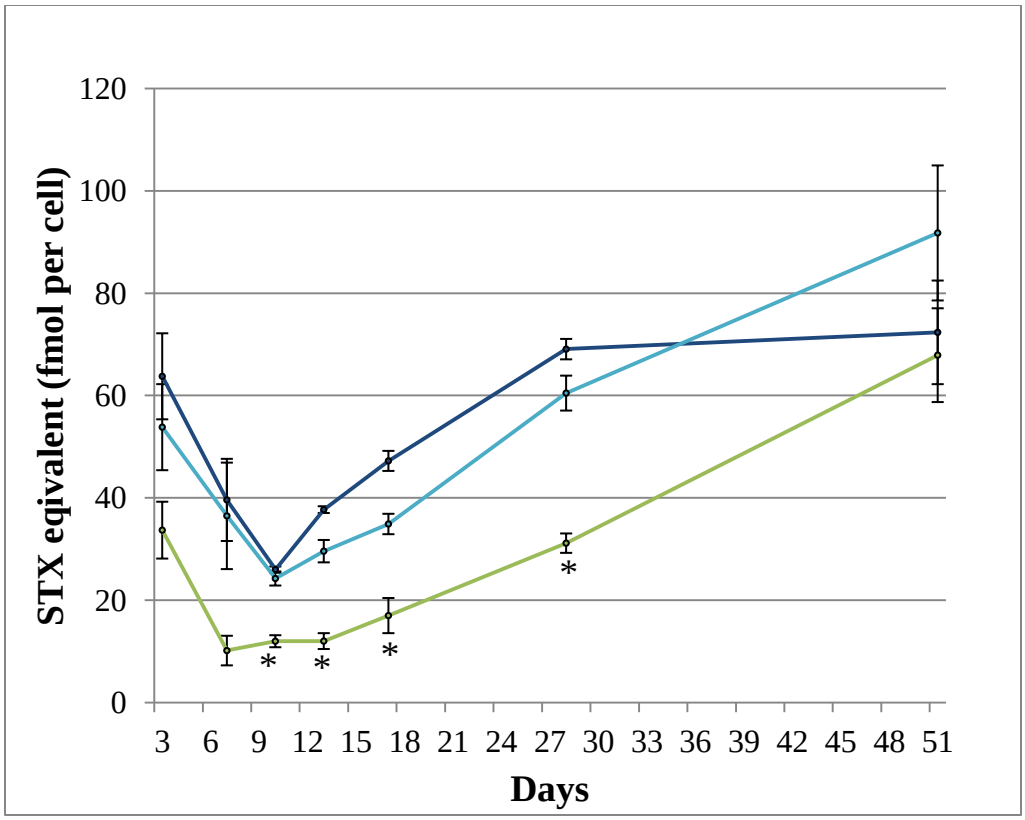



Figure 3.6. Saxitoxin equivalent averages for nitrogen conditions. Lines indicate nitrate (navy), ammonium (teal), and urea (green). Bars indicate standard error. Asterisks denote statistical significance at that time point.

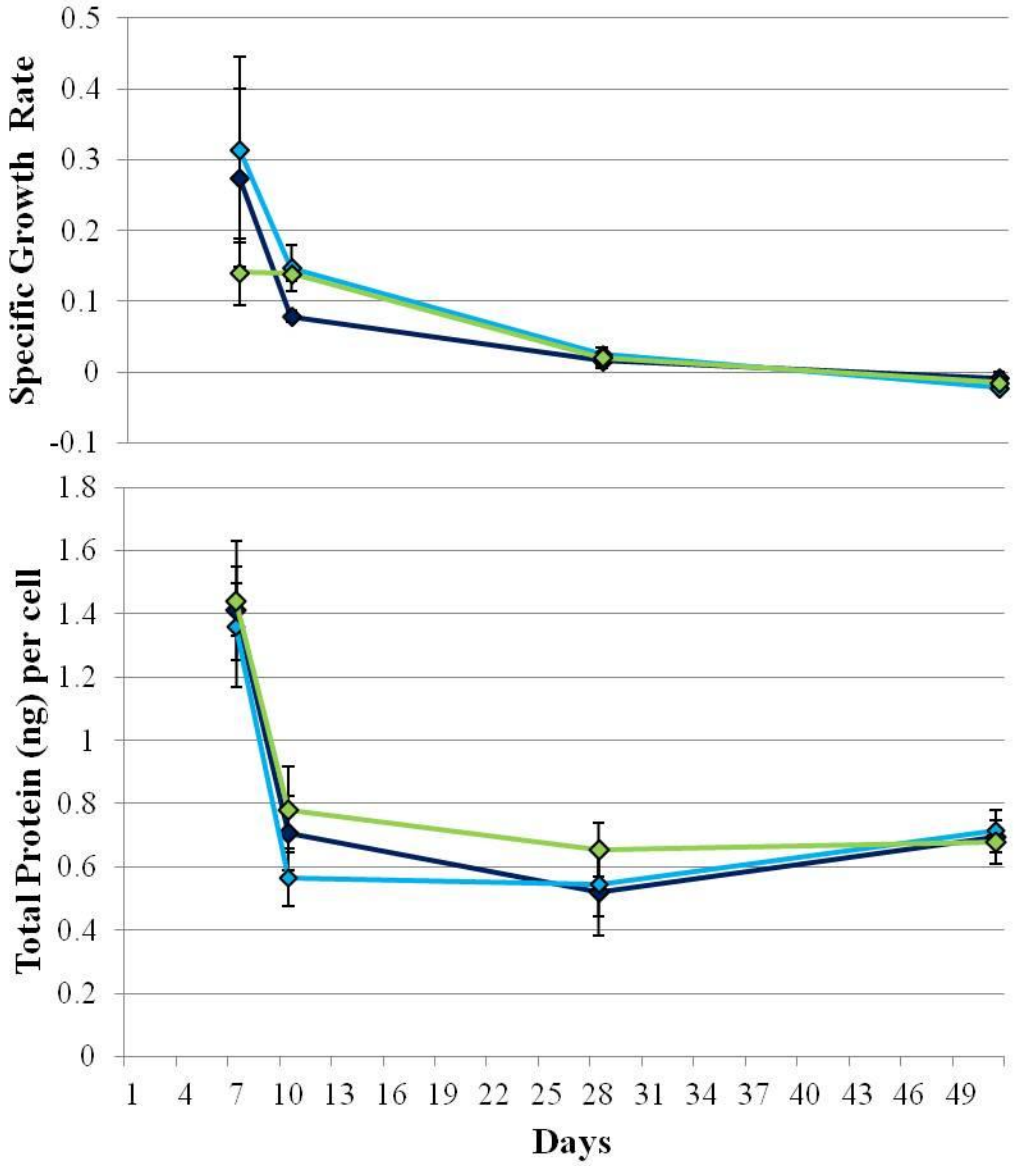



Figure 3.7. Specific growth rate and protein content at four selected timepoints. Lines indicate nitrate (navy), ammonium (teal), and urea (green). Bars indicate standard error.

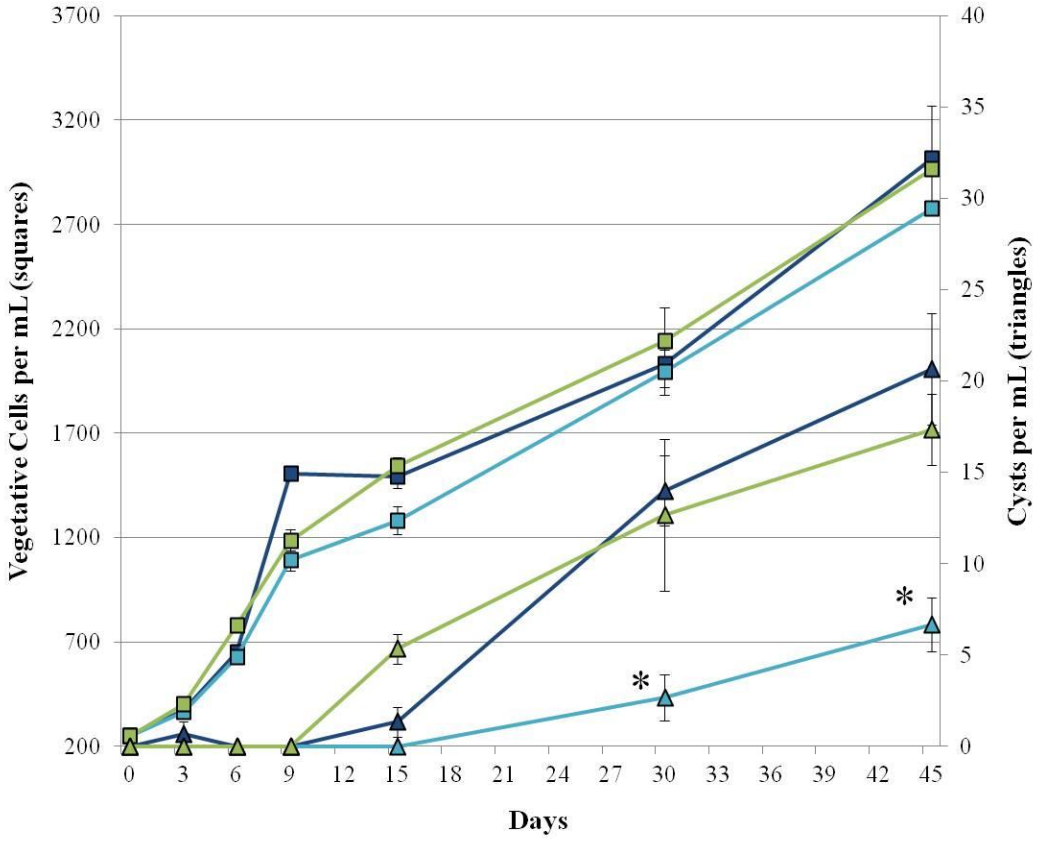



Figure 3.8. Vegetative cell and cyst counts of the second trial with five replicates. Bars indicate standard error. Lines indicate nitrate (navy), ammonium (teal), and urea (green). Squares indicate vegetative cell counts and triangles indicate cyst counts. Asterisks denote statistical significance.

## Discussion

Nitrogen source was not found to significantly change growth of *P.bahamense* in either the initial or the follow-up culture experiment, as found in many other phytoplankton (Solomon et al., 2010); Figures 3.3 and 3.8). Additionally, protein content remained similar across all conditions. The concentration of 0.5 ng per cell appears to be a threshold concentration for cell survival because it was maintained throughout stationary phase (Figure 3.7). In the var. *compressum* subspecies, protein content was reduced under nitrogen limitation (Usup and Anderson, 1996). A protein

concentration of nearly 1.5 ng per cell was found for day seven in culture during active growth and division. This value is comparable to total toxin values ranging from 1.3 to 1.7 ng per cell found during exponential growth in *Alexandrium tamarense* using the Bradford protein assay method in reference to BSA (Murata et al., 2006). A plot of protein concentrations bears similarity to a plot of growth rates, but was not found to be significant by Sperman correlation (Figure 3.7). Protein content was not found to be related to saxitoxin content in this species.

Cyst counts only began to increase once the specific growth rate dropped dramatically during the onset of stationary phase (Figures 3.8 and 3.9). This implicates nutrient limitation is a causative factor of cyst formation in culture, as has been found in other dinoflagellates. The bioavailability of ammonia may have delayed encystment before the onset of stationary phase and kept the cyst concentration lower than in the other conditions. In both experiments the cyst count in the ammonium condition remained zero longer (Figures 3.3 and 3.8). The concentrations of cysts during the two final time points in the ammonium culture were statistically significantly lower than in the other culture conditions (Figure 3.8). The stimulation of cyst production by nutrient limitation in culture studies cannot confirm this to be the causative environmental cue. Field studies have been less successful in linking cyst production to nutrient limitation. In *A. minutum*, nutrient stress may be an initial trigger for cyst production, but does not determine subsequent production (Garces et al., 2004).

Cultures growing on nitrate and ammonia proved similar in saxitoxin ratio and content. However, the urea condition generally contained the least total toxin and a smaller proportion of the most potent toxin, STX (Figure 3.5). Hence, the urea cultures

contained approximately half the STX equivalent toxin content per cell than did cells growing on nitrate or ammonia (Figure 3.6).

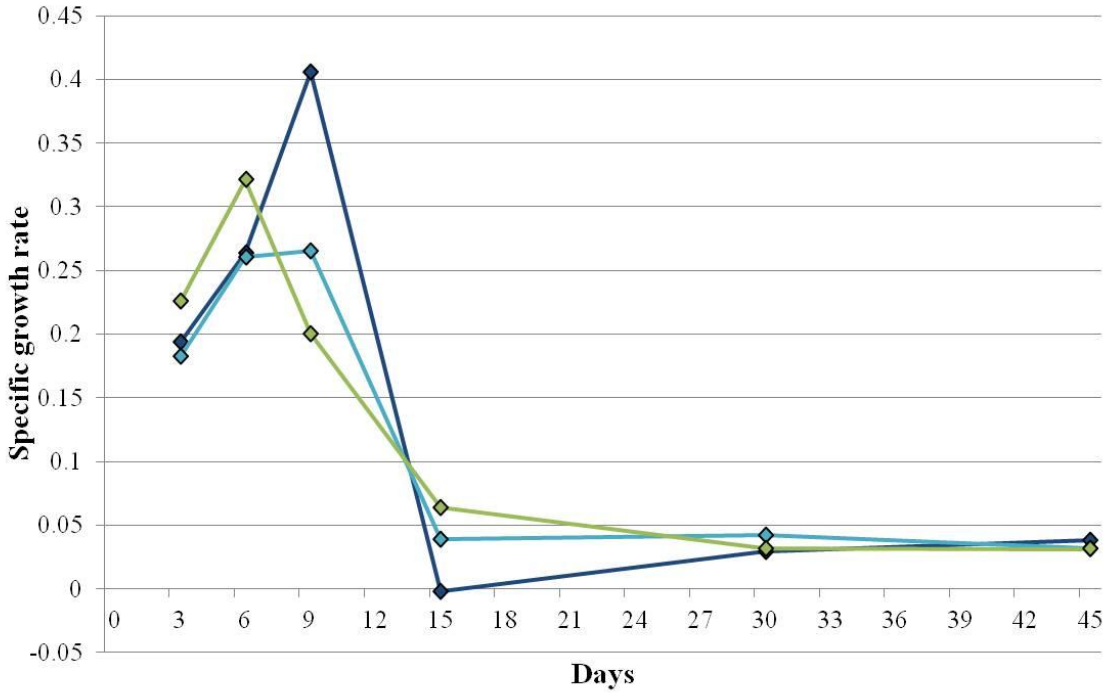



Figure 3.9. Average specific growth rate of each nitrogen condition in second growth experiment with five replicates. Lines indicate nitrate (navy), ammonium (teal), and urea (green).

Closely related dinoflagellates of the *Alexandrium* genus are known to utilize a nickel-dependent urease, but this *P. bahamense*'s urease enzyme has not been studied specifically. Nickel was not supplemented to the media (outside the nanomolar quantities present in the seawater base). Therefore, the toxin decrease in urea-containing media may have been associated with insufficient nickel. However, difficulty assimilating nitrogen would likely be manifested by decreased cell concentrations, in addition to decreased toxin content as was shown in *Alexandrium* (Dyhrman and Anderson, 2003). Cell concentrations were not found to be inhibited by nitrogen source in either the first or follow-up experiments (Figures 3.3 and 3.8), and protein concentration remained similar

for all nitrogen conditions (3.7). Though nitrogen may have been allocated preferentially to growth rather than toxin production in conditions of nitrogen stress, the impact of urea on toxin production but not on growth may indicate that a negative feedback process is at work.

Urea is broken down to two ammonia molecules, thus the difference between the urea and ammonium culture conditions can be directly attributable to the activity of urease enzymes and transporters. The biosynthesis of the essential saxitoxin precursor, arginine, in microorganisms and plants involves the enzymes of the urea cycle. This process consists of eight catalyzed steps beginning with glutamate and ending with the enzyme argininosuccinate lyase. Most enzymes of the urea cycle are highly conserved across phyla (Ouzounis and Kyrpides, 1994; Lawson et al., 1996; Qu et al., 2007), and when at very high concentrations in ill animals, urea inhibits the enzyme argininosuccinate lyase (Santhi Silambanan, 2009). A dearth of arginine would therefore impact toxin production but not as noticeably lower the growth rate. This explanation is further supported by the similarity of the toxin profiles of all three nitrogen conditions on the last day of sampling (Figure 3.5); when the excess of urea is depleted, the feedback inhibition on arginine would be lifted, making all have similar profiles.

This *P. bahamense* culture was non-axenic, but this is not thought to have appreciably affected the results, and may have led to a more accurate simulation of the natural environment. The effect of temperature and nickel on urease activity may significantly change the outcome of this experiment. Useful follow up experiments would be to replicate this experiment at a common bloom temperature of 30°C rather than the 25°C cultures were kept at here, and in the presence of nickel. Measurements of

enzymatic activity and individual amino acids, especially arginine, would also be practical in a duplication of this experiment.

Growth in *P.bahamense* with urea as a nitrogen source reduces toxicity in comparison with nitrate and ammonium: The total toxin content is reduced by reduction of the highly toxic STX form (Figures 3.4 and 3.5). In addition to normal biological inputs, urea is also a common constituent in fertilizers due to its high nitrogen content per molecule and rapid conversion to two ammonia molecules by soil bacteria. Recently, there has been increased concern that anthropogenic nutrients may expand and increase toxicity of HABs. This study indicates that urea depresses toxin production when compared to inorganic nutrients. Runoff of urea fertilizers therefore may encourage *P. bahamense* blooms, but is not enhancing toxicity of these blooms. It must be emphasized that this was found in culture experiments, and that further investigation is needed to understand the impact of nitrogen sources and anthropogenic runoff on growth and toxicity of *P. bahamense* in the field.

## Chapter Four: Toxin Production and Mole Composition of *Pyrodinium bahamense* var. *bahamense* under varied Environmental Conditions

### Introduction

The various forms of saxitoxins have distinct levels of toxicity. Environmental factors may lead not only to changes in production of saxitoxins, but also to changes in the forms of saxitoxins. *Pyrodinium bahamense* var. *bahamense* blooms in Florida under a variety of conditions. Knowledge of environmental effects on toxin production would be useful in predicting toxicity and potential impacts of these blooms.

*P. bahamense* is found in significant numbers in the Indian River Lagoon (IRL), exclusively when the water temperture approaches 25° to 30°C, and is not typically found when water temperature in the IRL is below 20° (Phlips et al., 2006). Temperature, therefore, is likely a limiting factor in its distribution, and future increases in temperature associated with climate change may lead to expansion of *P. bahamense* blooms (Phlips 2009). It is, however, not likely that higher temperatures would make *P. bahamense* more toxic; *Alexandrium sp.* are more toxic at higher latitudes (White, 1986; Cembella, 1988; Anderson et al., 1994), and the toxin content of *A. catenella* has been shown to be inversely related to temperature (Ogata et al., 1982; Cembella, 1998; Navarro et al., 2006). Low temperatures may reduce protein synthesis, resulting in a surplus of arginine that could be used for toxin synthesis (Shimizu et al., 1984; Anderson et al., 1990). In the Malaysian isolate, toxin content (toxin $cell^{-1}$) increased three-fold as the temperature decreased from the optimum growth (div $d^{-1}$) temperature at approximately $30^{o}C$ to $23^{o}C$ (Usup et al., 1994). Toxin depletion was suggested to be a product of dilution associated

with increased growth rate at higher temperatures. (Navarro et al., 2006) did not demonstrate a relationship of toxin to growth rate, implying the toxin disparity was due to metabolic processes. Toxin mole percents were found to be highly conserved through various temperature and salinity conditions in the Philippine isolate of *P. bahamense var compressum*, however, at high and low temperatures an increase in dcSTX and B1 and corresponding decrease in STX is observed (Gedaria et al., 2007). In the Malaysian var. *compressum* isolate, an increase in temperature from 22 $^{o}$C to 34 $^{o}$C was accompanied by a proportional increase in B1 and decrease of NEO (Usup et al., 1994).

*P. bahamense var. bahamense* is a euryhaline species, with tolerance to salinity from 14 to 46 and blooms reported over most of that range (Phlips et al., 2006). In the Indo-Pacific, a linkage has been suggested between increased rainfall and blooms (Azanza et al., 2001). The Philippine isolate of *P. bahamense var. compressum* survived in salinity 26 to 36 with optimum growth at 30 or greater (Gedaria et al., 2007) while the Malaysian isolate tolerated salinity 20-35 (Usup et al., 1994). In *P. bahamense var. compressum* and *A. tamarense,* toxicity tends to decrease with increasing salinity and the accompanied increase in growth rate (Cembella, 1998; Hamasaki et al., 2001; Gedaria et al., 2007). A trend of increasing dcSTX and equally decreasing STX occurred starting at salinities higher than 30 in the Philippine isolate (Gedaria et al., 2007). Usup et al. (1994) found that at salinities of 24 and higher in var. *compressum*, toxin content was maintained (Usup et al., 1994); however, at a salinity of 20 the toxin content was three-fold higher. Parkhill and Cembella (1999) found that the toxin variation in different of salinities of *A. tamarense* was due to salinity–dependent division, and not due to salinity directly (Parkhill and Cembella, 1999).

Cellular processes are strongly affected by fluctuations in pH. Euryhaline species like *P. bahamense* are exposed to a wider range of pHs than oceanic species. Saxitoxins have strongly alkaline guanidine groups, but the effect of pH on toxin production has not been investigated for saxitoxin-producing species. In *A. catenella*, however, pH was determined to play a role in population dynamics, with 8.5 being the optimal pH for growth at 25°C (Siu et al., 1997).

**Objectives**

All organisms respond to environmental change. Environmental effects on toxic species, such as *P. bahamense*, may have consequences to public health. If the saxitoxins are inflated in response to an environmental cue, the toxins absorbed by pufferfish caught in the IRL may lead to increased incidence of SPFP in humans who consume these fish.

The effects of varying temperature, salinity, and pH on the growth and toxin production in nutrient deplete and replete cultures of *P. bahamense* var. *bahamense* were investigated. All cultures were kept within varying conditions that are environmentally possible in the IRL.

**Materials and Methods**

A non-axenic clonal culture of *Pyrodinium bahamense* var. *bahamense* (CCFWC# 394) was isolated from the South Rockledge area of the Indian River Lagoon (IRL), Florida on October 15$^{th}$, 2002 and kept in culture at the FWC Fish and Wildlife Research Institute (FWRI; St. Petersburg FL). It was grown under full spectrum lights on a 12/12 light cycle at 25 °C in ESDK medium since its isolation. Deionized water and

seawater from offshore Florida were filtered and autoclaved to yield media with a salinity of 21±1

Nutrients were added to the starting culture one week prior to the onset of this experiment. Experimental conditions included temperatures of 20°C and 30°C, salinities of 17 and 27, pHs of 7.5 and 9. Measurements of pH were taken at 23°C using the total pH scale at the onset of the experiment. There were four replicates of each experimental condition. Control flasks were kept at a salinity of 22 at 25°C, and at a pH of 8.25. Cultures were kept on a 12/12 light cycle under full spectrum lights. In each experimental condition all other environmental conditions were kept identical to the control. At the onset of the experiment, sufficient seawater was added to 120 mL of culture so that the end result was 400 mL at the listed culture condition. The initial nutrient-deplete samples were taken at day six (Appendix C). Nutrient depletion was inferred from the absence of ample cell growth at day six. Concentrated media was then added to each experimental flask and the nutrient replete-samples were taken on day twelve. Again, nutrient enrichment was inferred through cell growth. At each sampling time, a Lugols sample for cell counts and 10mL culture was taken in duplicate and filtered onto 25mm glass fiber filters (GFFs). GFFs were ground in a Dounce homogenizer in one mL of 1% acetic acid and an additional one mL was used to rinse the homogenizer. The homogenate was then centrifuged at 4,500 x g for 10 minutes to obtain the supernatant. Extracts were analyzed by HPLC after peroxide oxidation as has been described (Lawrence et al., 2005).

**Results**

Only STX, dcSTX, and B1 were found in this *P. bahamense* var. *bahamense* culture, as was noted in the previous chapter and documented by Landsberg (2006); however, only the peroxide oxidation was performed so production of any hydroxylated saxitoxins would not have been detected. Nutrient deplete cultures generally contained more toxin than nutrient replete cultures (Figure 4.1). In deplete media, STX equivalent ranged between 26 and 46 fmol per cell, while in replete it ranged from 16 to 29 fmol per cell, with an outlier at 50 fmol per cell being associated with the T=20°C culture; this culture did not appreciably grow in replete media (Figure 4.2). The toxin profiles of all cultures remained largely unchanged: approximately 25% STX and the majority of the remainder being 72% B1, a very small 3% being dcSTX (Figure 4.3).

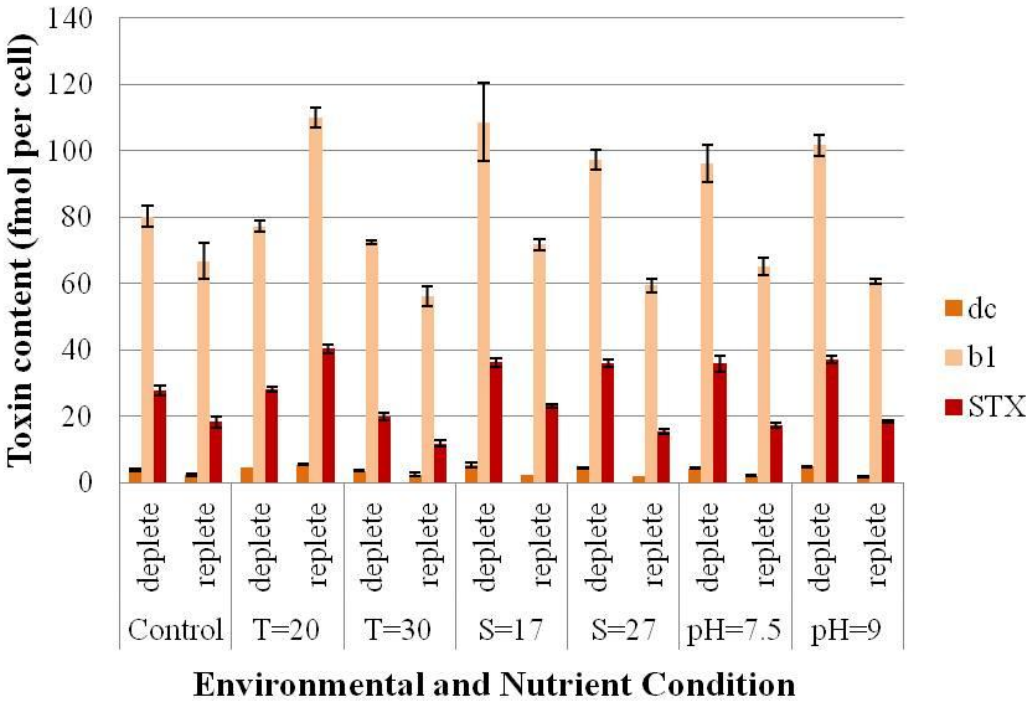



Figure 4.1. Toxin content for all experimental conditions. Bars indicate standard error.

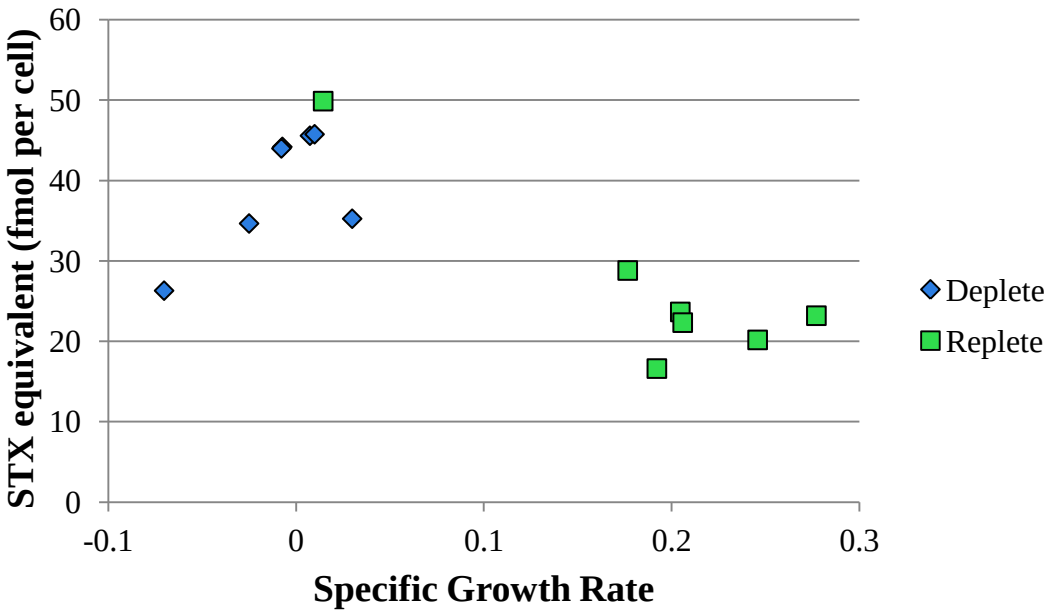



Figure 4.2. Relationship of specific growth rate to toxin content.

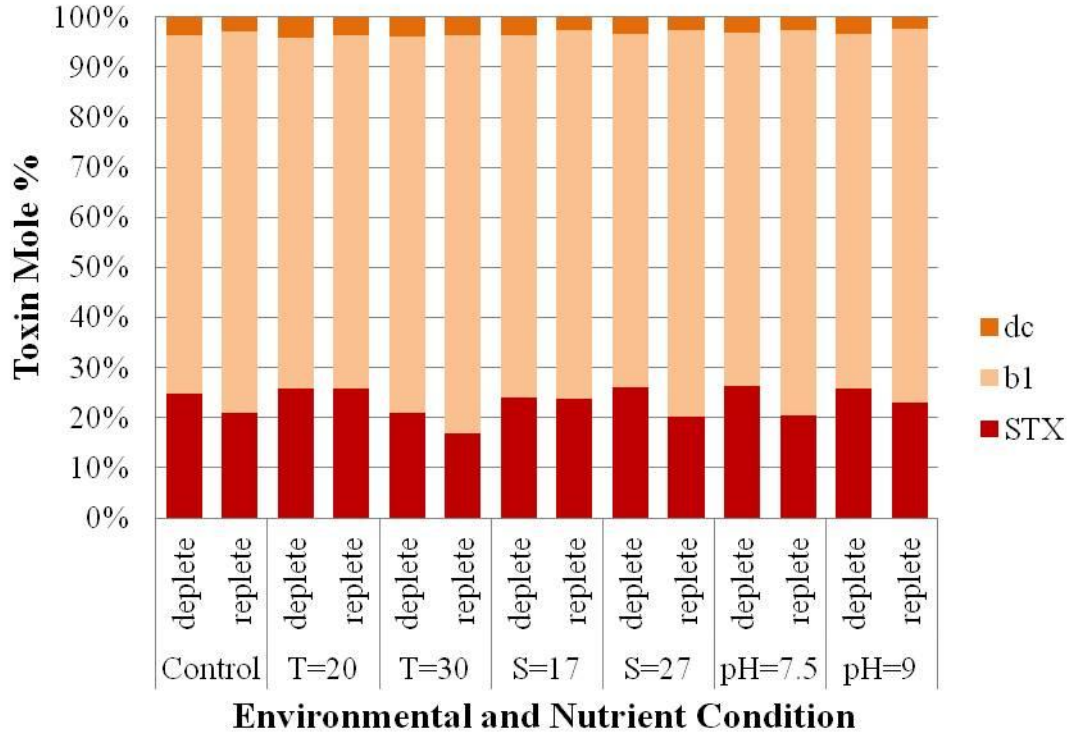



Figure 4.3. Average toxin mole percent of each environmental condition.

There was generally an equal or lesser percentage of STX in replete conditions when compared to the corresponding nutrient deplete condition. Toxin content was highest and cell counts were lowest for the 20°C culture followed by the S=17 culture (Figure 4.4). Toxin content was significantly lower and growth was higher in the 30°C culture when compared to these two culture conditions.

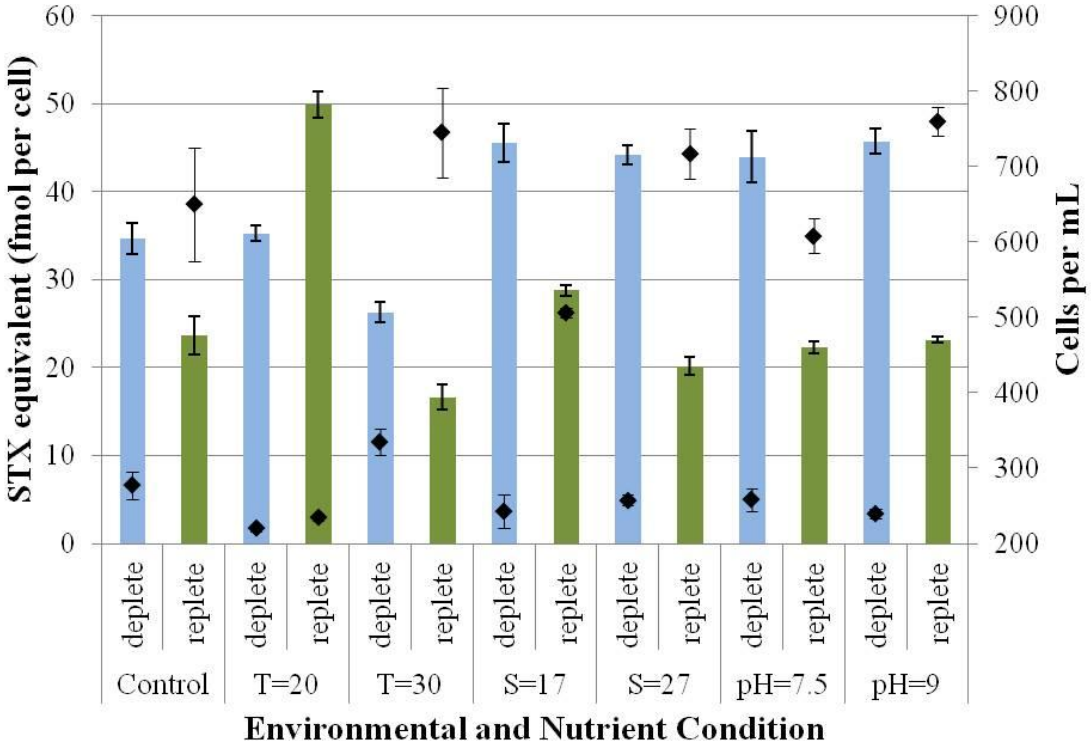



Figure 4.4. STX equivalent for all experimental conditions. Bar graph indicates STX equivalent, and diamonds indicate the cell count at that condition. Bars indicate standard error.

**Discussion**

The toxin profile of this clone closely matches those reported for field *P. bahamense* samples (Landsberg et al., 2006), and appears to be well conserved. Across

all experimental conditions approximately 25% of the toxin is the STX congener, the majority of the remainder being 72% B1, a very small 3% being dcSTX (Figure 4.2). The toxin profile most closely mirrors the Philippine isolate of *P. bahamense* var. *compressum*, which suggests its closer relation to this isolate than the Malaysian isolate which produces the GTX5 and NEO toxins additionally. However, the Philippine isolate contains a much greater proportion of the highly toxic STX at approximately 90% (Gedaria et al., 2007). These toxin signatures have been suggested to be a signature of each genetically different regional isolate (Cembella and Taylor, 1985; Boyer et al., 1987; Cembella et al., 1987; Ogata et al., 1987; Oshima et al., 1993; Anderson et al., 1994; Flynn et al., 1994). The toxin profile of *A. tamarense* is also conserved through changes in light, salinity, and nutrient changes (Parkhill and Cembella, 1999). In addition to toxin profiles, morphological studies between the two varieties have indicated that the distinction between the Atlantic and Pacific varieties of *P. bahamense* is warranted (Steidinger 1980, Badylak 2004).

Toxin content per cell was generally lower in replete media (Figure 4.1). The inverse relationship of growth and toxin content has been widely reported and is the main theme that emerges in this study. In nutrient deplete media, saxitoxins remained high, and seemed not to be appreciably catabolized to form other biomolecules when compared to the replete culture that shared their specific growth rate (Figure 4.2). This corroborates the finding that toxins remained constant in N-limited var. *compressum*, however in this isolate GTX5 increased while the other toxins remained constant (Usup and Anderson, 1996).

*P. bahamense* var. *bahamense* did not grow at 20°C (Figure 4.3). This corresponds to the observed temperature tolerance for other var. *compressum* isolates. The Philippine isolate was observed to grow from 23° C to 36°C with optimum growth at 25°C (Gedaria et al., 2007), while the Malaysian isolate grew from 22° C to 34°C with optimum growth at 28°C (Usup et al., 1994). This culture kept at 20°C, when nutrient replete, became the most toxic on a per cell basis due to continued saxitoxin production and halted cell division. *Alexandrium sp.* display an analogous latitudinal effect, in which northern species exhibit higher toxin content. This has been proposed to be due to slowed growth rate, or slowed protein synthesis, leaving arginine in excess to be assimilated into the saxitoxins. In nutrient deplete conditions, the culture at 20°C had a lower cellular toxin concentration compared to its replete counterpart presumably due to slowing of cellular processes with nutrient limitation.

The culture at 30°C grew at a greater rate than the control at 25°C, reflecting this isolate's propensity to bloom in hot summer temperatures (Figure 4.3). The optimal growth temperature for this var. *bahamense* isolate therefore is greater than the optima listed for both the Phillipine and Malaysian var. *compressum* isolates. This disparity in growth rate between experimental temperature conditions imparted a subsequent disparity in cell toxin content. Toxin content was lower in the culture kept at 30°C likely due to dilution into a greater number of daughter cells.

Growth of *P. bahamense* was slowed, and toxin per cell enhanced, when subjected to a salinity of 17 in comparison with salinities of 27 and the salinity of the control at 22. This outcome is not surprising, as this salinity is at the low range of reported occurrence of *P. bahamense* in the IRL.

Growth rate is the most important factor in predicting toxicity of *P. bahamense* on a per cell basis, although the conditions for optimum growth are the most important to bloom dynamics and potential impacts to the environment. The environmental factors tested had no quantifiable effect on toxin production alone; however, they did change growth rate and subsequently toxin per cell. Saxitoxin appears to be constitutively produced in this species through non-optimal conditions. Conditions for enhanced growth in *P. bahamense* var. *bahamense* were found to be 30°C, salinity = 22 to 27, and pH 9.

## Chapter Five: Biological activity of *Pyrodium bahamense* and *Karenia brevis* extracts

### Introduction

Marine algae have been used in Chinese, Egyptian, and Ayurvedic medicines since early history (Fujihara et al., 1984). They were some of the first organisms investigated by marine natural products chemists (Fujihara et al., 1984; Paul and Fenical, 1984; Folmer et al., 2010), and continue to be studied for their activities against common pathogens (Yi et al., 2001; Rios et al., 2009). The euphotic zone is characterized by fluctuations in oxygen concentrations, UV radiation, and temperature, making the bioactive compounds isolated from algae predominately anti-oxidants such as phlorotannins, ascorbic acid, carotenoids, and tocopherols (Heo et al., 2009; Zubia et al., 2009; Folmer et al., 2010). More than half of secondary metabolites reported from macroalgae are isoprenoids (Maschek and Baker, 2008).

Phytoplankton bridge algae and microorganisms, and bioactivity is found broadly throughout the group. Phytoplankton extracts have been shown to inhibit growth of some species of bacteria (del Pilar Sanchez-Saavedra et al., 2010), and emulsified microparticles of cyanobacteria in the order *Nostocales* may have potential for prophylaxis against methicillin-resistant *S. aureus* (MRSA) infections during surgery (Lukowski et al., 2008). Dinoflagellates specifically, have yielded valuable health food additives, laboratory tools, and show promise in drug discovery (Pietra, 1997); however, research on dinoflagellates has been predominantly related to their production of toxins. The capability of these species to produce bioactive toxins may make them superior

candidates to produce other bioactive and potentially useful compounds. The dinoflagellates *Gambierdiscus toxicus* and *Alexandrium tamarense* were found to possess anti-fungal properties (Accorinti, 1984; Nagai, 1992). Polysaccharide GA3P is an anticancer compound isolated from *Gymnodinium* species (Sogawa et al., 1998; Umemura et al., 2003). *Amphidium* species have been particularly prolific; karatungiol A was found to possess antifungal and antiprotozoan properties. Several types of macrolides, the amphidinolides (Kobayashi and Tsuda, 2004; Washida, 2006), the amphidinolactones (Takahashi et al., 2007), the caribenolides (Bauer et al., 1995), the iriomoteolides (Tsuda et al., 2007), have also been isolated. In *K. brevis*, one of the dinoflagellates explored in this study, the polyether compound brevenal has been found to oppose the action of brevetoxin on sodium channels (Bourdelais et al., 2004) and shows potential as a cystic fibrosis drug (Abraham et al., 2005).

**Objectives**

Bioactive substances have not been investigated in *Pyrodinium bahamense,* and have not been thoroughly explored in *K. brevis*. The primary objective of this study was to examine extracts of the dinoflagellates *K. brevis* and *P. bahamense* for *in vitro* activity against common infectious agents and to determine the potential of HAB dinoflagellate species as sources of natural products in comparison to nontoxic dinoflagellates.

**Materials and Methods**

Cultured *K. brevis* (see chapter 2) and *P. bahamense* (see chapter 3) were concentrated by a continuous flow-thru centrifuge system (Sorvall SS3 KSB). The

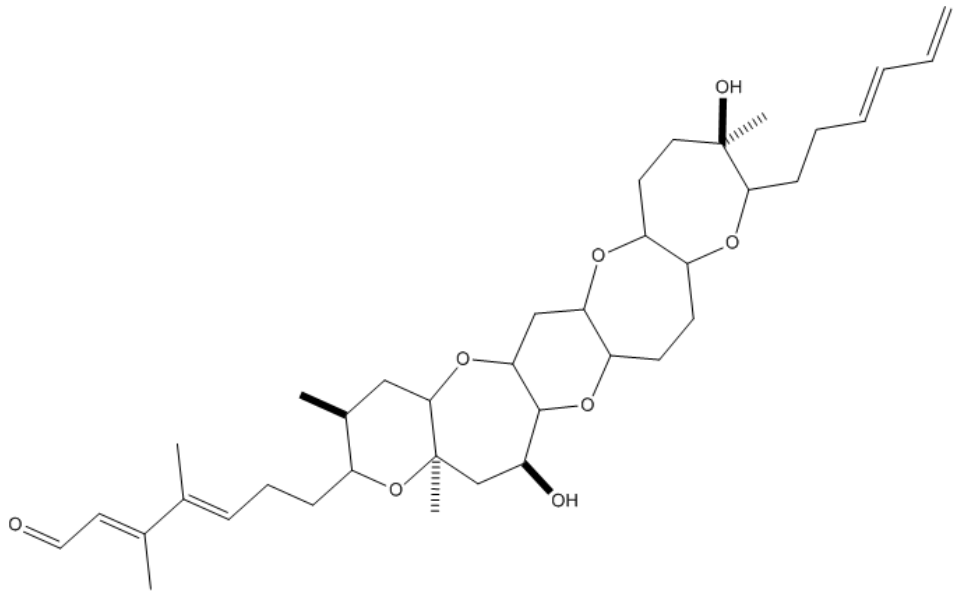



Figure 5.1. Structure of brevenal.

supernatant was set aside for further processing. The pellets were lyophilized and subsequently broken with methanol and sonication. The cell debris was removed by another centrifugation, and the solvent was dried by rotary evaporator. The residue was partitioned between water and hexane five times. These solvents were chosen to ensure the location of the toxins produced by *K. brevis* and *P. bahamense*. The saxitoxins produced by *P. bahamense* are hydrophilic and partition into water. The brevetoxins produced by *K. brevis*, though lipophilic, were found to partition preferentially into water as well.

A column was packed with Amberlite XAD18 polymeric resin (Rohm and Haas) and conditioned with methanol according to the sorbent recommended use. The supernatant from the flow-thru system (approximately 10 L) was allowed to pass through the column at two bed volumes per hour. The column was eluted with four bed volumes of methanol.

Extracts were assayed against bacteria *Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtillus, Enterococcus faecalis,Staphylococcus aureus,and* MRSA, the fungi *Aspergillus niger* and *Candida albicans*, and the protozoan responsible for malaria, *Plasmodium falciparum.* For each crude extract, 2 mg in dimethyl sulfoxide (DMSO) was applied to a 6mm paper disk. Disk diffusion assays were completed for ten bacterial and fungal strains (Bauer et al., 1966). A disk loaded with DMSO was used as a negative control. Bacterial zones of inhibition were measured at 24 hours of incubation at 37°C and checked again at 48 hours. Fungal zones of inhibition were assessed after incubation at 42°C for 48 hours. Extracts for anti-plasmodial activity were sent to the Global Health Infectious Disease Research Department in the College of Public Health at University of South Florida in Tampa for analysis by the SYBR Green method (Johnson et al., 2007).

The *P. bahamense* pellet hexane extract (120 mg) hexane extract was injected onto a Shimadzu HPLC with a YMC ODS C-18 column and PDA detector. Ultraviolet detection was at 254 nm, a typical wavelength for detection of unsaturated molecules. From 0 to 25 minutes, a 50% to 100% methanol gradient was used, and 100% methanol was held for an additional 30 minutes.

To scale up and maximize biomass, 400 L of seawater was collected from Tampa Bay, Florida, during the 2011 monospecific bloom of *P. bahamense*. The cells were concentrated by a continuous flow-thru centrifuge system (Sorvall SS3 KSB). Pellets were prepared as they were with culture extract. The crude extract was first fractionated by normal phase medium pressure liquid chromatography (MPLC) on a combi-flash RF using a 12g Redisep silica column (Figure 5.3). A solvent system of hexane and ethyl

acetate were from 0 to 19 minutes, 100% ethyl acetate was held for 4 minutes, and was followed by a methanol wash.

**Results**

Biological activity was first determined through a small scale extraction of cultured *K. brevis* and *P. bahamense*. In the extracts tested, inhibition was observed in *Klebsiella pneumoniae, Staphylococcus aureus*, MRSA, and *Plasmodium falsiparum* (Table 5.1). Growth of *K. pneumoniae* was inhibited by the hexane extract of both *K. brevis* and *P. bahamense* after incubation for one day. At two days, however, there was no longer any zone of inhibition. The hexane extract of *K. brevis* additionally inhibited MRSA, while the *P. bahamense* hexane extract additionally inhibited both *S. aureus* and MRSA as well as *P. falciparum*. The supernatants of both species were time intensive to extract and minimally active, and thus not further explored. No extracts in this study demonstrated activity against bacteria *E. coli, S. typhimurium, P. aeruginosa*, and *E. faecalis* or fungi *A. niger* and *C. albicans.* Additionally no activity was found against the bacterium *B. subtillus*, though this activity is common in phytoplankton (del Pilar-Sanchez-Saavedra et al., 2010).

Further separation of the *P. bahamense* hexane extract by reverse phase HPLC and subsequent assay showed that the compounds inhibiting *S. aureus* and that inhibiting *P. falciparum* eluted at approximately 27 and 29 minutes respectively in 100% methanol (Figure 5.2). Greater than 67% inhibition of *P. falciparum* was observed in 5 ug/mL fraction B.

To procure a large biomass of *P. bahamense*, a 2011 bloom of *P. bahamense* var. *bahamense* in Tampa Bay was sampled. The monospecificity of the bloom water sample and the exclusionary nature of slow centrifugation yielded a relatively clean environmental sample. Fractions obtained from the 2011 Tampa Bay *P. bahamense* bloom retained the antibacterial capacity and eluted at 4 minutes, immediately before chlorophyll by normal phase MPLC (Figure 5.3) The potent antiplasmodial activity foundin the IRL clonal culture was however not found in the bloom sample.

Table 5.1. Infectious agents, agar they were grown on, and zones of inhibition when exposed to *K. brevis* and *P. bahamense* extracts.

| Scientific Name | Description | Agar | *K. brevis* Pellet-Hexane | *K. brevis* Pellet-Water | *K. brevis* Supernatant | *P. bahamense* Pellet-Hexane | *P. bahamense* Pellet-Water | *P. bahamense* Supernatant |
|---|---|---|---|---|---|---|---|---|
| *Escherichia coli* | - rod bacteria | BBL brilliant green | - | - | - | - | - | - |
| *Salmonella typhimurium* | - rod bacteria | XLD | - | - | - | - | - | - |
| *Pseudomonas aeruginosa* | - rod bacteria | Pseudosel | - | - | - | - | - | - |
| *Klebsiella pneumoniae* | - rod bacteria | MacConkey | 1.3 cm* | - | - | 1.2 cm* | - | - |
| *Bacillus subtillus* | + rod bacteria | TSA | - | - | - | - | - | - |
| *Enterococcus faecalis* | + cocci bacteria | Vancomycin | - | - | - | - | - | - |
| *Staphalococcus aureus* | + cocci bacteria | Mannitol salt | - | - | - | 1 cm | 1 cm | - |
| MRSA | + cocci bacteria | Mannitol salt | 8 mm | - | 9 mm | 8 mm | - | - |
| *Candida Albicans* | fungus | SDA | - | - | - | - | - | - |
| *Aspergillus niger* | fungus | SDA | - | - | - | - | - | - |
| *Plasmodium falciparum* | protozoan | | - | - | - | + | - | - |

-No inhibition
+ Inhibition present, see results section.
* Growth reduced at 24 hours, but no inhibition at 48 hours

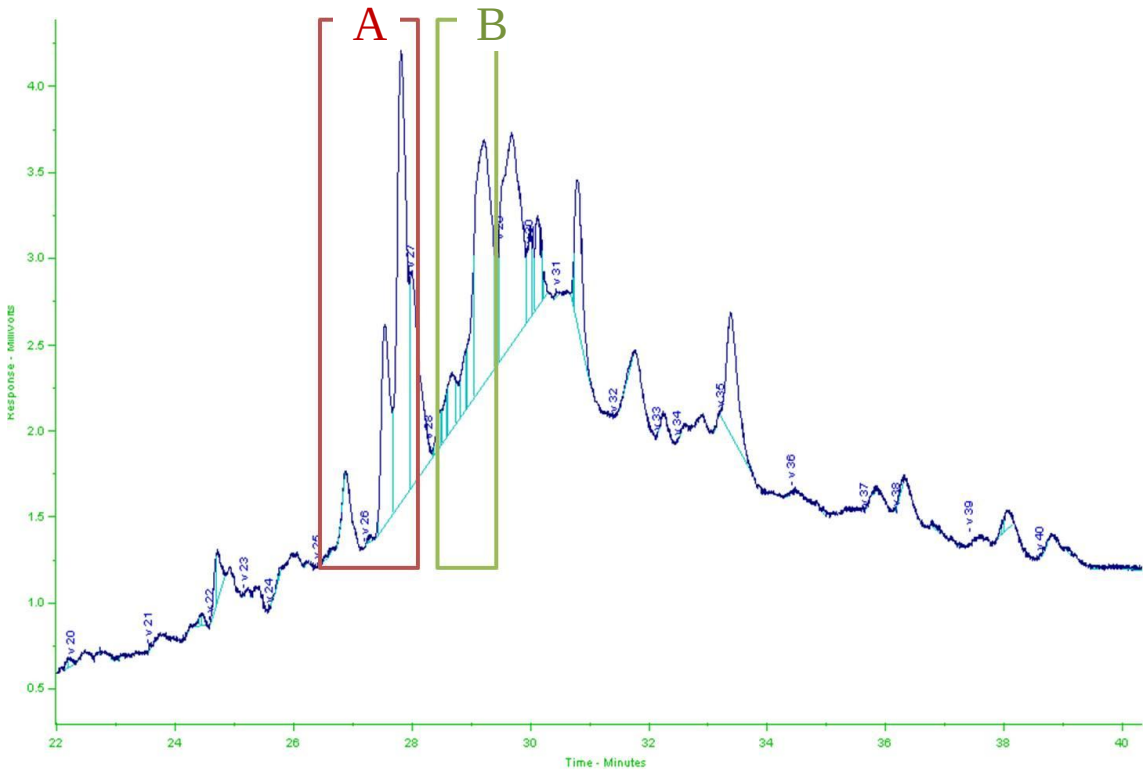



Figure 5.2. Reverse phase HPLC of cultured *P. bahamense* pellet hexane extract. The red box entitle fraction A denotes the fraction active against *S. aureus*, and the green box entitled fraction B denotes the fraction active against *P. falciparum.*

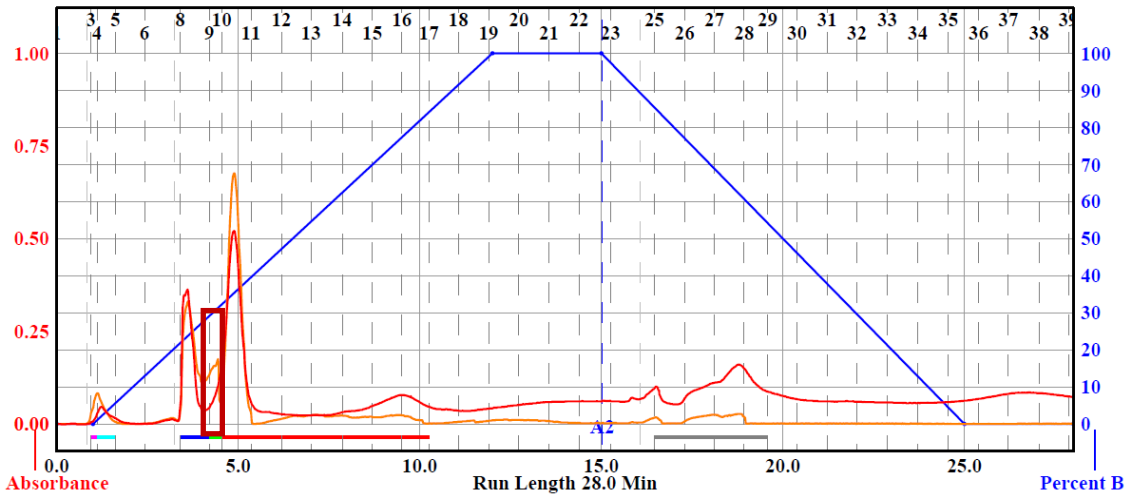



Figure 5.3. Normal phase MPLC of the pellet hexane extract of the 2011 bloom sample of *P. bahamense*. Solvent gradient is shown in blue. Red and orange chromatograms denote 254 nm and 200-360 nm scan respectively. The red box denotes the fraction active against *S. aureus*

**Discussion**

Activity against *S. aureus*, MRSA, *K. pneumoniae,* and *P. falciparum* were detected in extracts from cultures of *K. brevis* and *P. bahamense*. Growth of *K. pneumoniae* was inhibited by the hexane extract of both *K. brevis* and *P. bahamense* and perhaps is from a common compound more broadly produced than the phylum Dinoflagellata. The cyanobacteria *Synechococcus elongatus* and *Synechocystis* sp., the rhodophyte, *Porphyridium cruentum*, and some marine macroalgae also display activity against *K. pneumoniae* (Solomon and Santhi, 2008; Rios et al., 2009; del Pilar Sanchez-Saavedra et al., 2010); as do some terrestrial plants (Santhi and Annapoorani, 2010).

Activity against *S. Aureus* also appears to be common in plants, having been identified in the dinoflagellate *Alexandrium tamarense* (Accorinti, 1984), cyanobacteria *Synechococcus elongatus* and *Synechocystis* sp., diatoms *Amphiprora paludosa* and *Chaetoceros muelleri*, the chlorophyte *Dunaliella tertiolecta* (del Pilar Sanchez-Saavedra et al., 2010), some marine macroalgae (Yi et al., 2001; Rios et al., 2009), the water hyacinth, *Eichhornia crassipes* (Shanab et al., 2010), and some terrestrial plants (Zuridah et al., 2008). The extracts in the studies cited previously were not assayed against MRSA, however, it is likely that anti-MRSA properties are widespread as well. Anti-MRSA properties have been found in some macroalgae (Kim et al., 2007; Solomon and Santhi, 2008), in the diatom *Phaeodactylum tricornutum* (Desbois et al., 2009), and in the chlorophyte genera-mate of *D. tertiolecta, Duniella primolecta* (Ohta et al., 1994). Likewise, in this study *P. bahamense* displayed activity against both *S. aureus* and MRSA. *Karenia brevis* also showed activity against MRSA, however activity against *S. aureus* was not observed. The increased activity against MRSA may suggest that the

causative compound targets the genetic alteration making MRSA resistant to other antibiotics. The disparity in activities of *K. brevis* and *P. bahamense* against *S. aureus* suggests the production of differing antibacterial agents. The activity of the *K. brevis* supernatant against MRSA is most likely due to breakage of these fragile non-thecate dinoflagellates and subsequent leaking of intracellular compounds.

The hexane extract of *P. bahamense* was found to inhibit *P. falciparum*. Extracts of marine macroalgae have previously been found to inhibit *P. falciparum* in vitro (Chen et al., 2009; Ravikumar et al., 2011), and many anti-Plasmodial halogenated compounds have been isolated from marine macroalgae (Lane et al., 2009; Cabrita et al., 2010). Anti-Plasmodial calothrixins have also previously been characterized in cyanobacteria of the *Calothrix* genus (Rickards et al., 1999). The activity of a dinoflagellate against an Apicomplexan (*P. falciparum*) found in this study is especially interesting as the phyla are closely phylogenetically related (Grauvogel et al., 2007). Malaria continues to be the most important parasitic disease affecting humans. In developing countries it is a major cause of morbidity and mortality. According to the *World Malaria Report 2011*, there were 216 million cases of malaria and an estimated 655,000 deaths in 2010, most of these being African children (World Health Organization, 2011); these statistics are likely underestimates, as the Institute for Health Metrics and Evaluation indicated that far more adults were affected than the WHO reported (Murray et al., 2012). Activity against *P. falciparum* was not found in the 2011 bloom sample of *P. bahamense* var. *bahamense.* Many factors may have affected this outcome including genetic drift while in culture and variant production depending on growth stage or environmental condition. It is also likely that the clonal isolate in culture from the IRL produces different compounds or

quantities of compounds than the population that blooms in Tampa Bay. This may indicate that there is a considerable genetic difference between the two populations.

Activity in *P. bahamense* and *K.brevis* partitioned into the hexane fraction, and was therefore not related to the saxitoxins or brevetoxins respectively, which partitioned into the water fraction. In addition, other polyether dinoflagellate toxins have been found to not possess antibacterial properties when screened against common human infectious agents including *S. aureus* (Nagai et al., 1990).

Many drugs are losing effectiveness as infectious agents evolve. It is important to continue pursuing nature for new and potent drug sources. No published works were found previous to this study that assayed dinoflagellate extracts against *K. pneumoniae*, MRSA, *P. falciparum*, and many of the other infectious agents assayed that there was no activity against. While dinoflagellates do seem to be a promising source of diverse molecules, the HAB species do not seem to offer an uncharacteristic abundance of bioactivity in comparison to other phytoplankton. Phytoplankton in general may hold promise in drug discovery as algae are generally a sustainable source of secondary metabolites, able to be grown in the laboratory or outdoor culture fields (Folmer et al., 2010); and as knowledge of biosynthetic gene clusters continue to be elucidated, the potential for combinatorial biosynthesis grows (Zhang and Tang, 2008).

## Chapter Six: Summary and Conclusions

Mitigation of Harmful Algal Blooms (HABs) without knowledge of the ecological purpose and biochemical regulation of these toxins is highly unlikely. The research completed in these chapters dealt with production, function and potential of secondary metabolites from HAB species. Differential centrifugation and immunostaining demonstrated that brevetoxins locate within the cytosol of *K. brevis*. This location implicates the brevetoxins in cellular functions such as chelation and signaling. The lack of brevetoxins in the location of polyketide synthesis, the chloroplast, indicates that they are most likely cyclized in the cytosol.

Multiple culture experiments of *P. bahamense* var. *bahamense* were undertaken to establish environmental and nutrient parameters that may alter growth and toxin production in this species. Possible reasons for the reported increase in HABs worldwide include global warming, dumping of ballast water, and nutrient influx (Glibert et al., 2005). These and other studies that outline controls on toxin synthesis and production will aid in predicting environmental and human health effects in the face of these global changes. Saxitoxins in *P. bahamense* were found to be produced constitutively and kept in reserve during nutrient depletion. Saxitoxin content was found to vary most dramatically with growth rate. Toxin mole percent was largely unchanged over a suite of environmental parameters including typical temperature, salinity, and pH levels found in the IRL. However, growth of *P. bahamense* on a common nitrogen source in fertilizers, urea, was found to decrease the amount of the potent STX congener. This finding is important because anthropogenic nutrient loading has been implicated in increased

incidence and toxicity of algal blooms. Cyst production in *P. bahamense* was found to depend on nutrient limitation.

Many drugs are losing effectiveness as infectious agents evolve. It is important to continue pursuing nature for new and potent drug sources. Extracts from *K. brevis* and *P. bahamense* were shown to have action against the bacteria, *S. aureus*, MRSA, and *K. pneumoniae*, and the most common protozoan vector for malaria, *P. falciparum*. These results suggest that HAB dinoflagellates hold promise in drug discovery similar to other phytoplankton.

Many organisms produce bioactive chemical compounds, and the line between "toxin" and "drug" is often obscure and dose dependent. The saxitoxins, for instance, have potential as local anesthetics (Adams et al., 1976) and brevenal is a potential cystic fibrosis drug (Abraham et al., 2005). Examining the production of this unique metabolic artwork has applications to fields including chemistry, biochemistry, chemical ecology, epidemiology, and drug discovery.

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# Appendices

## Appendix A: Raw Cell Count Data for Chapter 3

| | Nitrate | | | Ammonium | | | Urea | | | volume filtered |
|---|---|---|---|---|---|---|---|---|---|---|
| flask | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | for toxin analysis |
| Day 3 Vegetative cells | 73 | 82 | 118 | 64 | 110 | 65 | 71 | 82 | 87 | 50 |
| Day 7 Vegetative cells | 85 | 170 | 175 | 130 | 87 | 161 | 130 | 326 | 112 | 50 |
| Day 7 cysts | 1 | 4 | 3 | 0 | 0 | 0 | 3 | 4 | 2 | 50 |
| Day 10 Vegetative cells | 252 | 254 | 219 | 252 | 267 | 192 | 195 | 359 | 164 | 25 |
| Day 10 cysts | 3 | 2 | 0 | 2 | 0 | 0 | 1 | 5 | 8 | 25 |
| Day 13 Vegetative cells | 294 | 292 | 267 | 345 | 322 | 292 | 272 | 475 | 212 | 25 |
| Day 13 cysts | 0 | 1 | 2 | 0 | 0 | 0 | 11 | 3 | 0 | 25 |
| Day 17 Vegetative cells | 312 | 336 | 354 | 481 | 466 | 340 | 352 | 450 | 294 | 25 |
| Day 17 cysts | 8 | 3 | 2 | 0 | 0 | 0 | 1 | 18 | 3 | 25 |
| Day 28 Vegetative cells | 305 | 345 | 363 | 400 | 504 | 353 | 355 | 446 | 332 | 25 |
| Day 28 cysts | 11 | 9 | 12 | 4 | 7 | 3 | 8 | 33 | 3 | 25 |
| Day 51 Vegetative cells | 334 | 318 | 235 | 276 | 340 | 254 | 247 | 353 | 297 | 50 |
| Day 51 cysts | 23 | 16 | 13 | 9 | 17 | 19 | 16 | 24 | 23 | 50 |

* cell and cyst counts are per 0.3 mL culture

## Appendix B: Raw HPLC Toxin Data for Chapter 3

| Day | 3 | | | 7 | | | 10 | | | 13 | | | 17 | | | 28 | | | 51 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Saxitoxin form (ng/mL) | dcSTX | B1 | STX | dcSTX | B1 | STX | dcSTX | B1 | STX | dcSTX | B1 | STX | dcSTX | B1 | STX | dcSTX | B1 | STX | dcSTX | B1 | STX |
| NitrateFlask1 | 2.01 | 376.8 | 113.8 | 2.57 | 315.1 | 103.9 | 2.92 | 436 | 132.2 | 2.39 | 375.5 | 119.1 | 2.9 | 454 | 180.3 | 5.52 | 840.8 | 228.8 | 12.91 | 1834 | 375.6 |
| NitrateFlask1 | 2.18 | 395 | 126.6 | 2.7 | 331 | 101.4 | 2.99 | 472 | 144.2 | 2.7 | 401.6 | 113 | 2.62 | 490.8 | 174.2 | 5.37 | 813.2 | 222.8 | 12.85 | 2060 | 381.3 |
| NitrateFlask2 | 2.28 | 386 | 123.7 | 2.64 | 356 | 128.4 | 3.16 | 528.36 | 158 | 2.48 | 409.6 | 129.1 | 2.77 | 468.4 | 149.6 | 4.91 | 796.2 | 238.6 | 14.08 | 2178 | 437.4 |
| NitrateFlask2 | 2.13 | 387.6 | 121.9 | 2.85 | 356 | 126.4 | 2.81 | 413.6 | 122.8 | 2.74 | 372.2 | 112.8 | 2.68 | 602.8 | 191.6 | 5.18 | 794.8 | 254.2 | 14.8 | 2394 | 472.8 |
| NitrateFlask3 | 2.14 | 410.9 | 120 | 3.13 | 327.2 | 110.4 | 2.9 | 448.37 | 139.7 | 2.8 | 310.6 | 91.5 | 2.62 | 501.4 | 185.4 | 5.24 | 829.6 | 263.4 | 13.39 | 2118 | 427.8 |
| NitrateFlask3 | 2.11 | 398.2 | 123.5 | 2.78 | 325 | 116.2 | 2.83 | 408.85 | 118.71 | 3.56 | 446.3 | 130.7 | 2.44 | 474 | 163.3 | 6.25 | 834.2 | 317.4 | 12.94 | 1871 | 457.5 |
| AmmoniumFlask1 | 1.83 | 223.6 | 94.2 | 2.68 | 197.8 | 79.9 | 2.56 | 365.8 | 138.8 | 2.64 | 334.8 | 96.8 | 2.63 | 486.6 | 167.4 | 6.15 | 722 | 288.8 | 19.74 | 2451 | 593.4 |
| AmmoniumFlask1 | 1.82 | 254.7 | 106 | 2.72 | 225.2 | 95.3 | 2.91 | 388 | 135 | 2.96 | 358.1 | 118.8 | 2.58 | 472 | 165.5 | 5.82 | 611.4 | 239 | 17.9 | 2115 | 533.1 |
| AmmoniumFlask2 | 2.22 | 253.6 | 92.5 | 3.17 | 324.9 | 118.6 | 2.94 | 388.4 | 121.8 | 2.33 | 359.2 | 111.1 | 2.63 | 505.8 | 186.9 | 7.42 | 891.6 | 309 | 15.9 | 2080.26 | 424.5 |
| AmmoniumFlask2 | 1.96 | 349.6 | 104.4 | 2.89 | 281.4 | 99.1 | 3 | 397.4 | 131.8 | 2.41 | 390.4 | 126.1 | 2.52 | 509.2 | 177.8 | 6.57 | 981 | 301 | 15.06 | 2349.62 | 506.7 |
| AmmoniumFlask3 | 1.57 | 220.4 | 71.8 | 2.47 | 196.5 | 79 | 2.63 | 287 | 110.6 | 2.45 | 240.8 | 91.5 | 2.91 | 318.6 | 128.6 | 6.17 | 747.6 | 250.8 | 15.17 | 2232.23 | 621.9 |
| AmmoniumFlask3 | 1.56 | 209.2 | 74 | 2.61 | 204 | 81.2 | 2.34 | 290.2 | 116.2 | 2.6 | 230.8 | 77.2 | 3.45 | 352.4 | 163.4 | 5.88 | 749.1 | 269.5 | 15.19 | 2158.52 | 591.9 |
| UreaFlask1 | 1.45 | 223.2 | 38.1 | 2.1 | 163.4 | 28.49 | 2.05 | 233.4 | 35.65 | 1.53 | 189.7 | 22.6 | 2.18 | 242 | 40.03 | 4.45 | 743.8 | 107.5 | 10.35 | 1547.91 | 406.5 |
| UreaFlask1 | 1.62 | 250.8 | 49.1 | 1.88 | 184.3 | 27.95 | 2.04 | 263.2 | 35.85 | 1.48 | 172.6 | 25.9 | 1.85 | 215.2 | 35.99 | 4.69 | 617 | 74.8 | 11.6 | 1632.54 | 399.6 |
| UreaFlask2 | 2.04 | 247.8 | 77.6 | 2.87 | 201.4 | 30.1 | 3.24 | 579.2 | 93.8 | 3.06 | 398.4 | 49 | 3.58 | 607.2 | 110 | 6.19 | 805.4 | 145.2 | 15.31 | 2186.73 | 318.6 |
| UreaFlask2 | 2.28 | 249 | 81.2 | 2.88 | 178.7 | 26.7 | 3.32 | 554.8 | 82.8 | 2.93 | 383.8 | 51.8 | 2.99 | 530.6 | 97.4 | 6.19 | 880.2 | 155 | 17.05 | 2397.85 | 346.8 |
| UreaFlask3 | 2.02 | 260.9 | 44.8 | 2.1 | 169 | 32.1 | 1.94 | 299.4 | 42.9 | 1.85 | 169 | 32.7 | 1.65 | 236 | 46.6 | 4.05 | 720.9 | 112.1 | 14.19 | 2068.43 | 480.9 |
| UreaFlask3 | 1.95 | 279.9 | 43.4 | 2.27 | 192.4 | 31.3 | 2.17 | 284.2 | 41.94 | 1.97 | 181.2 | 29.34 | 2.15 | 268.8 | 54.6 | 4.25 | 699.4 | 90.5 | 13.54 | 2171.26 | 469.5 |

* all toxin samples were in 2 mL final volume after filter extraction

## Appendix C: Raw Cell Counts and HPLC Toxin Data for Chapter 4

| | | 6 Day (Deplete) | | | | | | 12 Day (Replete) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Replicate 1 | | | Replicate 2 | | | Replicate 1 | | | Replicate 2 | | |
| | | count | dcSTX | B1 | STX | dcSTX | B1 | STX | count | dcSTX | B1 | STX | dcSTX | B1 | STX |
| Control | Flask 1 | 98 | 2.52 | 87 | 22.68 | 3.2 | 92.8 | 24.64 | 253 | 4.34 | 173.28 | 40.23 | 3.53 | 173.2 | 29.48 |
| | Flask 2 | 72 | 2.63 | 78.67 | 22.2 | 2.86 | 81.56 | 21.98 | 143 | 4.95 | 159.04 | 33.12 | 3.79 | 132.88 | 28.8 |
| | Flask 3 | 84 | 2.75 | 81.27 | 21.86 | 2.75 | 85.46 | 23.22 | 195 | 3.62 | 156.08 | 34.89 | 3.74 | 163.52 | 37.77 |
| | Flask 4 | 79 | 2.42 | 77.04 | 22.42 | 3 | 85.92 | 23.91 | 189 | 4.24 | 175.76 | 37.89 | 4.01 | 145.6 | 34.33 |
| T=20°C | Flask 1 | 67 | 2.41 | 61.41 | 19.6 | 2.76 | 66.94 | 20.3 | 67 | 3.21 | 99.98 | 29.35 | 3.02 | 99.52 | 29.23 |
| | Flask 2 | 62 | 2.18 | 60.31 | 16.9 | 2.69 | 68.01 | 19.44 | 70 | 3.72 | 101.39 | 30.11 | 3.15 | 92.49 | 26.04 |
| | Flask 3 | 67 | 2.63 | 63.38 | 17.75 | 2.55 | 61.85 | 17.58 | 70 | 3.4 | 93.84 | 26.61 | 3.48 | 100.57 | 29.87 |
| | Flask 4 | 69 | 2.58 | 65.09 | 18.47 | 2.74 | 69.19 | 19.33 | 74 | 3.38 | 97.07 | 28.49 | 3.41 | 95.76 | 26.57 |
| T=30°C | Flask 1 | 85 | 2.93 | 72.25 | 13.99 | 3.32 | 82.58 | 17.15 | 177 | 6.54 | 145.71 | 26.34 | 6.09 | 131.46 | 23.25 |
| | Flask 2 | 103 | 3.17 | 100.04 | 19.34 | 3 | 93.13 | 18.39 | 246 | 4.05 | 158.79 | 25.41 | 4.29 | 166.2 | 25.8 |
| | Flask 3 | 110 | 3.03 | 96.63 | 21.45 | 3.22 | 101.23 | 22.38 | 215 | 4.05 | 158.37 | 28.5 | 4.23 | 169.29 | 28.17 |
| | Flask 4 | 104 | 3.4 | 101.84 | 21.46 | 3.03 | 88.98 | 27.32 | 256 | 4.2 | 162.42 | 25.41 | 4.2 | 162.42 | 25.41 |
| S=17 | Flask 1 | 74 | 3.31 | 97.74 | 21.64 | 3.45 | 102.21 | 30.74 | 157 | 3.27 | 139.05 | 34.71 | 3.42 | 143.97 | 36.69 |
| | Flask 2 | 84 | 3.13 | 98.12 | 29.91 | 3.33 | 102.34 | 29.86 | 149 | 3.42 | 142.35 | 35.73 | 3.27 | 136.5 | 33.9 |
| | Flask 3 | 79 | 3.36 | 99.97 | 21.6 | 3.19 | 94.28 | 21.25 | 149 | 3.18 | 129.06 | 33.27 | 2.97 | 122.7 | 32.4 |
| | Flask 4 | 79 | 2.83 | 84.71 | 25.75 | 3.42 | 95.99 | 28.13 | 152 | 3.63 | 148.53 | 38.61 | 3.33 | 138.15 | 35.25 |
| S=27 | Flask 1 | 74 | 3.08 | 93.3 | 27.7 | 3.24 | 100.71 | 29.24 | 193 | 3.87 | 151.71 | 33.63 | 3.6 | 163.8 | 35.22 |
| | Flask 2 | 77 | 2.3 | 79.52 | 24.12 | 3.16 | 94.54 | 28.64 | 237 | 3.69 | 173.64 | 33.66 | 3.99 | 170.7 | 34.29 |
| | Flask 3 | 83 | 3.02 | 102.24 | 30.47 | 3.36 | 102.08 | 31.22 | 226 | 3.45 | 154.92 | 30.96 | 3.36 | 161.73 | 32.58 |
| | Flask 4 | 75 | 3.03 | 95.76 | 25.3 | 3.18 | 90.73 | 25.34 | 204 | 3.9 | 157.41 | 33.69 | 3.96 | 151.65 | 30.93 |
| pH=7.5 | Flask 1 | 65 | 2.8 | 88.72 | 25.9 | 2.88 | 91.08 | 29.08 | 201 | 3.54 | 149.94 | 35.01 | 3.63 | 146.55 | 32.34 |
| | Flask 2 | 77 | 2.64 | 92.16 | 26.36 | 3.16 | 99.36 | 27.4 | 180 | 3.45 | 143.19 | 31.65 | 3.57 | 144.96 | 31.56 |
| | Flask 3 | 86 | 2.84 | 86.74 | 24.28 | 2.88 | 93.78 | 28.38 | 167 | 3.63 | 150.57 | 32.67 | 3.21 | 139.95 | 30.24 |
| | Flask 4 | 82 | 2.6 | 88.66 | 28.68 | 3.26 | 105.72 | 29.7 | 182 | 5.08 | 165.28 | 28.88 | 0.08 | 156.4 | 29.32 |
| pH=9.0 | Flask 1 | 77 | 3.04 | 95.26 | 28.46 | 3.18 | 95.08 | 27.34 | 224 | 3.45 | 173.4 | 42.03 | 3.63 | 156.9 | 39.72 |
| | Flask 2 | 68 | 3 | 94.1 | 25.86 | 3 | 91.56 | 26.96 | 243 | 4.05 | 187.32 | 45.42 | 3.6 | 189.69 | 46.98 |
| | Flask 3 | 71 | 3 | 88.4 | 26.94 | 3 | 101.72 | 28.4 | 228 | 3.39 | 178.98 | 41.13 | 3.09 | 170.46 | 39.84 |
| | Flask 4 | 72 | 3 | 84.36 | 24.24 | 3 | 88.48 | 24.72 | 217 | 3.6 | 168.84 | 40.2 | 3.63 | 172.02 | 43.14 |

* Cell counts are per 0.3 mL of culture

* Toxin data resulted from filtration of 10 mL of culture and extraction of those filters into 2 mL