ABSTRACT

HARDISON, DONNIE RANSOM. Environmental Controls on the Toxicity of *Karenia brevis*. (Under the direction of Dr. Damian Shea).

Nearly annual blooms of the toxic dinoflagellate *Karenia brevis* have adversely impacted coastal ecology, human health, and local economies in coastal regions of the Gulf of Mexico. In experiments with several strains of this species, cells increased their toxin content in response to growth limitation by nutrients (nitrogen, phosphorus, and carbon dioxide) in accordance with the carbon:nutrient balance (CNB) hypothesis, originally formulated for terrestrial plants. The CNB states that as algae or plants slow in growth due to nutrient limitation, they will divert a greater fraction of their fixed carbon to defenses, such as toxins. This carbon diversion provides protection from overreduction of the photosynthetic apparatus and decreases grazing mortality rates as growth slows. A corollary to the CNB hypothesis is if the limiting nutrient element is contained in the toxin, then the toxin increase will also be limited. This was observed for the effect of CO₂ limitation on cellular brevetoxins which contain 67% C by weight. Under CO₂ limitation, increase in cellular brevetoxins was on average 45% less than observed under P limitation. Field measurements of the toxin content in *Karenia brevis* cells range from 1-68 pg cell⁻¹, with average values of 8-25 pg cell⁻¹. The toxin per cell values in the N- and P-limitation experiments account for the full range of brevetoxin values observed in the field, with these variations being related to the effects of nutrient limitation and interstrain differences.

Carbon diverted into the production of brevetoxins was documented in each study by presenting toxin values as a percent of total cellular carbon (%C-PbTx). This normalization of brevetoxins is preferred over the commonly used per cell or per cell volume
normalizations as these latter values can be affected by changes in cell size or cell carbon:volume ratios and are misleading. Under nutrient limitation, cell volume will increase or decrease depending on the nutrient thus affecting their toxin quotas. Such effects were observed under N- and P-limitation where P-limitation caused a 2-fold greater increase in brevetoxins per cell than N-limitation. However, when these toxins were normalized on a per cell volume basis, the percent toxin increase was similar, and the entire difference in toxin per cell was caused by a two-fold higher volume per cell in the P-limited cultures relative to the N-limited ones.

The results of these studies will benefit coastal managers, who initially judge the toxicity of *Karenia* blooms from cell count data alone. Our data show the toxin concentrations associated with a given cell can vary by up eight-fold depending on the genetic strain and whether or not the algal growth is limited by N, P or CO₂. For a given cell count P-limited cells should have higher toxin contents, and it is useful to know not only whether cell growth is nutrient-limited but also the nutrient element (N, P, or C), that is limiting. Nutrient status indicators thus need to be developed for better prediction of bloom toxicity by coastal managers. These could include simple pH measurements for predicting CO₂ limitation, or N:C or P:C of *Karenia* cells for assessing N- or P-limitation. Also, our data indicate that ongoing anthropogenic increases in atmospheric CO₂ may increase the toxicity of *Karenia brevis* blooms by increasing the CO₂ needed to support bloom growth and by increasing the likelihood that high biomass blooms will become limited by N or P rather than by CO₂. Using the data from this study, coastal managers should be able to make better predictions of the toxicity of *Karenia* blooms, allowing them to minimize the adverse effects of these blooms on human and ecosystem health and coastal communities.
Environmental Controls of Brevetoxin Production of the Dinoflagellate Karenia brevis

by
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A dissertation submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the degree of Doctorate of Philosophy

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DEDICATION

In memory of my father.
BIOGRAPHY

I was born and raised in a small farm town called Teachey, NC. I grew up as a farm hand until it was time to go to college. Of course, I had decided to not go to college as I wanted to be a farmer like my dad. My dad forced me to go to college, and so I chose the school closest to home which was the University of North Carolina at Wilmington. I graduated with a double major in Chemistry and Environmental Studies in 2000. Then I went on to attend graduate school to obtain my Master’s in Chemistry at the University of North Carolina at Wilmington, graduating in 2002. I was lucky enough upon graduation to meet Dr. Bill Sunda who convinced me that working for the NOAA would be a good career choice. Dr. Sunda has introduced me to a new world in phytoplankton and biology, as there is always something new to discover. Reflecting back, I am ever grateful that my dad forced me to go to college, as that was probably the best decision he ever made for me.
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INTRODUCTION

Karenia brevis is a highly toxic bloom forming dinoflagellate. Karenia blooms occur globally in coastal waters of the United States, New Zealand, Tasmania, Ireland, Japan, South Africa, Chile, and countries bordering the Mediterranean Sea (Brand et al. 2012). These blooms occur on a near annual basis in the region extending from the West Florida Shelf to the coast of Texas, USA. Karenia brevis produces at least 10 different congeners of the lipid soluble neurotoxins known as brevetoxins (PbTxs). These toxins activate voltage-sensitive sodium channels, which interfere with normal nerve transmission. Both the direct release of brevetoxins and their transfer through the food web can severely impact humans and many marine organisms (Tester et al. 2000, Naar et al. 2007). PbTxs have been shown to cause neurotoxic shellfish poisoning and respiratory distress in humans (Watkins et al. 2008). They have also caused massive fish kills and mortalities of marine mammals, sea turtles and sea birds (Landsberg et al. 2009, Fire & Van Dolah 2012, van Deventer et al. 2012). These effects in turn have caused severe ecosystem disruption and significant economic losses in affected coastal communities (Flewelling et al. 2005, Hoagland et al. 2009). Many of these adverse effects can be long lasting. Flewelling et al. (2005) showed that fish and animals were adversely impacted by PbTxs accumulated within seagrass epiphytes, and that these effects could occur weeks to months after a the bloom was no longer present (Hitchcock et al. 2012). Additional studies showed that PbTxs can persist in marine sediments for months to years confirming their prolonged stability in the environment (Mendoza et al. 2008). The
long-term accumulation of PbTxs in sediments may result in trophic transfer of toxins from benthic to pelagic communities.

*Karenia* blooms are often associated with wind driven currents that bring nutrients to the surface and concentrate cells along coastal fronts allowing development of high density blooms (Janowitz & Kamykowski 2006, Stumpf et al. 2008). Stumpf et al. (2008) were able to demonstrate that a combination of the geography of the west Florida shelf and the direction of winds responsible for downwelling and upwelling current determined the intensity of *Karenia brevis* blooms. Several other studies have cited nutrient inputs from groundwater and agricultural run-off, as well as fixed nitrogen inputs from *Trichodesmium* blooms as crucial drivers of bloom formation off the west coast of Florida (Walsh & Steidinger 2001, Hu et al. 2006, Walsh et al. 2006, Vargo et al. 2008). These alternative explanations have led to controversies concerning the relative importance of various factors in the development of high biomass *Karenia brevis* blooms. In reality, it is likely that multiple environmental factors are involved in bloom development, but that hydrodynamic concentration is always involved to some extent (Janowitz & Kamykowski, 2006; Stumpf et al., 2008).

Although the biology and ecology of *Karenia brevis* has been studied intensively for several decades, the effect of environmental factors on toxin production remains largely unknown. The reported range in total brevetoxin concentration per cell from field samples varies considerably from 1 to 68 pg·cell⁻¹, with average estimates typically varying between 6 and 26 pg·cell⁻¹ (Table 1.1). It is known from other studies of other toxin producing
dinoflagellates that nutrient limitation typically increases algal toxin content per cell. For example, hemolytic activity in the prymnesiophytes *Prymnesium parvum* and *Chrysochromulina polylepis* increases by up to 10-fold when growth is limited by nitrogen or phosphorus (Johansson & Graneli 1999b, Johansson & Graneli 1999a). Similarly, silica and phosphorus limitation increases cellular domoic acid concentrations in the diatom *Pseudo-nitzschia multiseries* (Pan & Rao 1996, Pan et al. 1998), and phosphorus limitation increases karlotoxin levels in the dinoflagellate *Karlodinium veneficum* (Adolf et al. 2009, Fu et al. 2010). Phosphorus limitation also increases intracellular concentrations of the phosphatase inhibitor nodularin in the cyanobacterium *Nodularia spumigena* (Sunda et al. 2006). The only exceptions to the relationship between nutrient limitation and increases in cellular toxins occur when the limiting nutrient is also a constituent of the toxin. Despite these previous findings with other toxic algae, the effect of nutrient limitation of growth rate in *K. brevis* has not yet been rigorously tested.

The underlying physiological basis for observed relationship between growth limitation by nutrients and toxin content was first determined in terrestrial plants. Numerous studies have shown that as plants became nutrient limited, they often increased their carbon investment in defenses against herbivory such as thorns, thick cuticles, toxic alkaloids, and compounds (e.g., tannins) that interfere with digestive processes (Lambers et al. 2008). Investing fixed carbon in this manner under low resource conditions helps reduce grazing by herbivores, thereby promoting increased survival during periods of low rates of growth and reproduction (Coley et al. 1985). In addition, there is often an increased production of toxic
compounds that adversely affect the growth of other plants (alleopathy), which decreases competition for scarce nutrients or other plant resources. This diversion of carbon into carbon-based, toxic secondary metabolites in response to reduced nutrient availability is commonly described as the carbon:nutrient balance (CNB) hypothesis (Bryant et al. 1983, Stamp 2003). Though generally applicable, there are some corollaries to the CNB hypothesis (Hamilton et al. 2001, Paul & Puglisi 2004). One is the optimal defense theory which describes how plants tissues most susceptible to herbivore grazing (leaves and reproductive parts) will have the highest defenses (Zangerl & Rutledge 1996). In this case, rather than generally investing in overall defense mechanisms, the plant may shift defense resources to leaves which are more readily accessed by the primary grazers. Given the structural complexity of terrestrial plants, up to twelve different corollaries to the CNB hypotheses have been developed to address slight differences in plant defense strategies (Berenbaum 1995).

The CNB hypothesis has recently been applied to phytoplankton, to explain the increased levels of toxins and other defenses such as thicker cell walls observed under nutrient limitation of growth rate (Ianora et al. 2006). Because phytoplankton generally consist of single cells or cell colonies and are thus much less complex than terrestrial plants, many of the CNB corollaries mentioned above do not apply to these organisms. However there is one CNB corollary that should apply to both: namely that if the toxin or defensive structure contains the growth limiting nutrient element (e.g. N or C), the resulting increase in grazing defenses will be more limited. Thus for example, *Alexandrium tamarense* and
*Alexandrium minutum* fail to show an increase in nitrogen containing saxitoxins under nitrogen limitation, but toxin concentrations increase dramatically under P-limitation due to the lack of phosphorus in the saxitoxin structure (Granéli & Flynn 2006). Such a restriction of toxin production should also apply to CO₂ limitation of *Karenia brevis* since brevetoxins contain 67% C by weight. However, since the toxins contain no N or P a restriction of toxin production would not apply to nitrogen or phosphorus limitation of growth rate.

The goal of this dissertation was to investigate the effect of environmentally important growth limiting nutrients, N-, P-, and CO₂ on brevetoxin production in several strains of *Karenia brevis*. A series of semi-continuous batch culture experiments were designed to impose growth-limitations by restricting the supply of available nutrients (N, P, and CO₂).

Based on previous observations in terrestrial plants and phytoplankton, N and P limitation was predicted to increase cellular toxin concentrations, despite a recent publication indicating otherwise (Lekan & Tomas 2010). Conversely, CO₂ limitation associated with high biomass blooms was hypothesized to severely limit increases in cellular toxins as C is the primary structural element in PbTxs. PbTx 1, 2 and 3 congeners; chlorophyll *a*; cellular C, N and P; specific growth rate, and cell concentrations and mean volume per cell were all measured. This allowed the comparison of how toxicity per cell, per biovolume and per unit of cell carbon biomass change with nutrient limitation. Few studies have previously collected the data needed to simultaneously normalize changes in toxin production on a per cell, per biovolume and per carbon basis. Normalizing observed changes in cellular PbTxs on a carbon basis allowed determination of how much of the total cellular fixed carbon was being
diverted to toxin production under each growth limiting condition. The resulting data were evaluated to determine if the CNB hypothesis held true for *Karenia brevis* as has been previously observed in other dinoflagellates.
CHAPTER 1

Nitrogen Limitation Increases Brevetoxins in *Karenia brevis*: Implications for Bloom Toxicity

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ABSTRACT

Laboratory and field measurements of the toxin content in *Karenia brevis* cells vary by >4-fold. These differences have been largely attributed to genotypic variations in toxin production among strains. We hypothesized that nutrient limitation of growth rate is equally or more important in controlling the toxicity of *Karenia brevis*, as has been documented for other toxic algae. To test this hypothesis, we measured cellular growth rate, chlorophyll *a*, cellular carbon and nitrogen, cell volume, and brevetoxins in four strains of *Karenia brevis* grown in nutrient-replete and nitrogen (N)-limited semi-continuous cultures. N-limitation resulted in reductions of chlorophyll *a*, growth rate, volume per cell and N:C ratios as well as a two-fold increase (1-4% to 5-9%) in the percentage of cellular carbon present as brevetoxins. The increase in cellular brevetoxin concentrations was consistent among genetically distinct strains. Normalizing brevetoxins to cellular volume instead of per cell eliminated much of the commonly reported toxin variability among strains. These results suggest that genetically linked differences in cellular volume may affect the toxin content of *Karenia brevis* cells as much or more than innate genotypic differences in cellular toxin content per unit of biomass. Our data suggest at least some of the >4-fold difference in toxicity per cell reported from field studies can be explained by limitation by nitrogen or other nutrients and by differences in cell size. The observed increase in brevetoxins in nitrogen limited cells is consistent with the carbon:nutrient balance hypothesis for increases in toxins and other plant defenses under nutrient limitation.
INTRODUCTION

B Blooms of the toxic dinoflagellate *Karenia brevis* adversely affect human and ecosystem health in the Gulf of Mexico. The most impacted region is the west coast of Florida, which experiences high biomass blooms on a near annual basis (Tester & Steidinger 1997). *Karenia brevis* is of concern because it produces at least ten structurally related neurotoxins, known as brevetoxins (PbTxs). These toxins activate voltage-sensitive sodium channels, which interferes with normal nerve transmission. When PbTxs are released into the environment, organisms from copepods to marine mammals are adversely affected (Asai et al. 1984, Purkerson et al. 1999, Cohen et al. 2007). During blooms, shellfish feeding on *K. brevis* can accumulate sufficient levels of brevetoxins to cause neurotoxic shellfish poisoning (NSP). NSP symptoms can include gastrointestinal distress, nausea, vomiting, dizziness, slurred speech, numbness of lips, mouth and tongue, and respiratory distress (Watkins et al. 2008, Heil 2009). Consequently, harvesting of shellfish beds is closed to protect human health when *K. brevis* concentrations exceed 5,000 cells·L⁻¹. Human exposure also occurs when *K. brevis* cells are disrupted in the surf zone and onshore winds transport toxic aerosols over land. These aerosols cause respiratory distress and illness in beach-goers and coastal inhabitants resulting in increased medical costs (Pierce et al. 2005, Hoagland et al. 2009, Morgan et al. 2009). Large-scale blooms also cause massive fish kills and the death of bottlenose dolphins and endangered manatees (Fire et al. 2007, Landsberg et al. 2009). The adverse environmental and health effects of *K. brevis* blooms, and the associated negative publicity, have resulted in significant loss of tourism revenues (Morgan et al. 2009). *Karenia*
*brevis* blooms are estimated to account for approximately one third of the $94 million (USD) in total annual economic losses attributable to HABs in the United States (Steidinger et al. 1999). Understanding and predicting the factors that control *K. brevis* blooms and their toxicity is therefore of great regional importance.

To date, most of our knowledge concerning the toxicity of *Karenia* blooms comes from a limited number of laboratory and environmental studies. The reported range in total brevetoxin concentration per cell varies from 1 to 68 pg·cell$^{-1}$, with average estimates typically varying between 6 and 26 pg·cell$^{-1}$ (Table 1.1). The extent to which this variation in toxin per cell is dependent on environmental factors versus inherent genetic differences among strains is controversial. Studies on other toxic algal species indicate that much of the observed variation in toxin content can be attributed to differences in the growth status of the cells, as regulated by macro-nutrient (N, P or Si) limitation or other environmental factors. For example, hemolytic activity in the prymnesiophytes *Prymnesium parvum* and *Chrysochromulina polylepis* increases by up to 10-fold under nitrogen or phosphorus limitation of growth (Johansson & Graneli 1999b, Johansson & Graneli 1999a). Similarly, silica and phosphorus limitation increases cellular domoic acid concentrations in the diatom *Pseudo-nitzschia multiseries* (Pan & Rao 1996, Pan et al. 1998), and phosphorus limitation elevates karlotoxin levels in the dinoflagellate *Karlodinium veneficum* (Adolf et al. 2009, Fu et al. 2010). Phosphorus limitation also increases intracellular concentrations of the phosphatase inhibitor nodularin in the cyanobacterium *Nodularia spumigena* (Sunda et al. 2006). In each case, nutrient limitation of growth increased cellular toxin content.
The physiological basis for a relationship between growth status and toxin content was first examined in terrestrial plants. Numerous studies have shown that as plants became nutrient limited, they often increased their carbon investment in defenses against herbivory such as thorns, thick cuticles, toxic alkaloids, and compounds (e.g., tannins) that interfere with digestive processes (Lambers et al. 2008). Investing fixed carbon in this manner under low resource conditions helps reduce grazing by herbivores, thereby promoting survival (Coley et al. 1985). In addition, there is often an increased production of toxic compounds that adversely affect the growth of other plants (alleopathy), which decreases competition for scarce nutrients or other plant resources. This diversion of carbon into carbon-based, toxic secondary metabolites in response to reduced nutrient availability is commonly described as the carbon:nutrient balance hypothesis (CNB) (Bryant et al. 1983, Stamp 2003). The CNB hypothesis was originally applied to terrestrial plants, and there is some controversy regarding its general applicability (Hamilton et al. 2001, Stamp 2003b, Paul and Puglisi 2004). However, it has recently been used successfully to explain increased toxin levels with nutrient limitation of growth rate in unicellular algae (Ianora et al. 2006).

We hypothesized that the CNB hypothesis also applies to *K. brevis*, and thereby promotes red tide blooms through relaxed grazing pressure and associated positive feedback interactions (Mitra & Flynn 2006, Sunda et al. 2006). The current understanding is that blooms primarily initiate when winds favorable for upwelling hydrodynamically concentrate nutrient-replete *Karenia* cells in near shore regions (Janowitz & Kamykowski 2006, Janowitz et al. 2008, Stumpf et al. 2008). As these cells grow, they frequently reach high densities in
excess of 1,000,000 cells·L⁻¹. During this process the blooms become progressively more nutrient-limited (Vargo et al. 2008, Vargo 2009). What remains controversial in this process is the relative importance of different nutrient sources in sustaining these blooms and at what point the blooms become nutrient-limited (Stumpf et al. 2008, Vargo et al. 2008). If *K. brevis* conforms to the CNB hypothesis, the brevetoxin content per cell should increase as a bloom becomes progressively more nutrient-limited. Increased toxin content of cells may result in positive feedback mechanisms via allelopathy and deterrence of grazers (Sunda et al. 2006). These mechanisms further increase bloom growth and decrease nutrient inputs from recycling due to reduced grazing, thereby further increasing cellular toxin levels. This prediction is in opposition to current thinking, which holds that nutrient-limitation has no effect on cellular brevetoxin concentrations and that changes in toxin content are likely attributable to genetic/strain variability (Baden & Tomas 1988, Lekan & Tomas 2010).

This study examined whether cellular brevetoxin concentrations increased with nitrogen limitation of growth rate in accordance with the carbon:nutrient balance hypothesis (CNB). To test this hypothesis *K. brevis* cells were grown in N-limited and N-replete long-term semi-continuous cultures and were carefully measured for total brevetoxins, total cellular carbon and nitrogen (where possible), mean volume per cell, cell densities, and chlorophyll *a* (chl *a*). Four separate strains of *K. brevis* from varying geographic locations were used in an effort to obtain results from genetically different strains. Because cells became significantly smaller under nutrient limitation, all results were normalized on a per cell volume as well as per cell basis.
MATERIALS and METHODS

Strains and culture conditions. The effect of nitrogen limitation on the production of individual brevetoxin congeners as well as total brevetoxin concentrations was studied in cultures of four different strains of Karenia brevis. Clone CCMP 2228 was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME, USA); clones SP2 and SP3 were obtained from Dr. Ed Buskey of University of Texas Marine Science Institute (Port Aransas, TX, USA); and the Wilson clone (CCFWC268) was acquired from the Fish and Wildlife Research Institute (St. Petersburg, FL, USA). Cells were cultured in an incubator (model I-36VLX, Percival Scientific, Boone, IA, USA) maintained at a constant temperature of 23°C and a 14h:10h daily light:dark cycle to simulate summer light conditions. Photosynthetically active radiation (PAR) was provided at an intensity of 120 µmol photons·m⁻²·s⁻¹ via vertically mounted fluorescent lamps (Vitalite bulbs, Duro-test Inc, Philadelphia, PA, USA). PAR intensity was measured with a Biospherical Instruments Inc. QSL -100 4π wand type light meter.

Media consisted of 1.0 L of 0.2 µm filtered Gulf Stream seawater (salinity 36) held in 2.5 L polycarbonate bottles. The media contained added vitamins (0.074 nM vitamin B₁₂, 0.4 nM biotin, and 60 nM thiamine), 10 nM Na₂SeO₃, and an EDTA-trace metal buffer system as described by Sunda et al. (2005), (100 µM EDTA, 1 µM FeEDTA, 50 nM MnCl, 100 nM ZnSO₄, 40 nM CuCl₂, and 40 nM CoCl₂). Nutrient replete culture media contained 64 µM NaNO₃ and 4 µM NaH₂PO₄ (N:P = 16:1, the Redfield ratio) while nitrate limited media contained 12 µM NaNO₃ and 4 µM NaH₂PO₄ (N:P = 3:1). Media were sterilized by
microwave treatment (Keller et al. 1988). Culture pH was monitored closely both initially and throughout experiment with a Thermo Orion 3 Star pH meter equipped with a Ross ultra combination pH electrode to ensure no carbon dioxide limitation occurred. Culture pH ranged from 8.10 to 8.35 for N-limited cultures and 8.10 to 8.30 for N-replete cultures.

*Karenia brevis* cells in our experiments were grown in semi-continuous batch cultures. Experimental cells in both high and low nitrate media were inoculated from acclimated nutrient-sufficient cultures that had been previously growing exponentially for several weeks at their maximum rates. The N-limited cultures were diluted at an average rate of 0.1 d\(^{-1}\) with fresh low-nitrate medium to obtain a continuous N-limited growth rate. Nutrient sufficient cultures growing at their maximum rates were diluted sequentially with nitrate-replete media well before they reached their maximum cell density to ensure no nutrient limitation of growth rate occurred. Specific growth rates and associated standard errors were calculated by linear regressions of the natural log of biovolume (µL\(_{\text{cells}}\) L\(_{\text{media}}\)^{-1}) versus time after correcting for serial culture dilutions (Sunda et al. 2007).

*Cell growth, chlorophyll a, nitrogen:carbon (N:C), and brevetoxin measurements.* At the middle of the light period (midday) culture aliquots were taken for measurement of cell density, mean volume per cell, chl \(a\), cellular carbon and nitrogen (CCMP 2228 and Wilson only), and brevetoxins every 2-3 days for N-limited cultures and as often as cell density allowed for N-replete cultures. At each sampling period triplicate subsamples were taken for measurement of chl \(a\), brevetoxins and cell numbers / cell volume. Due to volume restrictions, only duplicate subsamples were taken for cellular carbon and nitrogen. Results
were expressed as mean values ± standard deviation unless otherwise noted. Cell concentrations and mean volume per cell were measured with a Multisizer 3 electronic particle counter (0.5-mL sample volume) equipped with a 100-µm aperture (Beckman Coulter Inc., Brea, CA, USA). Ellipsoid and prolate spheroid equations for *Karenia* species were used to estimate cell volumes of the Wilson clone using published length, width, and thickness measurements (Haywood et al. 2004). The average volumes per cell calculated using these equations were 5,413 µm³ and 5,426 µm³, respectively. These calculated cell volumes agree with the average volume per cell of 5,643 µm³ obtained with the Coulter Counter, confirming the accuracy of cell volume measurements obtained with this method.

Cell growth curves were constructed as semi-log plots of cell concentration (cells·mL⁻¹) or total biovolume (µLcells·L⁻¹media) versus time in days. Total biovolume was calculated by multiplying cells·mL⁻¹ by volume per cell (µm³·cell⁻¹) and converting to µLcells·L⁻¹media.

Specific growth rates were computed from linear regressions of the natural log of total cell biovolume versus time after correcting for culture dilution (Sunda et al. 2007).

Chl *a* samples were processed by gentle vacuum filtration (<2.5 cm Hg) of the cells onto 25-mm GF/F filters (Whatman, Florham Park, NJ, USA) and then extracting the chl *a* with 90% acetone. The fluorescence of the extracted chl *a* was measured with a fluorometer (Turner Designs Inc., Sunnyvale, CA, USA) (Welschmeyer 1994). Due to the delicacy of *Karenia* cells, filtration efficiency was examined by measuring chl *a* recovery from culture aliquots of whole cells versus equivalent aliquots where the cells had been completely disrupted by sonication. The amount of chl *a* recovered from the disrupted cells was >98%
of that obtained from whole cells, indicating that loss of chl \(a\) due to gentle filtration was negligible. Cellular carbon and nitrogen were determined by gentle vacuum filtration (<2.5 cm Hg) of cells onto precombusted 13 mm GF/F glass fiber filters (Pall Corp., Ann Arbor, MI, USA) followed by fuming with HCl overnight to remove inorganic carbon (Liu et al. 2001). These filter samples were then analyzed for cellular nitrogen and carbon with an elemental analyzer (EAS 4010, Costech Analytical Technologies Inc., Valencia, CA, USA). The <2.5 cm Hg vacuum pressure used was the minimum needed to filter cells onto the 13 mm filters. Filtration efficiency of cellular N and C was evaluated by comparing the values obtained when equivalent aliquots of cells were either gravity filtered or filtered using the gentle filtration procedure described previously. The difference between the two methods was 9-10%. Cellular carbon values, used for toxin normalization, were adjusted to account for 10% filtration induced loss to provide a conservative estimate of brevetoxin (determined from whole water extraction) per unit carbon. Since the loss of cellular N and C was equivalent, their ratios were not affected by the filtration procedure.

Brevetoxins were extracted using liquid/liquid separations with ethyl acetate. Prior to separations, aliquots of cell cultures were mixed 1:1 by volume with ethyl acetate and the mixture was sonicated for 3 minutes with a microtip equipped Branson Sonifier 250. Complete cell disruption was confirmed by microscopy. Collected ethyl acetate fractions were desalted with Milli-Q water and concentrated with a rotovap. Extraction efficiency was determined in every fraction by the addition of an internal standard and typically ranged from 90-95% (Lekan & Tomas 2010). The internal standard (PbTx-42-acetate) was a synthetic
PbTx-3 methyl-acetate standard which was similar in structure and fragmentation to the other PbTx congeners (Errera et al. 2010). Concentrated fractions were measured for brevetoxins using an Agilent 1100 LC coupled to a Thermo-Finnigan TSQ Quantum triple quadrupole mass spectrometer with an electro spray ion source interface. LC-MS-MS conditions have been previously described in detail (Cheng et al. 2005, Mendoza et al. 2008). Briefly, a reverse phase (C18) Phenomenex, LUNA, 3 μm pore size, 2.0 × 50 mm column was employed with a gradient mobile phase of 50:50 acetonitrile:water (0.3% acetic acid) for the first 40 min, then changed to 5:95 acetonitrile:water for 2 min and finally back to 50:50 acetonitrile: water for 8 min. The flow rate was set at 0.2 mL·min\(^{-1}\). Nitrogen gas was used as both the drying gas and nebulizing gas. Structural information was determined in selective reaction monitoring (SRM) mode matching product ions and parent mass from standards to extracted brevetoxins. Optimized collision induced decomposition MS/MS spectra for each of the PbTx transitions were acquired at 12 V (PbTx-1), 4 V (PbTx-2), 18 V (PbTx-3), and 20 V (PbTx-42-acetate) using argon as the collision gas at a pressure of 1.0 mTorr. The SRM transitions of brevetoxins and internal standard were set at 867.5 m/z to 849.5 m/z (PbTx-1), 895.5 m/z to 877.5 m/z (PbTx-2), 897.5 m/z to 725.3 m/z (PbTx-3), and 939.5 m/z to 725.3 m/z (PbTx-42-acetate). Scan events of product ions were set at a scan width of 1.0 s with scan time of 0.25 s. Q1 and Q3 peak widths were set at 0.70 s. Total acquired segment time ran for 40 min. An external standard curve of purified brevetoxins 1, 2, and 3 (World Ocean Solutions, Wilmington, NC, USA) was used to quantify amounts of
extracted brevetoxins. Cell culture extracts were analyzed in triplicate and the concentrations are reported as mean values.

The above analytical procedure yielded total toxin in the culture, which provides a measure of cellular brevetoxins. Our preliminary experiments showed >90% of culture toxins were intracellular, consistent with previous findings (Tester et al. 2008, Lekan & Tomas 2010). PbTx 1 and 2 are found to be localized intracellularly while PbTx 3 is known to be an extracellular breakdown derivative of PbTx 2 (Roszell et al. 1989, Pierce et al. 2001). The conclusion that toxins were largely intracellular was supported by the low percentage (< 6%) of total brevetoxins present as PbTx-3 in this study.

**Batch Culture Experiment.** Batch culture methodology is commonly used to study the effects of nutrient limitation on algae. We conducted a nitrogen-limitation batch culture experiment with strain CCMP 2228 for comparison with results from our semi-continuous culture studies. The experimental culture was initiated by inoculating acclimated nitrogen replete cells growing at a rate of 0.44 d⁻¹ into 1L of the same low nitrate medium used for the N-limited semi-continuous culture experiments. The cells were then allowed to grow for 12 days through the exponential growth phase and the nitrogen-limited stationary phase with no culture dilution. Sample aliquots were taken during the middle and end of the exponential growth phase (days 4 and 7) and during the stationary phase (day 12) for measurement of cell nitrogen and carbon, cell counts and volume, and total brevetoxins as described above.
RESULTS

Growth rates. Nitrogen-replete maximum growth rates among the four Karenia strains varied from 0.25±0.008 d⁻¹ to 0.44±0.005 d⁻¹, with CCMP 2228 exhibiting the highest rate and strain SP3 the lowest (Table 1.2). Both high and low nitrate cultures had approximately the same specific growth rates for the first ~ 8-10 days of culture growth prior to the onset of N-limitation in the low (12 µM) NaNO₃ treatment on days 10-12 (Figs. 1.1-1.4A). After day 12, the growth of the low nitrate cultures was limited by nitrogen. The cultures were then resupplied by fresh medium addition at an average dilution rate of 0.1 d⁻¹. Measured specific growth rates of the N-limited, semi-continuous cultures ranged from 0.079±0.003d⁻¹ for strain SP2 to 0.095±0.005d⁻¹ for strain Wilson. N-limited growth rates for all four strains were 2.9- to 4.6-fold lower than rates for N-sufficient strains (Table 1.2; Figs. 1.1-1.4B).

Cell density and volume per cell. Cell density for each experiment ranged from 300 to 7,000 cells·mL⁻¹. These cell concentrations are low compared to those typically associated with cultures grown in f/2 or L1 media. We maintained lower densities in our cultures to minimize pH increases and associated carbon dioxide limitation and to minimize changes in trace metal nutrient availability (Sunda et al. 2005). Mean volume per cell for nutrient replete cultures ranged from 3,370 µm³·cell⁻¹ (CCMP 2228) to 5,643 µm³·cell⁻¹ (Wilson) and remained relatively constant for the duration of experiments (Table 1.2; Figs. 1.1-1.4C). All Karenia brevis strains exhibited a decrease in cell volume with the onset of N-limitation of growth rate (Figs. 1.1-1.4C), as is typically observed with growth limitation by nitrogen.
(Sunda & Hardison 2007). These reductions continued for the duration of the experiments, with some variability occurring in the SP2 strain. Mean decreases in volume per cell were 22%, 31%, 34%, and 35% for strains CCMP 2228, Wilson, SP2 and SP3, respectively.

Chlorophyll a. N-replete cultures of the four strains exhibited mean chlorophyll a (chl a) values of 1.5±0.3 fg·µm⁻³ to 2.3±0.3 fg·µm⁻³ normalized to cell volume and 6.1±2.4 pg·cell⁻¹ to 10.1±1.3 pg·cell⁻¹ on a per cell basis (Table 1.2). Cellular chl a began to decrease with the onset of N-limitation of growth rate and remained lower than N-replete values throughout the experiments (Figs. 1.1-1.2E, 1.3-1.4D). On average, these decreases were 56% for CCMP 2228, 66% for Wilson, 52% for SP2, and 48% for SP3 when expressed on a cell volume basis (Table 1.2). A greater reduction was observed when chl a was expressed on a per cell basis with a 66% decrease for CCMP 2228, 76% for Wilson, 68% for SP2, and 66% for SP3 (Table 1.2).

Cellular carbon and nitrogen. The SP2 and SP3 cultures contained visible associate microorganisms, some of which tightly adhered to the cell surfaces. The CCMP 2228 and Wilson clones may also have contained co-occurring microorganisms, but if so they were too small to be observed by light microscopy. The presence of “symbiotic” or “mutualistic” microorganisms that are essential for cell growth and viability is commonplace in dinoflagellates (Amin et al. 2009) and is commonly observed in Karenia cultures. The SP2 and SP3 associates were present at low levels during log phase growth, but proliferated in the cultures in late log and stationary phases. Filtration captured the associates in the media as well as those attached to the Karenia cells. The C and N contributed by these co-occurring
microorganisms was sufficient to bias the estimates of C and N in the SP2 and SP3 strains. Repeated attempts to remove the associates from SP2 and SP3 cultures failed, perhaps suggesting that they play a critical role in the growth or viability of *Karenia* cells. Since the associates in the SP2 and SP3 cultures could not be separated from the *Karenia* cells, there was no way to accurately assess the amount of N and C in the SP2 and SP3 strains. Hence, cellular C and N estimates were only shown for strains CCMP 2228 and Wilson. The Coulter Counter, however, was clearly able to distinguish between the small associates and much larger *Karenia* cells making it possible to accurately gauge the amount of brevetoxin per cell or per unit of cell volume.

Average cellular nitrogen to carbon (N:C) ratios for strains CCMP 2228 and Wilson in N-replete cultures were nearly identical, 0.13±0.01 mol·mol⁻¹ and 0.14±0.04 mol·mol⁻¹, respectively. Average N:C ratios were lower in N-limited cells (0.079±0.008 mol·mol⁻¹ for CCMP 2228 and 0.061±0.019 mol·mol⁻¹ for Wilson) due to a decrease in cellular N concentrations.

*Brevetoxins.* The dominant brevetoxins (PbTx) detected in three of the strains were PbTx 1, PbTx 2, and PbTx 3. An exception was the Wilson strain which only contained measurable levels of PbTx 2 and PbTx 3. In this study, total brevetoxins equal the sum of the three predominant congeners. For each of the strains tested, PbTx 2 was the major brevetoxin followed by PbTx 1 (when present), and a small amount of PbTx 3 (Figs. 1.5A and C). The relative distribution of the individual congeners was similar in both treatments even though there was a large increase in average total brevetoxin concentration in the N-
limited cells (Figs. 1.5A and C). Also, a small increase in the percentage of total PbTx present as PbTx 3 was observed as SP2 cells became N-limited; however, the percentage of PbTx 3 was small (6%) when compared to the other brevetoxin components (PbTx 1 and 2).

Nitrogen-replete cells growing at maximum rates in the high and low nitrate media near the beginning of the time course experiments exhibited similar total brevetoxin concentrations (Figs.1.1-1.2G, 1.3-1.4E). Nutrient replete controls in high nitrate media maintained similar levels of brevetoxins in all experiments except strain SP3, where brevetoxins actually decreased as the experiment progressed. As the N-replete cells in low nitrate media grew into N-limitation at approximately day 10, their brevetoxin levels on a cellular volume basis (fg·µm$^{-3}$) began to increase. These increases continued for 10 to 15 days until a peak or plateau in brevetoxin levels was reached on days 20-28. Elevated brevetoxins levels continued for the duration of the experiments.

When brevetoxins were normalized to cellular carbon, a similar trend was observed. CCMP 2228 showed an ~2.5-fold increase in the brevetoxin:C ratio from day 10 to 20 (Fig. 1.1F). The peak value at day 20 was equivalent to ~8.5% of the total cellular carbon. This contrasts with the 2-4% values observed in N-replete cells (Fig. 1.1F). After day 20, cellular brevetoxins gradually declined to levels approaching those observed in nutrient-replete control cultures by day 45. The Wilson strain exhibited an ~3-fold increase in the brevetoxin:C ratio starting around day 13 and peaking at day 20 (Fig. 1.2F). As previously observed in CCMP 2228, the cellular brevetoxin in the Wilson strain started to decrease after day 20. The N-replete brevetoxin values for the Wilson strain accounted for 1-2% of cellular
carbon in the maximally growing N-sufficient cells, and increased to a maximum of ~9% on
day 20 in the N-limited treatment, similar to the maximum observed under N-limitation in
strain CCMP 2228 (Fig. 1.2F).

To examine the relationship between cellular N:C ratios and brevetoxin values, cell
brevetoxin concentrations (fg·µm⁻³) and N:C (mol·mol⁻¹) were plotted against each other
(Figs. 1.1-1.2H). Both the CCMP 2228 and Wilson strains showed strong inverse
relationships between cellular brevetoxin levels and N:C ratios. The two strains exhibited
similar low cellular brevetoxin concentrations at N:C ratios of ~0.10 to 0.18, corresponding
with higher growth rates, and exhibited large increases in brevetoxin levels at the lower N:C
ratios associated with lower N-limited growth rates.

Average total cellular brevetoxin levels were normalized per cell and per cellular
volume (Figs. 1.5A and C). When total brevetoxins were expressed on a per cell basis, the
mean brevetoxin cell contents among strains were 48±38% higher in the N-limited cells
compared to those in the N-replete cells. By contrast, when the same changes in toxin
concentrations were normalized to cell volume, the relative increase was much higher
(111±38%) (Table 1.2, Figs. 1.5A and C). This apparent discrepancy is due to the fact that
increases in brevetoxin concentration per unit biovolume were accompanied by decreases in
cell size (Fig. 1.5B). The net result is that toxin per cell increased less than toxin per unit
biovolume (Figs. 1.5A and C). Given that toxin content per unit cell volume was similar
among the four strains under nutrient-sufficient conditions and also similar among the clones
under nutrient-limitation, the differences in total toxin per cell among the isolates was largely related to differences in cell volume.

**Batch culture experiment.** Changes in total brevetoxins, on a per cell and biovolume basis, were also determined in a batch culture experiment with strain CCMP 2228 (Fig. 1.6A) to determine how these changes compared with results obtained with acclimated semi-continuous cultures. The results using both methods were similar (Figs. 1.6B and C). As observed in semi-continuous experiments, batch culture brevetoxin levels were lowest during the middle of the exponential phase of growth when the cells were growing at their nutrient sufficient maximum rate. Cellular brevetoxins then increased as the cells transitioned into the stationary culture phase where growth was halted due to the onset of N-limitation (Fig. 1.6C). This increase continued during the N-limited stationary phase of growth. The transition period between the exponential and stationary growth phases is indicated by the vertical dashed lines in each plot. As in the semi-continuous culture experiments, the large increase in cellular brevetoxins (2.6-fold per unit of cell volume and 1.9-fold per cell), was accompanied by a decrease in the cellular N:C ratio (39%) and a decrease in mean volume per cell (28%) (Figs. 1.6B and C). A couple of brevetoxin studies have shown extracellular brevetoxins increase in stationary phase, however, this was attributed to cell death or lysis and is usually indicated by a decrease in PbTx 2 and a concomitant increase in PbTx 3 (Pierce et al. 2001, Roszell et al. 1989). We did not observe any significant cell death or lysis in stationary phase (Fig. 1.6A). Furthermore, the increase in total brevetoxin in
stationary phase was due overwhelmingly to intracellular PbTx 1 and 2 and not to extracellular PbTx 3 (Fig 1.6C).

**DISCUSSION**

*Consistency of results with the carbon:nutrient balance hypothesis.* Toxin concentrations in *Karenia brevis* increased as the growth rate of the cells became N-limited in accordance with the carbon:nutrient balance hypothesis (CNB) (Bryant et al. 1983, Stamp 2003). The CNB hypothesis predicts that as growth slows under limitation by nutrients (e.g., nitrogen and phosphorus), plants divert a greater portion of their fixed carbon from growth to defense, often in the form of increased levels of anti-grazing and allelopathic compounds (e.g., toxins) or physical defenses such as thicker cell walls. Our data for *K. brevis* show these same physiological patterns under nitrogen limitation, and are thus consistent with the CNB hypothesis. Reduced rates of N-assimilation and growth in N-limited *Karenia* cells resulted in excess fixed carbon which was then diverted into production of carbon-rich brevetoxins, which contain no nitrogen. Increasing N-limitation also caused cellular chl *a* to decrease by more than 50% in all strains examined (Figs. 1.1-1.2E, 1.3-1.4D), indicating a down-regulation of photosynthesis. Such a decrease is expected as phytoplankton invariably decrease their photosynthetic capacity and chl *a* under nutrient limitation (Falkowski & Raven 2007a). This allows the cells to achieve a balance between carbon fixation and growth and to prevent an over-reduction of the photosynthetic apparatus (Sakshaug et al. 1989, Sunda et al. 2007). Over-excitation of chlorophyll and over-reduction of the
photosynthetic apparatus give rise to the production of highly toxic reactive oxygen species, such as singlet oxygen, superoxide radicals, peroxides, and hydroxyl radicals, that can damage the cell (Niyogi 1999). To avoid over-reduction of the photosynthetic apparatus during early stages of nutrient limitation, the cells must continue to fix carbon in excess of that needed for reduced growth and thus require a means to ‘dispose’ of the excess fixed carbon. According to the CBNH, the excess carbon not needed for growth under N-limitation is reallocated to physical defenses and nitrogen-free toxic secondary metabolites such as brevetoxins. This carbon reallocation was confirmed in both strains Wilson and CCMP 2228, where carbon investment in brevetoxins increased from 1-4% of total cellular carbon in N-replete cells to a maximum of 9% under N-limitation (Figs. 1.1-1.2F). In all *Karenia brevis* strains investigated, this diversion of carbon was further indicated by the average two-fold increase in brevetoxin content per unit of cell volume (µm$^3$) as the cells became N-limited (Figs. 1.1-1.2G, 1.3-1.4E).

As suggested by the CNB hypothesis, the amount of secondary metabolites and overall defenses above baseline levels are determined by the amount of surplus carbon not needed for growth (Stamp 2003). If this is true, then a lower N:C ratio of *Karenia brevis* would be indicative of increased N-limitation stress and a greater amount of available carbon for brevetoxin production. This is exactly what our data showed with the cells with higher C:N ratios (lower N:C ratios) having higher brevetoxin concentrations (Figs. 1.1-1.2H). Growth rate, chl *a*, N:C ratios, and brevetoxin concentrations were all linked under N-limiting conditions. Brevetoxins appear to be an end product of a nutrient stress relief
mechanism for excess carbon resulting from nutrient limitation, as well as a way to provide increased defense against grazers. Such increases in defenses are especially beneficial during N-limitation of growth, because the decrease in growth rate limits the ability of a population to replace cells lost to grazing by herbivores. *Karenia* fits this pattern as brevetoxins have been shown to reduce grazing by copepods (Turner & Tester 1997, Cohen et al. 2007). This reduced grazing allows proliferation of the *Karenia brevis* cells and also reduces nutrient recycling, which is directly linked to grazing. The lower recycling inputs of nutrients leads to further nutrient limitation, further increases in cell toxicity, and thus even lower grazing rates, a positive feedback situation that can promote the formation of red tide blooms (Sunda et al. 2006).

**Implications of brevetoxin normalization to cell numbers and cell volumes under N-limitation.** An important question addressed in this study was whether differences in cellular brevetoxin levels in field populations of *K. brevis* were largely due to variations in cell physiology linked to variations in nutrient limitation or to inherent differences in cellular toxin concentrations among different genetic strains. Although the sample size examined was small, the results of this study indicate that the answer to this question is dependent on whether toxin content is expressed on a per cell or a per cell volume basis. When the changes in brevetoxin content were expressed per unit of cell volume there was a consistent average 2-fold increase as cells transitioned from N-replete to N-limited growth (Fig. 1.5C). In addition, variability among isolates within each experimental treatment was minimal. In the N-replete treatments, a one-way ANOVA showed no significant difference among
isolates in brevetoxins normalized to cell volume. In the N-limited treatments, toxin per cell volume values were significantly different among isolates, but this difference was due solely to a 2.5-fold increase in strain CCMP 2228, compared to the uniform 2.0-fold increase in the other isolates (df = 3, F = 10.7, p< 0.05).

In contrast, the same experimental results appear quite different when brevetoxin values were normalized per cell (Fig. 1.5A). The four strains all showed increases in brevetoxin per cell under nitrogen limitation, but the relative amount of the increase was less and was highly variable among strains, ranging from 12% for strain SP2 to 100% for strain CCMP 2228. Also, toxin per cell values among strains were significantly different under both N-replete (one-way ANOVA; df = 3, F = 7.85, p<0.05) and N-limited conditions (one-way ANOVA; df = 3, F = 4.03, p<0.05). The source of variability in most cases can be attributed to (a) inherent differences among isolates in average cell size, and (b) differences in the response of cell size to nutrient limitation. The cell size in all isolates declined with nitrogen limitation, but those declines were not uniform with some isolates experiencing more of a size change than others (Table 1.2; Fig. 1.5B). The importance of whether toxin normalization is based on cell number or cell volume is best exemplified by results from N-replete Wilson and CCMP 2228 strains. On a per cell basis the toxin content in the Wilson strain (16.8 pg·cell⁻¹) was 75% higher than that in CCMP 2228 (9.6 pg·cell⁻¹), while on a cell volume basis the toxin levels were virtually the same (3.0 and 2.9 fg·µm⁻³, respectively) (Table 1.2). Overall, our data indicate that some of the previously claimed genetic
differences in toxicity among strains could simply be attributed to inherent differences in their cell volumes.

*Changes in toxicity with growth phase.* The CBNH predicts that plants increase toxin levels in response to a reduction in growth rate under nutrient limitation. Thus, it should not matter in a general sense whether experimental cultures are grown as batch cultures or as semi-continuous cultures in their cellular response to nutrient limitation. This prediction was confirmed by experiments with strain CCMP 2228 conducted under both batch culture and semi-continuous experimental designs (Figs. 1.1A-H, 1.6A-C). In the batch culture experiment brevetoxins increased 2.6-fold on a per volume basis and 1.9-fold on a per cell basis between the nutrient-sufficient exponential growth phase and the N-limited stationary phase, while N:C ratios and cell volumes declined and growth rates decreased to zero. The increase in cellular brevetoxins during the stationary growth phase mimicked that observed in the semi-continuous culture experiment for this strain, further supporting the CNB hypothesis.

*Determination of growth phase in batch cultures.* Most previous experiments examining the effect of nutrient limitation on cellular toxin levels have relied on batch cultures. In these experiments, the determination of whether cells were growing exponentially, or had reached a nutrient-limited “stationary” stage of growth was based on plots of cell concentration versus time. However, plots of cell numbers versus time can provide a poor estimate of the growth phase of the culture, particularly the transition region between exponential and stationary phases of growth (Fig. 1.6A). By contrast, the growth
phase of the culture is more readily apparent when growth curves are based on total biovolume (Fig. 1.6A). The effect of this discrepancy on growth stage estimates and their effect on toxicity is evident in the batch culture experiment conducted in this study. The growth curves based on total biovolume per liter of media indicate a clear transition from exponential to the N-limited stationary phase between days 6 and day 8 (Fig. 1.6A; see region bounded by the vertical dashed lines). Based on the total biovolume data, the growth rate of the culture is clearly severely limited by nitrogen on day 7, which is confirmed by a sharp decrease in volume per cell between days 6 and 7 and a 27% decrease in the cellular N:C ratio from day 1 to day 7 (Fig. 1.6B). Declines in both volume per cell and N:C ratio are consistent with the onset of N-limited growth, as observed in our semi-continuous culture experiments (Table 1.2). Likewise, total brevetoxins per unit of cell volume increased 2-fold by day 7 further supporting N-limitation of growth (Fig. 1.6C). By contrast, the growth curve based on changes in cell numbers indicated that cells were still in the exponential growth phase on day 7 with complete growth limitation thereafter. This cell density “growth” curve, therefore, failed to identify the onset of N-limitation and the point of transition to the stationary phase of growth. The reason for this failure was that growth (i.e., the increase in biomass or biovolume) typically slows before the cellular division rate decreases, resulting in a decrease in cell size (Figs. 1.6A, B). Basing growth rate on changes in cell concentration does not take this decrease in cell size into account, and thus, can provide an inaccurate estimate of the growth phase of the culture.
This discrepancy between these two methods for determining growth has important implications for interpreting results of batch culture experiments, and in knowing whether the algae sampled were nutrient limited or not. It is all too common for researchers to sample batch cultures in very late log phase or even early stationary phase based on cell density growth curves, and conclude that cells are nutrient sufficient. In reality, these cells may already be experiencing nutrient limitation of growth, and toxin levels thought to be representative of exponentially growing cells may actually be elevated levels indicative of nutrient limitation, as seen in our results on day 7 of our batch culture experiment (Fig. 1.6C). It is also often assumed that because cells do not grow during stationary phase their cell composition is fixed. However, this assumption is not confirmed in the present experiment as the cell N:C ratio continued to decline and the brevetoxin to cell volume ratio continued to increase during the stationary phase of growth between days 7 and 12, where there was no apparent cell growth (Figs. 1.6B and C). Thus, the timing of sampling during "stationary" phase can significantly affect toxicity estimates associated with this growth phase. Based on biovolume growth curves, our data indicate that true baseline toxin measurements of nutrient sufficient cultures require measurement of exponentially growing cells at their fully acclimated maximum growth rate before they approach late log or early stationary phase. To ensure that cells become fully acclimated to nutrient sufficient or nutrient limited conditions, we recommend that batch culture experiments be run in a semi-continuous fashion to allow sufficient time for full acclimation to occur (e.g., Figs. 1.1-1.4A, B).
Comparison with previous batch culture results. The increase in toxin content per cell with nutrient limitation in *K. brevis* observed in this study is not consistent with those from a previous culture study, which concluded that nutrient limitation had no effect on cellular brevetoxin levels (Lekan & Tomas 2010). This apparent discrepancy, however, can be largely reconciled by examining the data for the two strains, Wilson and SP3, which were common to both experiments and grown at similar temperatures (20°C versus 23°C) and salinities (35 versus 36). Both studies showed an increase in brevetoxin per cell under nitrogen limitation in strain SP3 (Fig. 1.7). However, although our present study showed an increase in brevetoxin per cell in the Wilson strain under N-limiting conditions, Lekan and Tomas (2010) showed no apparent response, with both nutrient replete and N-limited treatments yielding the same cell brevetoxin levels. This inconsistency may indicate the “nutrient sufficient” culture in the Lekan and Tomas study was actually growth-limited. This conclusion is supported by the low 0.1 d^{-1} growth rate for the nutrient sufficient Wilson strain culture in the the Lekan and Tomas study compared to the nutrient-sufficient growth rate of 0.32 d^{-1} observed in this study. Also, both the N-sufficient and N-limited Wilson cultures reported by Lekan and Tomas (2010) contained ~22 pg·cell^{-1} brevetoxin, which matches the brevetoxin value of 21.6±2.7 pg·cell^{-1} obtained for our N-limited Wilson clone growing at the same rate (0.095 d^{-1}) under similar salinity and temperature conditions (Table 1.2). In contrast, the nutrient-sufficient Wilson strain culture in our study grew at a specific rate of 0.32 d^{-1} and contained 16.8±0.8 pg·cell^{-1} brevetoxin (Table 1.2). These data are all consistent with the “nutrient-sufficient” Wilson strain in the Lekan and Tomas study having been either
(a) growth-limited by some nutrient or (b) not fully acclimated to higher nutrient conditions following cell transfer from the stationary phase culture used to inoculate the experiment. Thus, at comparable specific growth rates, both studies yielded similar brevetoxin per cell values, suggesting that the same mechanism, the CNB hypothesis, was governing cellular toxin levels in both experiments.

Comparison with field data. In our present laboratory study, average total brevetoxin per cell for both nutrient sufficient and nutrient limited experiments ranged from 6.9 to 25.4 pg·cell⁻¹ with a mean for all samples of 16.8±4.4 pg·cell⁻¹. This mean value is similar to those reported in two large-scale field studies: 15.7±11.4 pg·cell⁻¹ (Tester et al. 2008) and 26±11 pg·cell⁻¹ (Pierce et al. 2008) (Table 1.1). The large range of values in the field study of Pierce et al. (2008) reflected differences in cell toxicity between surface waters (8-40 pg·cell⁻¹) and bottom waters (3-14 pg·cell⁻¹) (Table 1.1). Their bottom water values closely match those from the nutrient replete treatments in the present study (6.91 to 16.9 pg·cell⁻¹; Table 1.1), consistent with the often observed higher nutrient concentrations in bottom waters from benthic nutrient regeneration. By contrast, the surface waters, which typically have much lower nutrient concentrations, exhibited higher cellular brevetoxin levels consistent with nutrient limitation. The range in brevetoxin per cell values observed in our N-limited cultures, however, did not reach the upper range of cell brevetoxin values found in field populations. Therefore, other factors, such as limitation by other nutrients (e.g., phosphorus), should be examined to possibly account for the higher upper range in brevetoxins per cell observed in some field studies.
Management considerations for effects of N-limitation on brevetoxin production. Our results indicate that nitrogen limitation increases brevetoxin concentrations in *Karenia brevis* cells. This cellular response suggests that some blooms will be more toxic than others depending on nutrient status and the relative growth phase of the bloom. Our data indicate that as an older bloom grows into nutrient limitation, it has the potential to release two to three times more toxins into the water column for a given amount of cell carbon. These higher toxin levels can increase adverse impacts on ecosystems and their food web dynamics, even long after the bloom has dissipated (Flewelling et al. 2005). Therefore, coastal managers need to be aware of the potential increase in cellular brevetoxins and know what to expect should *Karenia brevis* blooms become nutrient limited. Indeed, our results suggest that at least some of the observed variation in the toxin content of *K. brevis* cells observed previously (Pierce et al. 2008, Tester et al. 2008) may be caused by limitation by nitrogen or other critical nutrients such as phosphorus.

The physiological responses to N-limitation, such as decreases in cellular volume or N:C ratios may serve as early indicators of N-limitation and alert regulators to possible increases in the toxicity of *K. brevis* cells. Flow cytometry technologies, such as the FlowCAM, have been used in the field and shown to provide accurate data for average volume per cell as well as growth phase distinction of *Karenia* cells (Buskey & Hyatt 2006). Thus, the use of flow cytometry technologies for cell size and elemental analyses of N:C ratios in *K. brevis* populations could prove to be important predictive tools for bloom toxicity in addition to microscopic counts of *K. brevis* cells.
CONCLUSIONS

This is the first study to relate the variability in toxin per cell among *Karenia brevis* strains to genetic differences in cellular volumes and to increases in cellular toxin to carbon ratios caused by nitrogen limitation of cell growth. Such nutrient-linked changes in cell toxin content may explain some of the greater than 5-fold variation in brevetoxin per cell observed in field toxin measurements. This increase in cellular brevetoxins appears to be a mechanism that diverts excess cellular fixed carbon away from growth during N-limitation of growth rate. The diversion of this otherwise unneeded carbon into the production of brevetoxins helps defend the cells against grazers such as copepods, thereby minimizing population losses to predation (Cohen et al. 2007). Thus, our results are in direct support of the carbon:nutrient balance hypothesis (CNB) proposed for terrestrial plants (Bryant et al. 1983). Since this study only focused on N-limitation, similar studies are needed to examine other potential limiting nutrients such as phosphorus to further test the CNB hypothesis and perhaps account for the full range of toxin values observed in the field. Our data also demonstrate the importance of normalizing brevetoxins not only to cell numbers but also to cell volume or carbon, to account for the effect of differences in cell size on the brevetoxin content of cells. Based on our results, coastal managers need to be aware that the toxicity of a bloom is not only determined by the number of cells but also by the average cell size and the degree of growth limitation by nitrogen or other nutrients.

Recent research focus on *Karenia brevis* seems to have shifted away from environmental and physiological controls on bloom toxicity and has been directed more
towards detection or enumeration of *Karenia* species or strains based on microscopic counts, optical characteristics, satellite data, and molecular probes (Heil & Steidinger 2009). While we do not discourage this direction in research, our data suggest more focus needs to be directed toward understanding previously ignored environmental influences as well as physiological conditions controlling toxicity of these blooms. This new direction of research in conjunction with nutrient monitoring, bloom initiation studies, and bloom forecasting models should provide for improved capabilities in the prediction, management, and mitigation of the harmful impacts of red tide blooms.
Table 1.1. Mean values, standard deviations (SD), and ranges (min and max) for total brevetoxins per cell (pg·cell\(^{-1}\)) from various published culture and field studies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>n</th>
<th>Study Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baden and Tomas 1988</td>
<td>11</td>
<td>4</td>
<td>6</td>
<td>16</td>
<td>6</td>
<td>culture strains</td>
</tr>
<tr>
<td>Backer et al. 2005</td>
<td>26</td>
<td>20</td>
<td>2</td>
<td>61</td>
<td>15</td>
<td>surf zone</td>
</tr>
<tr>
<td>Pierce et al. 2005</td>
<td>18</td>
<td>3</td>
<td>12</td>
<td>23</td>
<td>8</td>
<td>surf zone</td>
</tr>
<tr>
<td>Pierce et al. 2008</td>
<td>24</td>
<td>11</td>
<td>8</td>
<td>47</td>
<td>20</td>
<td>surface samples</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>16</td>
<td>26</td>
<td>bottom samples</td>
</tr>
<tr>
<td>Tester et al. 2008</td>
<td>16</td>
<td>11</td>
<td>1</td>
<td>68</td>
<td>118</td>
<td>surface and bottom samples</td>
</tr>
</tbody>
</table>
Table 1.2. Average values ± standard deviations for growth rate (d⁻¹), volume per cell (μm³), chlorophyll a (fg·μm⁻³) and (pg·cell⁻¹), total brevetoxins (fg·μm⁻³) and (pg·cell⁻¹), and cellular N:C (nitrogen:carbon) (mol·mol⁻¹) for nitrogen replete and nitrogen limited experiments with four strains of *Karenia brevis*. (NA = not available)

<table>
<thead>
<tr>
<th></th>
<th>CCMP 2228</th>
<th>Wilson</th>
<th>SP2</th>
<th>SP3</th>
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<tbody>
<tr>
<td><strong>Nitrogen Replete</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Growth rate</td>
<td>(d⁻¹)</td>
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<tr>
<td>Volume per cell</td>
<td>(μm³)</td>
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<tr>
<td>Chlorophyll a</td>
<td>(fg·μm⁻³)</td>
<td></td>
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<tr>
<td></td>
<td>(pg·cell⁻¹)</td>
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<tr>
<td>Total brevetoxin</td>
<td>(fg·μm⁻³)</td>
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<td></td>
<td>(pg·cell⁻¹)</td>
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<tr>
<td>Cellular N:C</td>
<td>(mol·mol⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.13±0.01</td>
<td>0.14±0.04</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Nitrogen Limited</strong></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Growth rate</td>
<td>(d⁻¹)</td>
<td>0.094±0.004</td>
<td>0.095±0.005</td>
<td>0.079±0.003</td>
</tr>
<tr>
<td>Volume per cell</td>
<td>(μm³)</td>
<td>2645±108</td>
<td>3873±150</td>
<td>2959±305</td>
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<tr>
<td>Chlorophyll a</td>
<td>(fg·μm⁻³)</td>
<td>0.8±0.2</td>
<td>0.6±0.07</td>
<td>1.1±0.1</td>
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<tr>
<td></td>
<td>(pg·cell⁻¹)</td>
<td>2.1±0.6</td>
<td>2.4±0.2</td>
<td>3.2±0.4</td>
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<tr>
<td>Total brevetoxin</td>
<td>(fg·μm⁻³)</td>
<td>7.3±1.2</td>
<td>5.6±0.5</td>
<td>5.8±1.0</td>
</tr>
<tr>
<td></td>
<td>(pg·cell⁻¹)</td>
<td>19.2±3.3</td>
<td>21.6±2.7</td>
<td>17.2±3.6</td>
</tr>
<tr>
<td>Cellular N:C</td>
<td>(mol·mol⁻¹)</td>
<td>0.079±0.008</td>
<td>0.061±0.019</td>
<td>NA</td>
</tr>
</tbody>
</table>
Figure 1.1. Results from acclimated semi-continuous batch cultures of *Karenia brevis* strain CCMP 2228 grown under nutrient-replete (closed circles) and N-limited conditions (open squares): A) Biovolume ($\mu$L$_{cells}$·L$_{media}^{-1}$), B) Curves for ln biovolume vs. time, correcting for culture dilution (Sunda et al. 2007), C) Mean volume per cell of N-replete and N-limited cells ($\mu$m$^3$·cell$^{-1}$), D) Cellular nitrogen to carbon molar ratios (N:C), E) Chlorophyll *a* normalized to cell volume (fg·µm$^{-3}$), F) Total molar brevetoxin concentrations normalized to cellular carbon (mmol·mol$^{-1}$) and brevetoxins as a % of total cell carbon, G) Total brevetoxins per unit of cell volume (fg·µm$^{-3}$), H) Total brevetoxins (fg·µm$^{-3}$) plotted against the cellular N:C ratio. Plots A-G have the same x-axis in days. Error bars represent the standard deviation of triplicate measurements, except for N:C ratios where samples were analyzed in duplicate.
Figure 1.2. Results from semi-continuous batch cultures of *Karenia brevis* strain Wilson grown under nutrient-replete (closed circles) and N-limited conditions (open squares): A) Biovolume ($\mu$L_{cells}·L_{media}$^{-1}$), B) Curves for $\ln$ biovolume vs. time, correcting for culture dilution (Sunda et al. 2007), C) Mean volume per cell of N-replete and N-limited cells ($\mu$m$^3$·cell$^{-1}$), D) Cellular nitrogen to carbon molar ratios (N:C), E) Chlorophyll $a$ normalized to cell volume (fg·µm$^3$), F) Total molar brevetoxin concentrations normalized to cellular carbon (mmol·mol$^{-1}$) and brevetoxins as % of total cell carbon, G) Total brevetoxins per unit of cell volume (fg·µm$^3$), H) Total brevetoxins (fg·µm$^3$) plotted against the cellular N:C ratio. Plots A-G have the same x-axis in days. Error bars represent the standard deviation of triplicate measurements except N:C ratios where samples were analyzed in duplicate.
Figure 1.3  Results from semi-continuous batch cultures of *Karenia brevis* strain SP2 grown under nutrient-replete (closed circles) and N-limited conditions (open squares):  

A) Biovolume ($\mu L_{\text{cells}} \cdot L_{\text{media}}^{-1}$) in semi-continuous batch cultures grown under N-replete and N-limited conditions, B) Calculated growth rates for the N-replete and N-limited cells in panel A using the method described in (Sunda et al. 2007), C) Volume per cell of N-replete and N-limited cells ($\mu m^3 \cdot \text{cell}^{-1}$), D) N-replete and N-limited chlorophyll $a$ on biovolume basis (fg·$\mu m^3$), E) Total brevetoxin concentration per biovolume (fg·$\mu m^3$). All plots have the same x-axis in days. Error bars represent the standard deviation of triplicate measurements.
Figure 1.4  Results from semi-continuous batch cultures of *Karenia brevis* strain SP3 grown under nutrient-replete (closed circles) and N-limited conditions (open squares):  A) Biovolume (µL cells·L media⁻¹) in semi-continuous batch cultures grown under N-replete and N-limited conditions, B) Calculated growth rates for the N-replete and N-limited cells in panel A using the method described in (Sunda et al. 2007), C) Mean volume per cell of N-replete and N-limited cells (µm³·cell⁻¹), D) N-replete and N-limited chlorophyll *a* on biovolume basis (fg·µm⁻³), E) Total brevetoxin concentration per unit biovolume (fg·µm⁻³). All plots have the same x-axis in days. Error bars represent the standard deviation of triplicate measurements.
SP3

(A) Biovolume (µL cells L⁻¹ media⁻¹)

(B) Ln biovolume (µL cells L⁻¹ media⁻¹)

N-replete

N-limited

R² = 0.989

R² = 0.953

(C) Volume per cell (µm³ cell⁻¹)

(D) Cell chlorophyll a (fg µm⁻²)

(E) Cell brevetoxin concentration (fg µm⁻²)

Day
Figure 1.5  A) Average levels of PbTx-1, -2, and -3 normalized per cell (pg·cell⁻¹) for all four strains of *Karenia brevis* under N-replete and N-limited conditions; B) Average volume per cell (µm³·cell⁻¹) of N-replete and N-limited cells; C) The data shown in panel A normalized on a cell volume basis (fg·µm⁻³). Error bars represent the standard deviations of triplicate measurements for average volume per cell and cellular toxin.
Figure 1.6 Results from a batch culture of *Karenia brevis* strain CCMP 2228 grown in low-nitrate media. A) Semi-log plot of cell concentration (left y-axis) and biovolume (right y-axis) versus time; B) Volume per cell (left axis) and cellular N:C ratio (right axis) versus time; C) Total brevetoxins normalized per unit of cell volume (fg·µm³, left y-axis) and normalized per cell (right y-axis) vs. time. The region between the vertical dashed lines represents the transition period from exponential phase to stationary phase for all plots. Error bars give standard deviation for three replicate measurements except cellular N:C ratios, which represented single measurements.
Figure 1.7  A comparison of this study’s brevetoxin per cell data (pg·cell⁻¹) with that of Lekan and Tomas (2010) for *Karenia brevis* strains Wilson and SP3. Data from Lekan and Tomas (2010) were estimated from Figure 4 of that paper for a salinity of 35 and a temperature of 20°C. The conditions for this study were a salinity of 36 and 23°C.
CHAPTER 2

Increased Toxicity of *Karenia brevis* During Phosphate Limited Growth: Ecological and Evolutionary Implications

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ABSTRACT

*Karenia brevis* is the dominant toxic red tide algal species in the Gulf of Mexico. It produces potent neurotoxins (brevetoxins [PbTxs]), which negatively impact human and animal health, local economies, and ecosystem function. Field measurements have shown that cellular brevetoxin contents vary from 1-68 pg cell$^{-1}$ but the source of this variability is uncertain. Increases in cellular toxicity caused by nutrient-limitation and inter-strain differences have been observed in many algal species. We examined the effect of P-limitation of growth rate on cellular toxin concentrations in five *Karenia brevis* strains from different geographic locations. Phosphorous was selected because of evidence for P-limitation of algal growth in some regions of the Gulf of Mexico. Depending on the isolate, P-limited cells had 2.3- to 7.3-fold higher PbTx per cell than P-replete cells. The percent of cellular carbon associated with brevetoxins (%C-PbTx) was ~0.7 to 2.1% in P-replete cells, but increased to between 1.6 to 5% under P-limitation. Because PbTx is a potent anti-grazing compound, this increased investment in PbTx should enhance cellular survival during periods of nutrient-limited growth. The %C-PbTx was inversely related to specific growth in both the nutrient-replete and P-limited cultures of all strains. This inverse relationship is consistent with an evolutionary tradeoff between carbon investment in PbTxs and other grazing defenses, and C investment in growth and reproduction. In aquatic environments where nutrient supply and grazing pressure often vary on different temporal and spatial scales, this tradeoff would be selectively advantageous as it would result in increased net population growth rates. The variation of PbTxs in this study can account for
the range of values observed in the field, including the highest values, which are not observed under N-limitation. These results suggest P-limitation is an important factor regulating cellular toxicity and adverse impacts during at least some *K. brevis* blooms.

**INTRODUCTION**

Blooms of the toxic dinoflagellate *Karenia brevis* produce a suite of structurally related neurotoxins, brevetoxins (PbTxs), which adversely affect both human and ecosystem health. These toxins bind to voltage-sensitive sodium channels which results in persistent activation of neuronal, skeletal muscle and cardiac cells (Baden & Adams 2000). Shellfish feeding on *K. brevis* accumulate PbTxs, which can lead to neurotoxic shellfish poisoning (NSP). NSP symptoms in humans include gastrointestinal distress, nausea, vomiting, dizziness, slurred speech, numbness of lips, mouth and tongue, and respiratory distress (Watkins et al. 2008, Heil 2009). Exposure is enhanced when *K. brevis* cells are disrupted by breaking waves and form toxic aerosols (Kirkpatrick et al. 2010). Onshore winds transport these aerosols over beaches and nearshore communities, causing respiratory related illnesses (Pierce et al. 2005, Kirkpatrick et al. 2006, Kirkpatrick et al. 2010). PbTxs produced by *K. brevis* also cause the intoxication and death of marine organisms including copepods, fish, bottlenose dolphins and manatees (Asai et al. 1984, Purkerson et al. 1999, Cohen et al. 2007, Waggett et al. 2012). The adverse environmental and health effects of *K. brevis* blooms, in conjunction with the associated negative publicity, result in significant economic losses in local communities that depend on tourism and recreational fishing (Hoagland & Scatasta 2006, Hoagland et al. 2009, Morgan et al. 2009). The most heavily impacted region is the
Gulf of Mexico, especially the west coast of Florida, which experiences toxic *K. brevis* blooms on a nearly annual basis (Tester & Steidinger 1997).

Several theories have been proposed concerning the factors that control the development and persistence of toxic *K. brevis* blooms along the west coast of Florida. It is generally accepted that the early phase of a bloom is initiated by northerly winds which promote upwelling events that transport nutrients and *Karenia* cells towards shore where they concentrate along frontal boundaries (Janowitz & Kamykowski 2006, Janowitz et al. 2008, Stumpf et al. 2008). In addition, nutrient assessments of cells from the field indicate that the growth of *K. brevis* blooms is limited by available nitrogen (N) or phosphorus (P), suggesting that these nutrients play an important role in the development and maintenance of blooms (Vargo et al. 2002, Vargo et al. 2008). What is less clear are the sources of N and P utilized by these blooms. Upwelling of subsurface nutrients, land runoff, N$_2$-fixation, drainage from phosphate mines and atmospheric deposition have all been proposed as important sources (Vargo et al. 2002, Stumpf et al. 2008, Vargo et al. 2008). In reality, some combination of these different sources likely controls the nutrient supply needed to support intense blooms.

Recent laboratory experiments indicate that N-limitation directly affects not only the growth potential of blooms, but also the toxicity of *K. brevis* cells (Hardison et al. 2012). Intracellular PbTx concentrations (fg µm$^{-3}$) increased by up to 2.5-fold during N-limited growth in laboratory cultures. In the field, this would translate into the potential for a significantly higher PbTx flux into the food chain and increased exposure of affected organisms during periods of N-limitation of algal growth (Bricelj et al. 2012, Echevarria et
al. 2012). Whether P-limitation causes a similar increase in cellular toxicity is unknown and was the focus of this study. Understanding the degree to which P-limitation regulates the toxicity of *K. brevis* cells is important given the apparent long-term shift from N- toward P-limitation in the Gulf of Mexico and other regions of the subtropical North Atlantic Ocean basin (Rabalais et al. 1996, Sylvan et al. 2006, Duce et al. 2008). P-limitation on the West Florida Shelf is controversial given the presence of phosphate containing soils on the northern and central Florida peninsula. The mining and drainage of these deposits may result in unknown inputs of phosphorus to local rivers and estuaries, but the amount that moves offshore appears to be relatively low (Vargo et al. 2008). Consistent with this observation, researchers have reported evidence for P-limitation of *Karenia brevis* blooms on the West Florida Shelf (Vargo et al. 2002, Heil et al. 2007).

Typically, toxin content per cell increases dramatically when the growth of harmful algae becomes P-limited. For example, hemolytic activity per cell in the prymnesiophytes *Prymnesium parvum* and *Chrysochromulina polylepis* increases by up to 10-fold under P-limitation (Johansson & Graneli 1999b, Johansson & Graneli 1999a). Similarly, P-limitation increases the cellular content of the neurotoxin domoic acid in the diatom *Pseudo-nitzschia multiseries* (Pan & Rao 1996, Pan et al. 1998), and karlotoxin in the dinoflagellate *Karlodinium veneficum* (Adolf et al. 2009, Fu et al. 2010). It also increases cellular levels of the potent phosphatase inhibitor nodularin in the cyaobacterium *Nodularia spumigena* (Sunda et al. 2006). Also, isolates of the dinoflagellates *Alexandrium tamarens* and *A. minutum*, which failed to produce significant levels of paralytic shellfish poisoning (PSP)
toxins under N-limitation, increased their cellular toxin contents under P-limitation (Granéli & Flynn 2006). In cases where cellular toxins increase under both N- and P- limitation, the increase in toxin per cell is often higher in P-limited cells (Granéli & Flynn 2006).

A collection of field measurements made in the Gulf of Mexico indicated that PbTx contents of *K. brevis* cells vary between 1 – 68 pg cell\(^{-1}\) (Table 2.1). In a previous study we found that N-limitation could only account for toxin values in the range of 7 - 25 pg cell\(^{-1}\) (Hardison et al. 2012). Observed patterns from the studies cited above suggests that P-limitation, rather than N-limitation might account for the upper range in PbTx contents per cell observed in the field. Currently, however, there are no data on the effect of P-limitation on the brevetoxin levels in *K. brevis* cells.

In this study we investigated the effect of P-limitation on cellular growth rate, and cellular content of chlorophyll \(a\) (chl \(a\)), carbon (C), phosphorus (P), nitrogen (N) and PbTx in laboratory cultures of *K. brevis*. Previous work showed that increases in cellular PbTx concentrations under N-limitation were consistent with the carbon:nutrient balance hypothesis (CNB) (Hardison et al. 2012). This hypothesis predicts that nutrient limited growth is accompanied by a diversion of fixed carbon into the increased levels of defensive compounds or structures (Bryant et al. 1983, Ianora et al. 2006, Ianora et al. 2011). This C diversion into toxic compounds has a dual advantage. It affords greater defense against grazers and pathogens to compensate for the reduced rates of growth and reproduction. It also provides the cell with a means disposing of unneeded fixed carbon during the onset of nutrient limitation of growth, thereby protecting the photosynthetic electron transport chain.
from over reduction and attendant oxidative stress (Sakshaug et al. 1989, Sunda et al. 2007, Schaeffer et al. 2009). The CNB hypothesis predicts an increase in cellular PbTx concentrations should also occur under P-limitation, and this prediction was examined in the present study.

Our experiments utilized five different strains of *K. brevis* from varying geographic locations: four from different locations on the west coast of Florida and one, SP2, from Texas. The strains were used to determine how the brevetoxin content of genetically distinct isolates responded to P-limited growth. Because cell size varies with nutrient limitation, PbTx was normalized on a per cell basis, a per biovolume basis, and cell carbon basis. Normalization of brevetoxins to cell volume or cell carbon made it possible to separate physiological changes in toxin per unit cell biomass from seeming changes in toxicity due solely to changes in cell size.

**MATERIALS and METHODS**

*Strains and culture conditions*- The effect of P-limitation on the cell content of individual brevetoxin congeners and total PbTx concentrations was studied in five *Karenia brevis* strains with different specific growth rates, cell sizes, and P:C ratios under nutrient sufficient growth (Table 2.2). Strains CCMP 2228, CCMP 2229, and CCMP 2820 were obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (West Boothbay Harbor, ME, USA); strain SP2 was obtained from Dr. Ed Buskey of University of Texas Marine Science Institute (Port Aransas, TX, USA); and the Wilson strain
(CCFWC268) was acquired from the Fish and Wildlife Research Institute (St. Petersburg, FL, USA).

Cells were cultured in a Percival Scientific model I-36VLX incubator maintained at a constant temperature of 23°C and on a 14h:10h daily light:dark cycle to simulate summer light conditions. Photosynthetically active radiation (PAR) was provided at an intensity of 120 µmol quanta m⁻² s⁻¹ via vertically mounted fluorescent Duro-test Vita-lites. PAR intensity was measured with a Biospherical Instruments Inc. QSL-100 4π wand type light meter.

Media consisted of 1.0 L of 0.2 µm filtered Gulf Stream seawater (salinity 36) held in 2.5 L polycarbonate bottles. The media contained added vitamins (0.074 nM vitamin B₁₂, 0.4 nM biotin, and 60 nM thiamine), 10 nM Na₂SeO₃, and an EDTA-trace metal buffer system (Sunda et al. 2005) (100 µM EDTA, 1 µM FeEDTA, 50 nM MnCl₂, 40 nM CuCl₂, 100 nM ZnSO₄, and 40 nM CoCl₂). Nutrient replete culture media contained 64 µM NaNO₃ and 4 µM NaH₂PO₄ (N:P = 16:1, the Redfield ratio) while P-limited media contained 64 µM NaNO₃ and 0.5 µM NaH₂PO₄ (N:P=128:1). Media were sterilized by microwave treatment (Keller et al. 1988). Culture pH was measured initially and throughout experiment with a Thermo Orion 3 Star pH meter equipped with a Ross ultra-combination pH electrode to ensure no carbon dioxide limitation occurred. Culture pH ranged from 8.10 to 8.35 for P-limited cultures and 8.10 to 8.30 for nutrient-replete cultures.

*Karenia brevis* cells in our experiments were grown in semi-continuous 2.5L batch cultures. Experimental cells in both high and low phosphate media were inoculated from
acclimated nutrient-sufficient cultures that had been growing exponentially for several weeks at their maximum rates. The P-limited cultures were diluted with fresh low-phosphate medium every 2 to 3 days at an average rate of 0.1 d\(^{-1}\) to obtain a continuous P-limited growth rate. Nutrient sufficient cultures growing at their maximum rates were diluted sequentially with high phosphate medium well before they reached their maximum cell density to ensure no nutrient limitation of growth rate occurred. Specific growth rates and associated standard errors were calculated by linear regressions of the natural log of biovolume (µL\(_{\text{cells}}\) L\(_{\text{media}}\)\(^{-1}\)) versus time after correcting for serial culture dilutions (Sunda et al. 2007).

*Cell concentrations, mean volume, growth rate, chlorophyll a, nutrient element stoichiometry, and brevetoxins*- In the middle of the light period (midday) culture aliquots were taken for measurement of cell concentrations, mean volume per cell, chlorophyll a (chl \textit{a}), brevetoxins, and cellular carbon, nitrogen, and phosphorus every 2-3 days for P-limited cultures and as often as cell density allowed for P-replete cultures. All analyses were conducted in triplicate except for cellular carbon and nitrogen which were measured in duplicate subsamples due to volume limitations. Results were expressed as mean values ± standard deviation unless otherwise noted. Cell concentrations and mean volume per cell were measured with a Beckman Coulter Inc. Multisizer 3 electronic particle counter (0.5-mL sample volume) equipped with a 100-µm aperture. Cell growth curves were constructed as semi-log plots of total biovolume (µL\(_{\text{cells}}\) L\(_{\text{media}}\)\(^{-1}\)) versus time in days. Total biovolume (µL\(_{\text{cells}}\) L\(_{\text{media}}\)\(^{-1}\)) was calculated by multiplying the cellular concentration (cells L\(^{-1}\)) by the
mean volume per cell (µL cell⁻¹). Specific growth rates were computed from linear regressions of the natural log of total cell biovolume versus time after correcting for culture dilution (Sunda et al. 2007).

Chl a was measured by filtering cells onto 25-mm GF/F filters and extracting the cells with a 90:10 acetone:water mixture. The fluorescence of the extracted chl a was measured with a Turner Design 10-AU fluorometer (Welschmeyer 1994). Cellular carbon (C) and nitrogen (N) were determined by gentle filtration of cells onto precombusted 13 mm GF/F glass fiber filters (Hardison et al. 2012) followed by fuming with HCl overnight to remove inorganic carbon (Liu et al. 2001). These filter samples were then analyzed for cellular N and C with an EAS 4010 Costech elemental analyzer. Cellular phosphorus (P) samples were prepared by gently filtering culture samples onto precombusted 25 mm GF/F filters and analyzing the collected cells for particulate P (Solorzano & Sharp 1980).

Brevetoxins were extracted using liquid/liquid separations with ethyl acetate. Prior to separations, aliquots of cell cultures were mixed 1:1 by volume with ethyl acetate and the mixture was sonicated with a microtip-equipped Branson Sonifier 250 for 3 minutes. Complete cell disruption was confirmed by microscopy. The analysis gave total culture toxin, which was deemed appropriate since preliminary experiments showed >90% of culture toxins were intracellular, corroborating previous findings (Tester et al. 2008, Lekan & Tomas 2010). Collected ethyl acetate fractions were desalted with Milli-Q water and concentrated with a rotovap. Extraction efficiency was determined in every fraction by the addition of an internal standard; efficiency typically ranged from 90-95% (Lekan & Tomas 2010).
Concentrated fractions were measured for brevetoxins using an Agilent 1100 LC coupled to a Thermo-Finnigan TSQ Quantum triple quadrupole mass spectrometer with an electrospray ion source interface. LC-MS-MS conditions have been previously described in detail (Cheng et al. 2005, Mendoza et al. 2008). An external standard curve of purified brevetoxins 1, 2, and 3 (World Ocean Solutions, Wilmington, NC, USA) was used to quantify amounts of extracted brevetoxins.

RESULTS

Growth rates- Nutrient replete maximum growth rates among the five K. brevis strains varied from 0.26 d\(^{-1}\) to 0.47 d\(^{-1}\), with CCMP 2228 exhibiting the highest rate and strain SP2 the lowest (Table 2.2). The low phosphate cultures had approximately the same specific growth rates as the high-P ones for the first ~8-10 days prior to the onset of P-limitation on days 10-12 (Figures 2.1A-2.5A). After day 12, the growth of the low-P cultures was limited by phosphate. These P-limited cultures were then resupplied with fresh low-P medium at an average dilution rate of 0.1 d\(^{-1}\). Measured specific growth rates of the P-limited, semi-continuous cultures ranged from 0.09d\(^{-1}\) for the Wilson strain to 0.15 d\(^{-1}\) for strain CCMP 2229 (Table 2.2). P-limited growth rates for all four strains were 2.6- to 4.8-fold lower than rates in the nutrient sufficient cultures (Table 2.2; Figures 2.1B-2.5B).

Cell density and volume per cell- The cultures were maintained at low total cell volumes (≤ 20 µL\(_{\text{cells/L culture}}\)) to minimize pH increases and attendant decreases in CO\(_2\) concentrations and changes to trace metal nutrient availability (Sunda et al. 2005). Average
volume per cell for nutrient replete cultures ranged from 3,650 µm$^3$ cell$^{-1}$ for strain CCMP 2228 to 5,668 µm$^3$ cell$^{-1}$ for the Wilson strain. These volumes remained relatively constant throughout the experiments (Table 2.2; Figures 2.1D-2.5D). The volume per cell in the *K. brevis* strains increased with the onset of P-limitation of growth rate (Table 2.2; Figures 2.1D-2.5D), as is typically observed with growth limitation by phosphate (Rhee 1978). These increases in mean volume per cell ranged from 6% for strains SP2 and CCMP 2820 to 64% in strain CCMP 2228 (Table 2.2). The cell volume increase for the latter strain continued for the duration of the experiment, but the other four strains exhibited oscillations in cell volume following the onset of P-limitation (Figures 2.1D-2.5D). Although some of the volume increases were quite variable, all strains exhibited significant differences in volume per cell between P-replete and P-limited treatments (one-way ANOVA: $p<0.001$ for strains CCMP 2228, CCMP 2229, CCMP 2820 and Wilson; $p=0.03$ for strain SP2).

*Cellular C and P:* Cellular P:C ratios decreased by 2.5- to 3.0-fold with decreasing growth rate in the low-P cultures (days 8-12), indicating that these cultures were P-limited (Figures 2.1C-2.5C). Average cellular P:C ratios for P-replete strains ranged from 6.9±0.9 mmol mol$^{-1}$ for strain CCMP 2229 to 20.0±7.5 mmol mol$^{-1}$ for the Wilson strain (Table 2.2). Average P:C ratios were lower in P-limited cells and ranged from 3.5±1.8 mmol mol$^{-1}$ for CCMP 2228 to 6.2±2.0 mmol mol$^{-1}$ for CCMP 2820 (Table 2.2). In all strains tested, P:C values for P-limited cultures remained lower than those in the respective P-replete cultures.

*Chlorophyll a:* P-replete cultures of the five *K. brevis* strains exhibited mean cellular chl $a$ of 1.1 to 1.6 fg µm$^{-3}$ normalized to cell volume (Table 2.2). Cellular chl $a$ decreased
with the onset of P-limitation of growth rate and remained lower than P-replete values throughout the experiments (Figures 2.1F-2.5F). Average decreases ranged from 44% to 68% when expressed on a cell volume basis (Table 2.2).

*Brevetoxins*—The dominant brevetoxins (PbTxs) detected in four of the strains were PbTx-1, -2, and -3. The only exception was the Wilson strain which only contained measurable levels of PbTx-2 and PbTx-3. In this study, reported total brevetoxins equal the sum of these three dominant congeners. For each of the strains tested, PbTx-2 was the most abundant brevetoxin, followed by PbTx-1 (when present) and trace amounts of PbTx-3 (<1 pg cell⁻¹). PbTx-1 and PbTx-2 are intracellular congeners whereas PbTx-3 is primarily an extracellular breakdown product of PbTx-2 (Pierce & Henry 2008). The relative distribution of the individual toxin congeners was similar in P-limited and P-replete cultures (data not shown).

Phosphate-replete cells growing at their maximum rates in the high-P media and in the low-P media at the beginning of the time course experiments exhibited similar total brevetoxin concentrations (Figures 2.1 E,G,H – 2.5 E,G,H). The percent of cell carbon present as brevetoxins (referred to hereafter as %C-PbTx) varied among the nutrient sufficient cultures and was highest (2.1 and 1.5%, respectively) for the Wilson and SP2 strains and lowest (0.75%) for strain CCMP 2229 (Table 2.2). As the initially P-replete cells in the low-P media transitioned into P-limitation of growth at approximately day 10, their brevetoxin levels increased on a cell volume (fg µm⁻³), per cell (pg cell⁻¹), and a per cell carbon basis (%C-PbTx ). The pattern of increase, however was often complex, and
depended on both the strain and the cellular attribute (cell number, volume, or C) the brevetoxins were normalized to (Figures 2.1 E,G,H – 2.5 E,G,H). All but one of the strains (SP2) showed peaks in brevetoxin values at varying times after the onset of P-limitation, followed by decreasing values thereafter. For strain SP2, the cellular toxin per cell and per unit of cell volume continued to increase from the onset of P-limitation after day 7 until the end of the experiment on day 29, while the %C-PbTx remained nearly constant after day 12 (Figures 2.3 E, G and H). Peak values for total brevetoxin per cell for the strains examined were 65 pg cell\(^{-1}\) for strain CCMP 2228, 43 pg cell\(^{-1}\) for the Wilson strain, 36 pg cell\(^{-1}\) for SP2, 16 pg cell\(^{-1}\) for CCMP 2820, and 48 pg cell\(^{-1}\) for CCMP 2229 (Figures 2.1 E-2.5 E). These peak values were 2.9- to 7.3-fold higher than the mean values observed in nutrient replete cells, with strains CCMP 2228 and CCMP 2229 showing the highest increases. A similar trend was observed when toxins were normalized to biovolume and cell carbon (Figures 2.1 G,H-2.5 G, H). Biovolume normalized brevetoxins exhibited maximum increases of 1.9- to 4.6-fold under P-limitation relative to nutrient sufficient values and %C-PbTx exhibited maximum increases of 2.3- to 4.9-fold. As with toxin values per cell, brevetoxins per unit of cell volume for strains CCMP 2228 and CCMP 2229 showed the largest increase in maximum values (4.6-fold), strains SP2 and CCMP 2820 had intermediate increases (2.7- and 2.8-fold), whereas the Wilson strain had the least increase (1.9-fold). Maximum increases in brevetoxins normalized to cell C (%C-PbTx) were also largest for strains CCMP 2229 and 2228 (4.9- and 4.8-fold), intermediate for strains SP2 and CCMP2820 (3.4- and 3.1-fold), and smallest for the Wilson strain (2.3-fold). A noticeable
difference in the magnitude of the cellular brevetoxin increase is observed depending on which normalization factor is used. On a per biovolume or cell C basis, brevetoxin increases were not as substantial as when toxins were normalized per cell. This behavior reflects the fact that the P-limited cells increased in size and thus had higher volumes and carbon mass per cell. This size increase meant that a given increase in toxin per unit cell volume or carbon, would automatically translate to an even larger increase in toxin per cell. Regardless of how brevetoxins were normalized, P-limited toxins remained elevated relative to nutrient replete cultures for the duration of the experiments. Nutrient replete controls maintained similar low levels of brevetoxins throughout the experiments (Figures 2.1 E, G, H – 2.5 E, G, H).

Cellular brevetoxins normalized per cell, cell volume, and cell C showed consistent relationships with the cellular P:C ratio among all strains under both nutrient sufficiency and P-limitation, however the trend in these relationships varied with the P:C ratio (Figures 2.6 A-C). The lowest brevetoxin values were seen at intermediate P:C values of 5 to 8 mmol mol\(^{-1}\) observed in nutrient sufficient strains CCMP 2228, 2229, and 2820. The brevetoxin values were higher in the nutrient sufficient cultures of strains SP2 and Wilson, which had the highest P:C ratios. Overall, the %C-PbTx values were positively correlated with the cellular P:C ratio in nutrient sufficient cultures of the five isolates with a linear regression \(r^2\) of 0.67. However, at low P:C ratios associated with P:limitation of growth rate the opposite behavior occurred, and decreases in P:C values below 5 mmol mol\(^{-1}\) were accompanied by a
sharp rise in cellular brevetoxins. Once again the functional relationship between cellular brevetoxins and P:C ratios was similar for all of the strains (Figure 2.6 A-C).

Cellular brevetoxins as a percent of cell C (%C-PbTx) appear to be less related to P:C ratios and more closely related to growth rate (d⁻¹) (Figure 2.7 A). A polynomial fit was applied to plots of %C-PbTx vs. specific growth rate, and vs. cellular N:P and P:C ratios for both P-replete and P-limited cultures (Figures 2.7 A-C). A better non-linear regression fit was observed for %C-PbTx vs. growth rate (r² = 0.65) than for %C-PbTx vs. cellular N:P (r² = 0.44) or P:C (r² = 0.40). Furthermore, the toxin vs. growth rate plot showed a monotonic decrease in %C-PbTx values with increasing growth rate rather than a change in curve slope as observed in plots of %C-PbTx vs. P:C (Figure 2.6 C). Thus, as growth rates decrease, there is an increased investment of cellular carbon in brevetoxins irrespective of whether the growth rate change was related to inter-strain variations in maximum growth rate or to decreases in growth rate among the strains caused by phosphate deficiency (Figure 2.7 A).

**Comparison of N- and P-limitation** - Data in Figure (2.8 A, B) show a comparison of average brevetoxin values under nutrient replete, N-limited, and P-limited conditions based on data from strains CCMP 2228, Wilson, and SP2 in the present study and an N-limitation study we previously conducted (Hardison et al. 2012). Results vary depending on how the toxin values are normalized. Both N- and P-limitation increased cellular brevetoxin per unit of cell volume in all strains tested, but to varying degrees. Strain CCMP 2228 exhibited the same increase in toxin per unit of cell volume under N- and P-limitation, but the Wilson and SP2 strains showed a slightly higher increase under N-limitation although the differences
were within the measurement error limits. N-limitation caused an average 29\% decrease in volume per cell in the three strains (Hardison et al. 2012) while P-limitation caused an average 35\% increase (Table 2.2). Because of these opposing changes in cell size, the P-limited cells on average had a 2.0-fold higher volume per cell and consequently had higher values of brevetoxin per cell than the N-limited cells (Figure 2.8 B).

DISCUSSION

*Evolutionary tradeoffs between grazing defense and growth*—Karenia brevis forms large ecosystem disruptive algal blooms in the Gulf of Mexico that adversely affect human and animal health (Kirkpatrick et al. 2004, Sunda et al. 2006, Backer 2009). The role of nutrients in initiating and maintaining blooms, and in controlling cellular toxicity remains controversial (Stumpf et al. 2008, Vargo et al. 2008, Hardison et al. 2012). This study was undertaken to examine how phosphate limitation regulates the growth and carbon investment in brevetoxins in different Karenia brevis strains. P-limitation is of interest because evidence indicates that regions where Karenia blooms occur can become transiently phosphate limited (Heil et al. 2007, Rivera-Monroy et al. 2011).

Evolutionary theory predicts that as microalgal growth slows in response to nutrient limitation, cells will apportion a greater percentage of their fixed carbon into defense mechanisms (Ianora et al. 2006). This hypothesis is often referred to as the carbon:nutrient balance (CNB) hypothesis and was originally based on observations from vascular plants, which were shown to divert a greater portion of their fixed carbon from growth to defense
under resource limitation (Bryant et al. 1983, Bryant et al. 1985). Plant and algal defenses often involve the production of anti-grazing compounds (e.g., toxins) as well as the formation of thicker cell walls, spines, or other structures (Lambers et al. 2008, Ianora et al. 2011). In *Karenia brevis* brevetoxins (PbTxs) have been shown to be important anti-grazing compounds (Hong et al. 2012, Waggett et al. 2012). These potent voltage-sensitive sodium channel activators disrupt nerve function (Poli et al. 1986), which accounts for why *Karenia brevis* blooms cause neurotoxic shellfish poisoning, massive fish kills, marine mammal and bird mortalities, and the formation of toxic aerosols along affected beaches (Shumway et al. 2003, Watkins et al. 2008, Landsberg et al. 2009, Kirkpatrick et al. 2010). In the present study average PbTx per unit of cell volume increased 1.5- to 2.9-fold from P-replete to P-limited growth conditions and similar increases were observed under N-limitation in a previous study (Hardison et al. 2012). These findings are consistent with the CNB hypothesis and indicate that P- and N-limited blooms are more likely to create greater adverse ecosystem and human health effects than those which are nutrient sufficient.

The data also indicated that some strains were more toxic than others (Figure 2.7 A). Under nutrient-replete growth conditions, the investments of cell C in PbTx varied between 0.75 and 2% of total cellular C. The corresponding range of mean %C-PbTx for P-limited cells was from ~1.5 to 5.3% (Table 2.2), with maximum values ranging from 3 to 6% (Figures 2.1 H-2.5 H). These latter values represent a significant carbon investment in anti-grazing defenses as growth slowed. Interestingly, although all strains increased their investment in PbTxs under P-limitation, some diverted more of the total carbon pool to
PbTxs than others (Figures 2.6 A-C). The factors governing the differences in this investment are not immediately obvious until %C-PbTx is plotted against growth rate (Figure 2.7 A). That plot showed that growth rate and %C-PbTx were inversely related for both P-replete and P-limited cells (p=0.005; Figure 2.7 A). Thus, the slower the growth rate under all conditions (P-replete and P-limited) the greater the cellular investment in PbTxs, known grazing defense toxins (Hong et al. 2012, Waggett et al. 2012). These data are consistent with evolutionary tradeoffs between C invested in supporting growth and reproduction and that invested in the production of PbTxs and other grazing defenses (Sunda & Hardison 2010, Ianora et al. 2011). It is likely that if we assessed more strains or varying degrees of P-limitation of growth rate, there would be a continual gradation in growth rates versus the %C-PbTx. Given that net algal population growth is largely dependent on rates of growth and reproduction minus rates of grazing mortality losses, this variation would be selectively advantageous at the population level in aquatic environments where grazing pressures and growth limiting nutrients continually fluctuate on different temporal and spatial scales (Figure 2.7 A) (Hong et al. 2012, Waggett et al. 2012). This trade-off linked variability among strains promotes genetic diversity within populations of algal species, which in turn permits the adaptation of populations to changing conditions. Similar evolutionary tradeoffs have been observed in terrestrial plants (Lambers et al. 2008) and in other phytoplankton (Sunda & Hardison 2010). For example, a study of nutrient acquisition in 13 algal strains representing 11 separate species, the species or strains with lower nutrient uptake rates and growth rates for their size were also poorly grazed or assimilated, suggesting higher levels of
grazing defenses (Sunda & Hardison 2010). These results are consistent with the growth and defense relationship exhibited by the different *K. brevis* strains under nutrient-sufficient and P-limited growth conditions, where the least amount of carbon was associated with brevetoxins in strains growing at the fastest rates, and more carbon was allocated to these defensive compounds under reduced growth rates (Figure 2.7 A). Furthermore, the C contained in PbTxs likely represents only a portion of the total C expended for cellular defenses. Additional C (and N) would be needed for the production of PbTx biosynthetic enzymes and for the synthesis of unidentified toxic compounds produced by *K. brevis*, that inhibit the growth of other algae (Kubanek et al. 2005). Similar production of multiple defensive compounds is widespread in terrestrial plants (Lambers et al. 2008).

These results also raise an interesting question of whether the increased PbTx to cell C ratios under P-limited growth represents a net up-regulation of cellular toxin production rates. To answer this question we note that during steady state growth, the cellular toxin:C ratio equals the C-normalized production rate divided by the specific growth rate, the effective rate of biodilution. Because of this relationship increases in cellular toxins can be caused by an increase in production rates, a decrease in growth rate, or by changes in both. Computed steady-state PbTx production rates in the P-limited cells were generally the same or lower than those in the faster growing nutrient-replete cells (Table 2.2). A possible exception was strain CCMP 2229, where there was a 26% increase in the production rate under P-limitation, but the increase was not statistically significant (p=0.15, t-test). The results indicate that the increased toxin to C ratios in the P-limited cells are achieved by
down regulating C investment in other compounds, such as those associated with photosynthesis (e.g. chl a; Figures 2.1-2.5 F), while maintaining the same PbTx production or down-regulating PbTx production to a lesser degree than other cellular constituents (Table 2.2). Thus, the cells preferentially maintain production of PbTx over other cellular constituents when growth slows leading to an increase in cellular toxin concentration.

The CNB hypothesis also predicts that the investment in PbTx will be highest during the period when growth rate initially slows from the onset of nutrient limitation. During this time the growth slows more quickly than the photosynthetic apparatus can be down regulated, which results in a temporary imbalance between photosynthetic C-fixation and the cellular C demand for growth (Figures 2.1 F-2.5 F). To avoid over-reduction of the photosynthetic apparatus, and consequent production of toxic reactive oxygen species (Niyogi 1999), the CNB states that cells divert some of the “excess” fixed carbon into defensive compounds. In this study, the %C-PbTx rapidly increased in all five of the strains during this unbalanced growth period, but then subsequently declined to different degrees in all strains except SP2 once photosynthetic C-fixation and growth began to come back into balance (Figures 2.1-2.5 H). The same trends were observed whether the data were expressed as PbTx per cell or per unit of cell volume (Figures 2.1-2.5 E, G). The timing and magnitude of the maxima, however, were not always the same as for %C-PbTx because of concomitant changes in volume per cell (Figures 2.1-2.5 D) and cell C:volume ratios (Table 2.2). The transient peaks in PbTx per cell in the P-limited cells were 16 - 65 pg cell⁻¹, depending on the strain, with the higher of these values corresponding to the upper values observed in the field
during blooms (Table 2.1). This observation suggests that blooms will reach their maximum toxicity in the early phases of nutrient limitation.

As P-limitation progresses past early transient phases into the later phases of growth limitation, toxin levels began to stabilize as the cells reach an acclimated (relatively constant) P-limited growth rate. Interestingly, at this point, when cells no longer need to “dump” excess photosynthetically fixed carbon, toxin levels (%C-PbTx), remain significantly higher than observed in the P-replete cells, but the degree to which this occurs varies among strains. As noted previously, these data indicate that even after down regulation of chl a brings C-fixation and growth back into balance, K. brevis cells continue to invest a greater portion of their total cellular C in PbTx, apparently to lower grazer-linked mortality rates when P-limitation significantly reduces cellular rates of growth and reproduction.

The thoroughness of the data obtained in this paper provides an unusual opportunity to examine how well N:P and P:C ratios predict cellular toxicity, as has been typically used in past to demonstrate relationships between toxin (or toxicity) per cell and N and P limitation (Granéli & Flynn 2006). For example, culture studies with Alexandrium tamarense, A. minutum, Prymnesium parvum, and Chrysochromulina polylepis have consistently shown that as the cellular N:P ratio increases above ~16 (the Redfield ratio (Redfield 1958)) under P-limitation of growth rate toxin per cell increased steadily as long as P is not an essential component of the toxin being synthesized (Granéli & Flynn 2006). However, relationships between cellular toxicity and specific growth rate were not presented. Conversely, the results of our study indicate that growth rate is the best predictor of toxin
amounts per unit of cell carbon or biomass (Figure 2.7 A). N:P and P:C ratios are less accurate predictors (Figure 2.7 B,C), but they may be useful in the field where specific growth rates are often difficult to quantify.

Brevetoxin levels in cultures versus those observed in the field- A survey of field and laboratory measurements show a cellular brevetoxin range of 1-68 pg cell\(^{-1}\) (Table 2.1). Our previous study of N-limitation effects indicated a range of PbTx in nutrient-sufficient and N-limited \textit{K. brevis} of only 7-25 pg cell\(^{-1}\) (Hardison et al. 2012). By contrast, the range of values in this study ranged from 5-65 pg cell\(^{-1}\) (Figures 2.1 E-2.5 E). This larger range in toxicity per cell was due to both inherent strain differences and to higher PbTx cell\(^{-1}\) under P-limitation of growth rate. PbTx per cell in the five \textit{K. brevis} strains investigated ranged from 5-15 pg cell\(^{-1}\) under nutrient sufficiency (Table 2.2) and increased by up to 2.9- to 7.3-fold under P-limitation (Figures 2.1 E-2.5 E). These data indicate that P-limitation, rather than N-limitation can account for the full upper range of PbTx per cell observed in the field. It is also consistent with transient P-limitation having occurred during the time when some of the cells were collected in the field (Pierce et al. 2008).

The influence of P-limitation on toxin per cell observations in the field is only relevant if bloom waters in the Gulf of Mexico are indeed P-limited, either on a continuing or intermittent basis. Generally, the world’s ocean waters are considered to be N-limited except in the few regions where the ratio of net P- to N-inputs from various sources exceeds the average value in nutrient sufficient phytoplankton (generally 16:1) (Hecky & Kilham 1988, Moore et al. 2004). In the Gulf of Mexico there appears to be a number of factors operating
simultaneously, which may be fueling a long-term transition from a predominantly N-limited to a P-limited system. These factors include increases in atmospheric dust deposition which supplies iron and promotes increased N-fixation by cyanobacteria, N-enriched river inputs, and atmospheric deposition of reactive N. In the Gulf of Mexico, all these factors are shifting the N:P stoichiometry towards P-limitation of algal growth rate.

An increasing body of evidence indicates that iron limitation of N$_2$-fixation by cyanobacteria is a major contributor to the N-limited state of most surface ocean waters (Wu et al. 2000, Sohm et al. 2008, Sohm et al. 2011). However, in the tropical and subtropical North Atlantic and adjacent oligotrophic waters of the Gulf of Mexico and West Florida Shelf, atmospheric deposition of iron from Saharan dust promotes N$_2$-fixation by the cyanobacterium *Trichodesmium*, thereby driving these waters towards P-limitation of algal growth (Wu et al. 2000, Lenes et al. 2001). *Trichodesmium* has both behavioral and biochemical mechanisms that allow effective uptake of inorganic and organic phosphorus, even when present at extremely low concentrations (Dyhrman et al. 2006). In oligotrophic waters, N$_2$-fixation and concomitant depletion of P during *Trichodesmium* blooms may intensify P-limitation in co-occurring *Karenia brevis* populations (Walsh & Steidinger 2001, Vargo et al. 2008). Long term climate monitoring has shown that atmospheric deposition of Saharan dust to the ocean has increased 3- to 4-fold since the 1960’s as a result of drought conditions in the sub-Saharan region (Mahowald et al. 2009). If these trends continue, enhanced N$_2$-fixation should accelerate the shift from N- to P-limited phytoplankton growth currently occurring in the subtropical North Atlantic and Gulf of Mexico.
Another important source of nutrients to the Gulf of Mexico (GOM) is the Mississippi River. Shifts in agricultural practices and regulation of phosphate inputs over the past 50 years have led to both a significant increase in nutrient inputs to the GOM (Turner & Rabalais 1991, Simpson et al. 2008) and a fundamental shift toward higher N:P ratios, thereby favoring P-limitation of algal growth. Specifically, N:P ratios increased from 9:1 in 1966 to 38:1 in 1996, and to 20-60:1 in 2003-2008 (Howarth et al. 1996, Rabalais et al. 1996, Hill et al. 2011). Long term data from the Mississippi outflow in the GOM shows that N:P loading ratios rose from 16 in 1960 to 24 in 1987 (Rabalais et al. 1996). Currently, the Mississippi River is considered to be P-limited (Rabalais et al. 1996, Hill et al. 2011). This assessment was confirmed by seasonal bioassays in the Mississippi plume in the Gulf of Mexico in 2001, where >75% of the stations surveyed in the summer and early fall were found to be P-limited (Sylvan et al. 2006). Although the outflow of the Mississippi advects towards Texas from winter to spring, these nutrient-rich waters are transported towards the West Florida Shelf in summer and fall as a result of a shift in prevailing winds and may represent a significant nutrient source (Walker et al. 2005, Stumpf et al. 2008).

Increasing atmospheric inputs of reactive nitrogen from anthropogenic sources, such as NO\textsubscript{x} inputs from fossil fuel burning, may also contribute to a shift from N- to P-limitation of algal growth in the Gulf of Mexico. Atmospheric deposition from anthropogenic sources has become a major contributor of biologically available fixed nitrogen to the oceans and is projected to increase 4-fold by 2030 and may surpass N\textsubscript{2}-fixation as the primary source of N.
for the world’s oceans in the next 50 years (Duce et al. 2008). In contrast, atmospheric deposition of P is relatively small (Baker et al. 2003, Jickells 2006).

A recent study of nutrient inputs and ratios present on the west and southwest Florida shelf provides possible evidence for how frequently P-limitation is likely to occur in the different regions along the shelf (Heil et al. 2007). Based on particulate N:P ratios Heil et al. (Heil et al. 2007) concluded that the northern areas of the West Florida Shelf (WFS) were N-limited and that P-limitation became increasingly more common toward the western Florida Bay and Florida Keys. They proposed that the observed gradient was partly due to P inputs from the drainage of phosphate mines east of Tampa Bay resulting in N-limitation on the northern shelf. It was further hypothesized that the greater P-limitation in the south was caused by the outflow of waters from the Everglades with high N:P ratios, and the presence of carbonate sediments in Florida Bay and the southwest Florida shelf that bind and sequester phosphate from the overlying waters (Rivera-Monroy et al. 2011). Therefore, *K. brevis* blooms may become P-limited as they advect with southward flowing coastal currents along the WFS. These nutrient limitation dynamics also suggest that for a given cell concentration, blooms might be expected to be most toxic in southern Florida, and less toxic farther north.

_Ecosystem effects and management implications-_ The 2- to 5-fold increase of PbTx per mole cell carbon or unit of cell volume during periods of transient P-limitation in this study and N-limitation in a previous study (Hardison et al. 2012) means that marine ecosystems are likely exposed to significantly different toxin levels depending on the nutrient status of the cells. Given that cellular PbTxs released into the environment often absorb onto
organic surfaces such as sea grasses and accumulate in consuming organisms, high toxin levels may persist in the food chain long after a bloom has subsided (Flewelling et al. 2005, Hitchcock et al. 2012). Higher cellular toxin levels can also disrupt grazer-prey interactions. Copepod grazing on _K. brevis_ has been shown to decrease as PbTx per cell increases (Hong et al. 2012, Waggett et al. 2012). Reduced rates of grazing and grazing mortality provide _K. brevis_ with a selective advantage over competitors. In addition, as grazing on _Karenia_ is reduced or eliminated, grazer-mediated nutrient recycling is diminished. Lower recycling causes increased nutrient limitation of _Karenia brevis_ growth rates, ultimately yielding even more toxic cells. This sets up a positive feedback interaction with increasing _K. brevis_ cell densities having progressively greater negative impacts on the grazing, nutrient recycling and nutrient availability. This dynamic may foster the development and persistence of _Karenia brevis_ and other ecosystem disruptive algal bloom (EDAB) species (Mitra & Flynn 2006, Sunda et al. 2006).

The data presented here and previously (Hardison et al. 2012) indicate that brevetoxins increase under both N- and P-limitation, but that the increases in brevetoxins per cell under P-limitation are roughly twice those under N-limitation due to differential changes in cell size. Thus, for the same concentration of cells, a P-limited bloom would be twice as toxic as an N-limited one for the same degree of growth rate limitation. This difference is of significance since the severity of a bloom for the management of shellfish bed closures is based upon _Karenia brevis_ cell concentrations, with the false assumption that brevetoxin concentrations per cell do not vary (Heil 2009). Therefore public safety could be
compromised if a bloom became nutrient limited, and the risk could be much higher if the
bloom was P-limited. The potential for N- and P-limitation to increase bloom toxicity
provides further support for the need to directly measure brevetoxins as well as cell numbers
to accurately assess the potential impact of *Karenia brevis* containing waters to human health
and to enable proper regulation of shellfish bed closures by coastal managers (Plakas &
Dickey 2010).
Table 2.1. Mean values, standard deviations (SD), and ranges (min and max) for total brevetoxins per cell (pg cell\(^{-1}\)) from various published culture and field studies.

<table>
<thead>
<tr>
<th>Study Origin</th>
<th>Brevetoxin Content (pg cell(^{-1}))</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>culture strains (Baden &amp; Tomas 1988)</td>
<td></td>
<td>11</td>
<td>4</td>
<td>6</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>surf zone (Backer et al. 2005)</td>
<td></td>
<td>26</td>
<td>20</td>
<td>2</td>
<td>61</td>
<td>15</td>
</tr>
<tr>
<td>surf zone (Pierce et al. 2005)</td>
<td></td>
<td>18</td>
<td>3</td>
<td>12</td>
<td>23</td>
<td>8</td>
</tr>
<tr>
<td>surface samples (Pierce et al. 2008)</td>
<td></td>
<td>24</td>
<td>11</td>
<td>8</td>
<td>47</td>
<td>20</td>
</tr>
<tr>
<td>bottom samples</td>
<td></td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>surface and bottom samples (Tester et al. 2008)</td>
<td></td>
<td>16</td>
<td>11</td>
<td>1</td>
<td>68</td>
<td>118</td>
</tr>
</tbody>
</table>
Table 2.2. Average values ± standard deviations for growth rate (d⁻¹), volume per cell (μm³ cell⁻¹), chlorophyll \( a \) (fg μm⁻³) and (pg cell⁻¹), total brevetoxins (fg μm⁻³) and (pg cell⁻¹), percent brevetoxin as cellular C (%PbTx as cell C), PbTx production rate ([mmol PbTx C mol cell C⁻¹] d⁻¹), cellular phosphate (P):carbon (C) ratio (mmol/mol), and cellular carbon normalized to biovolume (mol C L⁻¹cell⁻¹) for phosphate replete and phosphate limited experiments with five strains of *Karenia brevis*.

<table>
<thead>
<tr>
<th></th>
<th>CCMP 2228</th>
<th>Wilson</th>
<th>SP2</th>
<th>CCMP 2820</th>
<th>CCMP 2229</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phosphate Replete</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth rate</td>
<td>(d⁻¹)</td>
<td>0.473±0.002</td>
<td>0.327±0.006</td>
<td>0.259±0.004</td>
<td>0.417±0.002</td>
</tr>
<tr>
<td>Volume per cell</td>
<td>(μm³ cell⁻¹)</td>
<td>3650±249</td>
<td>5668±416</td>
<td>5245±126</td>
<td>3826±249</td>
</tr>
<tr>
<td>Chlorophyll ( a )</td>
<td>(fg μm⁻³)</td>
<td>1.28±0.09</td>
<td>1.33±0.02</td>
<td>1.14±0.17</td>
<td>1.57±0.15</td>
</tr>
<tr>
<td>Total brevetoxin</td>
<td>(fg μm⁻³)</td>
<td>2.51±0.63</td>
<td>2.68±0.61</td>
<td>2.34±0.33</td>
<td>1.43±0.35</td>
</tr>
<tr>
<td></td>
<td>(pg cell⁻¹)</td>
<td>8.90±2.65</td>
<td>15.2±3.8</td>
<td>12.1±1.6</td>
<td>5.45±1.26</td>
</tr>
<tr>
<td></td>
<td>(%C-PbTx)</td>
<td>1.43±0.47</td>
<td>2.1±0.54</td>
<td>1.53±0.35</td>
<td>1.05±0.22</td>
</tr>
<tr>
<td>PbTx production rate</td>
<td>([mmol PbTx C mol C⁻¹] d⁻¹)</td>
<td>6.76±2.22</td>
<td>6.87±1.77</td>
<td>3.96±0.91</td>
<td>4.38±0.92</td>
</tr>
<tr>
<td>Cellular P:C</td>
<td>(mmol mol⁻¹)</td>
<td>7.40±1.73</td>
<td>20.0±7.5</td>
<td>12.9±0.8</td>
<td>12.1±2.1</td>
</tr>
<tr>
<td>Cellular C/biovolume</td>
<td>(mol C L⁻¹cell⁻¹)</td>
<td>10.78±1.43</td>
<td>7.48±1.42</td>
<td>7.43±1.41</td>
<td>7.67±0.74</td>
</tr>
</tbody>
</table>
### Phosphate Limited

<table>
<thead>
<tr>
<th></th>
<th>Growth rate (d⁻¹)</th>
<th>Volume per cell (µm³ cell⁻¹)</th>
<th>Chlorophyll a (fg µm⁻³)</th>
<th>Total brevetoxin (fg µm⁻³)</th>
<th>PbTx production ([mmol PbTx C mol C⁻¹] d⁻¹)</th>
<th>Cellular P:C (mmol mol⁻¹)</th>
<th>Cellular C/biovolume (mol C L_cell⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.099±0.004</td>
<td>5993±316</td>
<td>0.69±0.22</td>
<td>7.26±2.37</td>
<td>5.25±1.56</td>
<td>3.46±1.82</td>
<td>9.92±2.73</td>
</tr>
<tr>
<td></td>
<td>0.090±0.017</td>
<td>7687±1358</td>
<td>0.94±0.04</td>
<td>4.04±0.67</td>
<td>2.52±1.01</td>
<td>5.21±1.21</td>
<td>8.35±1.75</td>
</tr>
<tr>
<td></td>
<td>0.100±0.006</td>
<td>5551±438</td>
<td>0.58±0.11</td>
<td>4.90±0.75</td>
<td>4.47±0.50</td>
<td>4.49±2.20</td>
<td>6.36±1.52</td>
</tr>
<tr>
<td></td>
<td>0.135±0.007</td>
<td>4039±478</td>
<td>0.80±0.18</td>
<td>3.06±0.77</td>
<td>4.47±0.50</td>
<td>6.23±1.97</td>
<td>11.3±1.80</td>
</tr>
<tr>
<td></td>
<td>0.152±0.006</td>
<td>5571±395</td>
<td>0.71±0.17</td>
<td>4.89±1.39</td>
<td>2.21±0.94</td>
<td>3.76±1.12</td>
<td>10.6±3.11</td>
</tr>
</tbody>
</table>
Figure 2.1. Time-dependent results for semi-continuous batch cultures of *Karenia brevis* strain CCMP 2228 grown under nutrient-replete (open squares) and P-limited conditions (closed circles): (A) Curves for biovolume ($\mu L_{\text{cells}} L_{\text{media}}^{-1}$)(log scale) vs. time, (B) Curves for the natural log (ln) biovolume vs. time, correcting for culture dilution (Sunda et al. 2007), (C) Cellular phosphorus to carbon molar ratios (mmol mol$^{-1}$), (D) Mean volume per cell ($\mu m^3$ cell$^{-1}$), (E) Total brevetoxins per cell (pg cell$^{-1}$), (F) Chlorophyll $a$ normalized to cell volume (fg $\mu m^3$), (G) Total brevetoxins normalized to cell volume (fg $\mu m^3$), (H) Percent of total cellular carbon associated with brevetoxins (%C-PbTx). Error bars represent the standard deviation of triplicate measurements.
Biovolume ($\mu$L cells L$^{-1}$media)

P:C (mmol mol$^{-1}$)

Volume per cell ($\mu$m$^3$ cell$^{-1}$)

Total cellular brevetoxins (pg cell$^{-1}$)

Chlorophyll a (fg. $\mu$m$^{-3}$)

% C-PBTx

Day

P-replete

P-limit

$\mu$P-replete = 0.473 ± 0.002 d$^{-1}$

$\mu$P-limited = 0.099 ± 0.004 d$^{-1}$

$R^2$ = 0.999

$R^2$ = 0.973
Figure 2.2. Time-dependent results from semi-continuous batch cultures of *Karenia brevis* strain Wilson grown under nutrient-replete (open squares) and P-limited conditions (closed circles): (A) Curves for biovolume ($\mu$L$_{cells}$ L$_{media}^{-1}$) (log scale) vs. time, (B) Curves for ln biovolume vs. time, correcting for culture dilution (Sunda et al. 2007), (C) Cellular phosphorus to carbon molar ratios (mmol mol$^{-1}$), (D) Mean volume per cell ($\mu$m$^3$ cell$^{-1}$), (E) Total brevetoxins per cell (pg cell$^{-1}$), (F) Chlorophyll $a$ normalized to cell volume (fg $\mu$m$^3$), (G) Total brevetoxins normalized to cell volume (fg $\mu$m$^3$), (H) Percent of total cellular carbon associated with brevetoxins (%C-PbTx). Error bars represent the standard deviation of triplicate measurements.
Wilson

A  Biovolume (L cells L media$^{-1}$)

B  ln biovolume (L cells L media$^{-1}$)

C  P:C (mmol mol$^{-1}$)

D  Volume per cell ($\mu m^3$ cell$^{-1}$)

E  Total cellular brevetoxins (pg cell$^{-1}$)

F  Chlorophyll a (fg $\mu m^{-3}$)

G  Total cellular brevetoxins (fg $\mu m^{-3}$)

H  %C-PBTX

\[ \mu_P_{\text{replete}} = 0.327 \pm 0.006 \text{d}^{-1} \]
\[ R^2 = 0.997 \]

\[ \mu_P_{\text{limited}} = 0.090 \pm 0.017 \text{d}^{-1} \]
\[ R^2 = 0.870 \]
Figure 2.3. Time-dependent results from semi-continuous batch cultures of *Karenia brevis* strain SP2 grown under nutrient-replete (open squares) and P-limited conditions (closed circles): (A) Biovolume (µL(cells L-media⁻¹)) (log scale) vs. time, (B) Ln biovolume vs. time, correcting for culture dilution (Sunda et al. 2007), (C) Cellular phosphorus to carbon ratios (mmol mol⁻¹), (D) Mean volume per cell (µm³ cell⁻¹), (E) Total brevetoxins per cell (pg cell⁻¹), (F) Chlorophyll *a* normalized to cell volume (fg µm⁻³), (G) Total brevetoxins normalized to cell volume (fg µm⁻³), (H) Percent of total cellular carbon associated with brevetoxins (%C-PbTx). Error bars represent the standard deviation of triplicate measurements.
SP2

A

B

Biovolume (L cells L media⁻¹)

\[ P_\text{replete} = 0.259 \pm 0.004 \text{d}^{-1} \]
\[ R^2 = 0.998 \]

\[ P_\text{limited} = 0.100 \pm 0.006 \text{d}^{-1} \]
\[ R^2 = 0.977 \]

C

D

P:C (mmol mol⁻¹)

E

F

ln biovolume (L cells L media⁻¹)

G

H

Volume per cell (cm³ cell⁻¹)

Volume per cell (cm³ cell⁻¹)

% C-PbTx

Day

Day
Figure 2.4. Time dependent results from semi-continuous batch cultures a *Karenia brevis* strain CCMP 2820 grown under nutrient-replete (open squares) and P-limited conditions (closed circles): (A) Biovolume (µL<sub>cells</sub> L<sub>media</sub><sup>-1</sup>) (log scale) vs. time, (B) Ln biovolume vs. time, correcting for culture dilution (Sunda et al. 2007), (C) Cellular phosphorus to carbon ratios (mmol mol<sup>-1</sup>), (D) Mean volume per cell (µm<sup>3</sup> cell<sup>-1</sup>), (E) Total brevetoxins per cell (pg cell<sup>-1</sup>), (F) Chlorophyll $a$ normalized to cell volume (fg µm<sup>3</sup>), (G) Total brevetoxins normalized to cell volume (fg µm<sup>3</sup>), (H) Percent of total cellular carbon associated with brevetoxins (%C-PbTx). Error bars represent the standard deviation of triplicate measurements.
CCMP 2820

A. Biovolume (L cells L media⁻¹)

B. ln biovolume (L cells L media⁻¹)

C. P:C (mmol mol⁻¹)

D. Volume per cell (µm³ cell⁻¹)

E. Total cellular brevetoxins (fg cell⁻¹)

F. Chlorophyll a (µg  g⁻¹)

G. Total cellular brevetoxins (fg µm⁻³)

H. %C-PbTx

\[
\mu_{P\text{-replete}} = 0.417 \pm 0.002 \text{d}^{-1} \\
R^2 = 0.999
\]

\[
\mu_{P\text{-limited}} = 0.135 \pm 0.007 \text{d}^{-1} \\
R^2 = 0.965
\]
Figure 2.5. Time dependent results from semi-continuous batch cultures of *Karenia brevis* strain CCMP 2229 grown under nutrient-replete (open squares) and P-limited conditions (closed circles): (A) Biovolume ($\mu$L-cells L-media$^{-1}$) (log scale) vs. time, (B) Ln biovolume vs. time, correcting for culture dilution (Sunda et al. 2007), (C) Cellular phosphorus to carbon ratios (mmol mol$^{-1}$), (D) Mean volume per cell ($\mu$m$^3$ cell$^{-1}$), (E) Total brevetoxins per cell (pg cell$^{-1}$), (F) Chlorophyll $a$ normalized to cell volume (fg $\mu$m$^3$), (G) Total brevetoxins normalized to cell volume (fg $\mu$m$^3$), (H) Percent of total cellular carbon associated with brevetoxins (%C-PbTx). Error bars represent the standard deviation of triplicate measurements.
CCMP 2229

A. Biovolume (L cells L\text{-}media^{-1})

B. ln biovolume (L cells L\text{-}media^{-1})

C. P:C (mmol mol^{-1})

D. Volume per cell (\mu m^3 cell^{-1})

E. Total cellular brevetoxins (pg cell^{-1})

F. Chlorophyll \text{a} (fg m^{-2})

G. Total cellular brevetoxins (fg m^{-2})

H. %C-PbTx

\mu_{\text{P-replete}} = 0.467 \pm 0.002\text{d}^{-1}

\mu_{\text{P-limited}} = 0.152 \pm 0.006\text{d}^{-1}

R^2 = 0.999

R^2 = 0.971
Figure 2.6. Toxin relationships to P:C ratios. (A) Total brevetoxin per cell (pg cell⁻¹), (B) total brevetoxins normalized to cell volume (fg µm⁻³), and (C) % total cell carbon present as brevetoxins (%C-PbTx) plotted vs. cellular P:C ratio (mmol mol⁻¹). Error bars represent standard deviations of triplicate measurements for both x and y values. Data from P-replete and P-limited cultures are indicated by open and closed symbols, respectively.
Total brevetoxins (fg cell⁻¹)

Total brevetoxins (fg μm⁻³)

% C-PbTx

P:C (mmol mol⁻¹)
Figure 2.7. Predictors of carbon diversion to brevetoxins. Relationships between brevetoxin-carbon as a % of total cell carbon (%C-PbTx) and (A) specific growth rate (d⁻¹), (B) cellular N:P ratio (mol mol⁻¹), and (C) cellular P:C ratio (mmol mol⁻¹). Results from P-replete and P-limited cultures are indicated by open symbols and closed symbols, respectively. Error bars indicate standard deviations of triplicate measurements. Polynomial fits were applied to the data in all plots with their corresponding regression and p-values.
Figure 2.8. Comparison of N- and P-limitation studies of *Karenia brevis*. (A) Average total brevetoxin normalized to cell volume (fg um$^{-3}$), and (B) Average total brevetoxin per cell (pg cell$^{-1}$) in nutrient-replete and P-limited cultures in the present study and nutrient-replete and N-limited cultures in a previous study (Hardison et al. 2012). Data are presented for the three *Karenia brevis* strains common to both studies.
CHAPTER 3

Effects of Carbon Dioxide Limitation of Growth Rate on Cellular Brevetoxins in the Dinoflagellate *Karenia brevis*

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**ABSTRACT**

*Karenia brevis* forms high biomass toxic blooms in coastal regions of the Gulf of Mexico which can reach population densities of up to 20 million cells L\(^{-1}\). These blooms produce potent neurotoxins called brevetoxins (PbTxs), which adversely impact human health, marine ecosystems, and coastal economies. Recent evidence has shown that nitrogen and phosphorus limitation of growth rate substantially increases cellular brevetoxin concentrations in accordance with the carbon:nutrient balance (CNB) hypothesis, initially formulated for terrestrial plants. This hypothesis also predicts that increases in cellular toxins should be lessened if the toxin in question contains the limiting nutrient element. In the present study we tested this prediction by examining the effect of carbon dioxide (CO\(_2\)) limitation of *Karenia brevis* growth on cellular levels of brevetoxins, which contain 67% carbon (C) by weight. Brevetoxin content, expressed as percent cellular carbon (%C-PbTx), was ~2-fold higher in CO\(_2\)-limited cells than in control cells growing at their maximum rates, but were 20-40% lower than values previously observed for similar levels of growth rate limitation by phosphate. All strains experienced decreases in growth rate at CO\(_2\) concentrations < 2.4 µmol L\(^{-1}\) (pH 8.7) and ceased growing at ~1.0 µmol L\(^{-1}\) CO\(_2\) (pH ~9.0). The CO\(_2\) limited cells exhibited 50% higher cellular N:C ratios and 2-fold higher chl a:C ratios than observed for an equivalent reduction of growth rate under P-limitation. This effect was apparently largely due to an increased demand for photosynthetically produced ATP (and associated increase in photosynthetic capacity) to supply the energy needed to fuel the cell’s CO\(_2\) concentrating mechanism. Our findings indicate that current and future
anthropogenic increases in CO$_2$ concentrations in surface ocean waters are likely to increase
the toxicity of *K. brevis* blooms due to two interacting factors: 1) they will tend to increase
bloom biomass by decreasing CO$_2$ limitation of bloom growth, and 2) they should increase
the likelihood that a high density bloom will become N- or P-limited rather than CO$_2$-limited,
which will increase toxin levels per unit of cell biomass. By increasing bloom toxicity these
effects should increase the adverse effects of *K. brevis* blooms on human and ecosystem
health and coastal economies.
INTRODUCTION

The carbon:nutrient balance (CNB) hypothesis predicts that as phytoplankton growth slows due to limitation by nutrients such as N or P, an imbalance is created in which fixed carbon that would have otherwise gone into growth is no longer needed. The cells then divert this excess fixed carbon into defensive compounds or structures (Bryant et al. 1983, Ianora et al. 2006, Ianora et al. 2011). This hypothesis was originally developed from observations of the effects of growth limitation by nutrients on the grazing defense systems in terrestrial plants (Bryant et al. 1983). Diversion of excess fixed carbon into defensive compounds or structures as growth slows also helps protect the photosynthetic electron transport chain from over reduction and attendant oxidative stress by diverting surplus photosynthetic reductive capacity (NADPH) and energy (ATP) into the production of these compounds while simultaneously providing greater protection from grazers and pathogens (Hong et al. 2012, Waggett et al. 2012). Production of defensive compounds allows cells to make use of surplus carbon to increase survival during periods of low growth and reproduction (Sakshaug et al. 1989, Sunda et al. 2007, Schaeffer et al. 2009).

Recent experiments have shown that the production of brevetoxins (PbTxs) in the toxic dinoflagellate *Karenia brevis*, under both N and P limitation, conforms to the predictions of the CNB hypothesis. These experiments demonstrated that PbTxs normalized to cell volume concentrations as a percent of total carbon increased respectively by 2.8- and 2.2-fold when growth was limited by N and P (Hardison et al. 2012, Hardison et al. submitted). The greatest increase in cellular toxin content occurred during the transition
period from nutrient-replete to nutrient-limited growth when the imbalance between photosynthetic capacity and growth was greatest. As the cells down regulated their photosynthetic apparatus to bring photosynthesis and growth back into balance, the toxin concentrations in the cells declined, but remained significantly higher than observed during nutrient replete exponential growth. These data indicate that as blooms of *Karenia brevis* become nutrient limited due to high biomass demand for nutrients, they will become substantially more toxic (Hardison et al. 2012, Hardison et al. submitted). Such blooms frequently exceed 1,000,000 cells L\(^{-1}\) along the west Florida shelf so the combination of high biomass and nutrient limitation can generate extremely toxic blooms.

Unpublished measurements taken during high density blooms indicate that pH can exceed 8.8 to 9.0 (Steidinger personal communication) which corresponds to a five- to seven-fold decrease in CO\(_2\) concentrations due to high demand for CO\(_2\). Such CO\(_2\) depletion can also limit algal growth during blooms (Riebesell et al. 1993). The potential for nutrient vs. CO\(_2\) limitation raises an interesting question concerning the effective toxicity of high density blooms. The CNB hypothesis postulates that if a defensive compound contains the limiting nutrient element, less of the toxin will be produced than when growth is limited by an element not found in toxin (Granéli & Flynn 2006). For example, under nitrogen limitation, *Alexandrium tamarense* and *Alexandrium minutum* fail to show an increase in nitrogen containing saxitoxins (Granéli & Flynn 2006). In contrast, toxin concentrations increase dramatically under P-limitation due to the lack of phosphorus in the saxitoxin structure. Given that brevetoxins contain 67% carbon by weight and that they contain no nitrogen or
phosphorus, the CNB hypothesis predicts that growth limitation by CO$_2$ should cause a lower increase in cellular toxin levels than an equivalent limitation by N or P. The goal of this study was to test this prediction by determining the extent to which growth limitation by CO$_2$ affects PbTx content in otherwise nutrient-replete *Karenia brevis* cells compared to the toxicity increases observed in N- and P-limited, CO$_2$-sufficient cultures. Understanding this relationship has important implication for predicting and modeling the adverse public health and ecosystem effects of *Karenia* blooms and in predicting the effects of anthropogenic increases in CO$_2$ levels in surface seawater on bloom growth and toxicity, and associated adverse impacts. Brevetoxin production by these blooms cause neurotoxic shellfish poisoning (NSP) and respiratory distress in humans (Watkins et al. 2008, Backer 2009). These blooms are also responsible for massive fish kills, marine mammal mortalities, and significant economic losses in coastal communities (Flewelling et al. 2005, Hoagland & Scatasta 2006).

To examine the effect of CO$_2$-limitation on PbTx content in *K. brevis* cells, a series of semi-continuous and batch culture experiments were undertaken using 3 different isolates. The semi-continuous culture experiments employed two treatments. In the first control treatment, cells were diluted at a rate which allowed maximum rates of nutrient- and CO$_2$-sufficient growth. In the second treatment, cells were grown to a sufficiently high biomass that growth became limited by insufficient CO$_2$. The cells were then diluted with new medium at a rate of 0.1 d$^{-1}$ which imposed a constant CO$_2$-limited specific growth rate of 0.1 d$^{-1}$. This CO$_2$-limited specific growth was maintained for several weeks. In a second set of
experiments, the semi-continuous cultures growing at their maximum rates were split evenly. The first half served as a control and was maintained at maximal nutrient- and CO₂-sufficient growth rates in semi-continuous cultures. The other half was maintained as batch culture where the cells were allowed to grow until their growth rates slowed to zero (during stationary phase) due to insufficient CO₂ levels. Both control and batch cultures were periodically sampled for a period of four to six weeks. Sufficient nutrients were provided in the CO₂-limited batch cultures to ensure that the growth was limited by CO₂ rather than by nutrients such as N or P. Cells in the batch culture experiments experienced greater CO₂-limitation of growth rate than those in the corresponding 0.1 d⁻¹ semi-continuous treatment. Results in both sets of CO₂ limited cultures were compared with those observed in the control cells growing at their maximum growth rates. CO₂ concentrations in all cultures were computed from measured pH values. CO₂ concentrations were 5- to 10-fold lower in the CO₂-limited cultures relative to the controls. The nutrient status of the cells in the experimental cultures was confirmed by measuring cellular N:C ratios for each sampling period. For comparison with other studies, brevetoxin concentrations were normalized per cell, per unit of cell volume, and as a percent of total cellular fixed carbon.

Our results showed that CO₂-limited growth occurred as CO₂ concentrations declined below 2.4 µmol L⁻¹ (> pH 8.7). Brevetoxin:C ratios increased in the CO₂-limited cells, but not as much as occurred at comparable reduced growth rates under N- and P-limitation. However, in each isolate tested the cells still diverted more cellular carbon into PbTx than
found in exponentially growing CO₂-replete cells, indicating a strong selection pressure for diversion of carbon into defensive compounds even when CO₂ was the limiting nutrient.

MATERIALS and METHODS

Strains and culture conditions – Three *K. brevis* strains were examined in this study. Strains CCMP 2228 and CCMP 2229 were obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota (West Boothbay Harbor, ME, USA). Strain SP3 was kindly provided by Dr. Ed Buskey of University of Texas Marine Science Institute (Port Aransas, TX, USA).

Experimental cultures were grown in a media prepared by filtering Gulf Stream seawater (salinity 36) through 0.2 µm-pore Nuclepore filters to remove particles. The culture medium contained added vitamins (0.074 nmol L⁻¹ vitamin B₁₂, 0.4 nmol L⁻¹ biotin, and 60 nmol L⁻¹ thiamine), Na₂SeO₃ (10 nmol L⁻¹), and an EDTA-trace metal buffer system (100 µmol L⁻¹ EDTA, 1 µmol L⁻¹ FeEDTA, 50 nmol L⁻¹ MnCl₂, 40 nmol L⁻¹ CuCl₂, 100 nmol L⁻¹ ZnSO₄, and 40 nmol L⁻¹ CoCl₂) (Sunda et al. 2005). It also contained, 128 µmol L⁻¹ NaNO₃ and 8 µmol L⁻¹ NaH₂PO₄ to generate a Redfield N:P ratio of 16:1. Media were sterilized by microwave treatment (Keller et al. 1988). Once inoculated, cultures were placed in a Percival Scientific model I-36VLX incubator maintained at a constant temperature of 23°C and on a 14h:10h daily light:dark cycle to simulate summer light conditions. Photosynthetically active radiation (PAR) was provided at an intensity of 120 µmol quanta m⁻² s⁻¹ via vertically mounted fluorescent Duro-test Vita-lites. Light intensity was measured
with a Biospherical Instruments Inc. QSL-100 4π wand type light meter. Culture pH was measured throughout each experiment using a pH meter (Thermo Orion 3 Star Plus) equipped with a Ross ultra-combination pH electrode (resolution - 0.001; relative accuracy - ±0.002 pH units). A two-point calibration was performed daily before and after analysis using certified NIST traceable pH 7 and 10 buffers. The concentrations of CO₂ present in the media were calculated from measured pH values and alkalinity of Gulf Stream seawater (2.36 mmol kg⁻¹) using the CO2SYS computer program (Sunda & Cai 2012).

In a first set of experiments cells were grown semi-continuously under two sets of conditions. To initiate the experiment cells were inoculated into fresh media and were diluted every two to seven days at an average dilution rate equal to their maximum growth rates, which varied from 0.36 to 0.50 d⁻¹ depending on the isolate. The growth of these cultures was maintained at their maximal nutrient- and CO₂-sufficient rates by diluting them with new medium at low biomass (see Figs. 3.1-3.3A) and never letting their growth to become nutrient- or CO₂-limited (Tables 3.2 & 3.3). The lack of CO₂ limitation was indicated by the ambient computed CO₂ concentrations of between 15.3 and 4.72 µmol L⁻¹ which correspond to pH values of 8.1 to 8.5. The dilution process was continued until maximal steady state growth was observed for several weeks. Once this steady state growth had been achieved, each clonal culture was divided and diluted. One half of the culture for each isolate was grown under the same ~0.36 to 0.50 d⁻¹ dilution regime to maintain maximal nutrient- and CO₂-sufficient growth rates. The other half was allowed to grow into early stationary phase where the biomass was sufficiently high to cause CO₂ limitation of the
cellular growth rate. As soon as the cells reached early stationary phase, new medium was added every 2 to 3 days at an average dilution rate of 0.1 d⁻¹ which allowed maintenance of continuous CO₂ limited growth at a rate of 0.1 d⁻¹. The average measured pH in these cultures was 8.89-8.91 which corresponded to CO₂ concentrations of 1.2 to 1.3 µmol L⁻¹. These low values are consistent with CO₂ limitation of growth rate (Table 3.2). Cell concentrations, mean cell volume, growth rate, chl a, cell C and N, and brevetoxins were measured for each sampling period as described below. Specific growth rates and associated standard errors were calculated by linear regressions of the natural log of the total cell volume (µL_cells L_media⁻¹) versus time after correcting for serial culture dilutions (Sunda et al. 2007).

In a second set of experiments, semi-continuous cultures growing at their maximum nutrient- and CO₂- sufficient rates were diluted into two sets of media. The first set of cultures served as controls and were continued to be grown semi-continuously at their maximum rates as described above. The second set of cultures were not diluted further and were grown from CO₂- and nutrient-sufficiency into complete CO₂-limitation of growth as batch cultures. These batch cultures allowed us to assess the effects CO₂-limitation severe enough to cause a complete cessation of growth. The same parameters were measured at each sampling period as listed above in both sets of cultures. Supplemental L1 nutrients and trace metals were added to cultures after stationary phase was reached in the batch cultures to ensure that nutrient limitation was not occurring at the ambient CO₂ limiting concentrations (0.9 to 1.3 µmol L⁻¹) and pH (8.90-9.01) (Table 3.2). The addition of supplemental nutrients
caused no further increase in culture cell carbon or total cell volume confirming that cellular growth was limited by CO₂.

*Cell concentrations, mean volume, growth rate, chl a, cell C and N, and brevetoxins.*

Culture aliquots for determining cell concentration, mean volume per cell, growth rate, chl *a*, cellular N and C, and brevetoxins were collected in the middle of the light period (midday). The analyses were conducted in triplicate except for cellular carbon and nitrogen which were measured only in duplicate due to culture volume limitations. Results were expressed as mean values ± standard deviation unless otherwise noted.

Cell concentrations and mean volume per cell were measured with an electronic particle counter (Beckman Coulter Inc. Multisizer 3) equipped with a 100-µm high resolution aperture using a 0.5 mL sample volume. Cell growth curves were constructed as semi-log plots of total cell volume (µL<sub>cells</sub>L<sub>media</sub>⁻¹) versus time in days. Total cell volume (µL<sub>cells</sub>L<sub>media</sub>⁻¹) was calculated by multiplying the cellular concentration (cells L⁻¹) by the mean volume per cell (µL cell⁻¹). Specific growth rates were computed from linear regressions of the natural log of total cell volume versus time after correcting for culture dilution (Sunda et al. 2007).

Chlorophyll *a* was determined by filtering cells onto 25-mm GF/F filters, extracting them with a 90:10 acetone:water mixture, and measuring the fluorescence of the extracted chl *a* with a fluorometer (Turner Design 10-AU) (Welschmeyer 1994). Cellular carbon (C) and nitrogen (N) were determined by gentle filtration of cells onto precombusted 13 mm GF/F glass fiber filters followed by fuming with HCl overnight to remove inorganic carbon. These filter samples were then analyzed for cellular N and C with an elemental analyzer (EAS 4010...
Costech) (Hardison et al. 2012). The resulting N:C ratios were compared with those from previous CO₂-replete, N-limited and P-limited culture studies to confirm that the cultures were not nutrient limited (Table 3.3) (Hardison et al. 2012).

Brevetoxins were analyzed by the same procedures as in previous N-limitation and P-limitation experiments (Hardison et al. 2012, Hardison et al. submitted). Brevetoxins were extracted from whole culture aliquots using liquid/liquid separations with ethyl acetate. Prior to separations, aliquots of cell cultures were mixed 1:1 by volume with ethyl acetate and the mixture was sonicated with a microtip-equipped sonicator (Qsonica, Q700) for 1 minute. Complete cell disruption was confirmed by microscopy. The analysis gave total culture toxin, which was deemed appropriate since preliminary experiments showed >90% of culture toxins were intracellular, corroborating previous findings (Tester et al. 2008, Lekan & Tomas 2010). Collected ethyl acetate fractions were desalted with Milli-Q water (18.2 MΩ) and concentrated with a rotovap (Büchi R-210). Extraction efficiency was determined in every fraction by the addition of a synthetic PbTx-3 methyl-acetate internal standard (PbTx-42-acetate) (Lekan & Tomas 2010). Recovery efficiency typically ranged from 90-95%. Concentrated fractions were measured for brevetoxins using an Agilent 1100 LC coupled to a triple quadrupole mass spectrometer (Thermo-Finnigan TSQ Quantum) with an electrospray ion source interface. LC-MS-MS conditions have been previously described in detail (Cheng et al. 2005, Mendoza et al. 2008). An external standard curve of purified brevetoxins 1, 2, and 3 (World Ocean Solutions, Wilmington, NC, USA) was used to quantify amounts of extracted brevetoxins.
Calculations- All CO2-limited values presented in Tables 3.1, 3.2, and 3.3 were means of measurements taken after CO2 limitation of growth occurred, typically by days 10-15 depending on strain tested. Brevetoxin production rates in Table 3.1 ([mmol PbTx-C mol C⁻¹] d⁻¹) were calculated dividing the amount of C associated with the brevetoxins (mol L⁻¹) by the total cellular C (mol L⁻¹) and multiplying this value by specific growth rate (d⁻¹). In Figure 3.7, the maximum growth rates and associated chl \( \alpha \):C values were determined by averaging values from the control cultures of the CO2 semi-continuous and stationary phase experiments.

RESULTS

The maximal growth rates in the CO2-replete Karenia brevis cultures varied from 0.30 d⁻¹ for strain SP3 to 0.55 d⁻¹ for strain CCMP 2228 (Table 3.1; Figs 3.1-3.6 B). The corresponding cell volume varied from 3710-4231 µm³ for CCMP 2228, the fastest growing strain, to 5075-5443 µm³ for SP3, the slowest growing strain (Table 3.1, Figs 3.1-3.6 D). Average CO2 concentrations in the CO2-replete exponentially growing cultures ranged from 9.6 to 12.3 µmol L⁻¹ (Table 3.2). Average CO2 levels in the CO2-limited cultures growing at a specific rate of 0.1 d⁻¹ were substantially lower and ranged from 1.2 µmol L⁻¹ (SP3) to 1.3 µmol L⁻¹ (CCMP 2229)(Table 3.2). The CO2 concentrations in the stationary phase batch cultures were slightly lower and ranged from 0.9 µmol L⁻¹ (SP3) to 1.29 µmol L⁻¹ (CCMP 2229).
As CO₂ concentrations decline, one might expect the cells to become smaller to increase their surface to volume ratios and the diffusive flux of CO₂ across the cells’ surface boundary layers (Sunda & Hardison 2010). Strain CCMP 2228 followed this expected pattern and the cells decreased in size in both the CO₂-limited semi-continuous cultures and the batch cultures relative to values in the CO₂-sufficient control cultures (Figs. 3.1A, 3.4A). The mean volume per cell in CO₂-limited semi-continuous cultures of isolate CCMP 2229 was 7% lower than that of the control treatment, but the cell volume declined to 46% of the control value during stationary phase in the batch culture experiment (Figs. 3.2A, 3.5A). Likewise, the mean volume per cell in the SP3 isolate was 9% less than the control value in the CO₂-limited semi-continuous culture, but decreased by a much larger amount (34%) relative to the control during stationary phase in the batch culture experiment (Figs. 3.3A, 3.6A). These results indicated that under the most CO₂-limited conditions, cellular volumes decreased as expected, but that the amount of decrease varied among isolates and with the degree to which CO₂ limits growth rate.

Studies on nitrogen or phosphorus limitation of growth in Karenia brevis showed that as growth slowed, chl a was consistently down regulated to bring carbon fixation into balance with growth (Hardison et al. 2012, Hardison et al. submitted). In the present study a similar decrease was observed in chl a:cell volume ratios with the onset of CO₂-limitation of growth rate (at approximately days 12-13) in the semi-continuous cultures of the two faster growing strains CCMP 2228 and 2229 (Table 3.1). On average in these strains, chl a per unit of cell volume decreased to 45-50% of the control values in the CO₂-limited semi-continuous
cultures, and 55-61% in the stationary phase (0 d\(^{-1}\)) cultures (Table 3.1). By contrast, chl \(a\):cell volume ratios in the CO\(_2\)-limited semi-continuous and stationary phase cultures of strain SP3 were 7% and 16% higher than observed in the CO\(_2\)-sufficient control cultures, indicating no down regulation of the photosynthetic apparatus (Table 3.1). In contrast, somewhat different results are seen when chl \(a\) is normalized to cellular carbon (mmol chl \(a\):mol C\(^{-1}\)) owing to variations in C:cell volume ratios (Table 3.1; Figs. 3.1-3.6 F). Strains CCMP 2228 and 2229 showed slight decreases in chl a:C on the order of 10-30% in semi-continuous cultures (Table 3.2; Figs. 3.1F,3.2F). However, chl a:C ratios in these two strains decreased on average by 60-64% in the CO\(_2\) limited batch cultures (Table 3.2; Figs. 3.4F,3.5F). Chl a:C ratios in strain SP3 increased by 10-12% in both semi-continuous and batch CO\(_2\) limited experiments (Table 3.2; Figs. 3.3F,3.6F). Chl \(a\) per mole of cell C is plotted as a function of specific growth rate for CO\(_2\)-limited cultures in the present study and P-limited cells in a previous study (Hardison et al. submitted)(Fig. 3.7A). Collated data for the two controls of the semi-continuous and batch CO\(_2\) experiments are shown as an average for each strain in Figure 3.7A. These data show no substantial down-regulation of chl a:C ratios in clone CCMP 2228, a slight (27%) decrease in CCMP2229, and a slight (16%) increase in clone SP3 for CO\(_2\) limited cells growing at a specific rate of 0.1 d\(^{-1}\). By comparison, P-limited CCMP 2228 and 2229 cultures growing at rate of 0.1 d\(^{-1}\) down regulated their chl a:C ratios by roughly two-fold (Fig. 3.7A). A similar effect is seen for cellular N:C ratios, which exhibited 3-21% higher values in the CO\(_2\) limited cultures than in the nutrient and CO\(_2\) sufficient controls (Table 3.3; Fig. 3.7B). By comparison the cellular
N:C ratios in P-limited cells of strains CCMP 2228 and CCMP 2229 growing at the same low specific rate (0.1 d\(^{-1}\)) were 20-21% lower than values in the CO\(_2\) and nutrient sufficient cultures (Fig. 3.7B). These results indicate that all strains respond to CO\(_2\) limitation by requiring higher chl \(a:C\) and N:C ratios to support a given growth rate than under limitation by nutrients such as N and P. These increases are likely due to much higher demand for photosynthetically produced ATP under CO\(_2\) limitation needed to fuel cellular carbon concentrating mechanisms, as has been argued previously for similar effects on chl:C ratios in diatoms (Sunda & Huntsman 2005). This effect appears to be greatest in clone SP3 where the chl \(a:C\) ratio actually increased with CO\(_2\) limitation of growth rate and the chl \(a:C\) and N:C values were higher in the stationary phase cultures (\(\mu = 0\) d\(^{-1}\)) than in the other two strains (Fig. 3.7A,B). Again, we argue that this increase in chl \(a:C\) and N:C ratios under CO\(_2\)-limitation of growth rate relative to those observed under P- or N-limitation of growth is due to an increase in the photosynthetic apparatus and attendant increases in chl \(a\), light harvesting protein-pigment complexes, and proteins involved in primary photosynthesis, electron transport, and photosynthetic ATP synthesis. The increase in N:C ratios may also be caused by up-regulation of proteins directly involved in the carbon concentrating mechanism such as carbonic anhydrase (Badger & Price 1994, Sunda & Huntsman 2005).

In previous nutrient limitation experiments, brevetoxin per unit cell volume (fg \(\mu m^3\)), per cell (pg cell\(^{-1}\)), and as a percent of cellular fixed carbon (%C-PbTx) all increased initially as cells became growth limited and then declined by varying amounts as the photosynthetic apparatus was down regulated to acclimate to the new lower growth rate, as predicted by the
CNB hypothesis (Bryant et al. 1983). Growth limitation by CO₂ would be expected to decrease the ability of cells to divert fixed carbon into PbTxs thereby potentially altering the magnitude and timing of the PbTx response to slowed growth compared to that observed in nutrient limited, CO₂-replete cells. In this study, the cellular PbTx response varied depending on how the toxin data were normalized. This occurs because the various ways of normalizing the PbTx data are influenced by variations in volume per cell and fixed carbon per unit of cell volume (Table 3.1). Brevetoxin expressed as a percent of cell carbon biomass provides a measure of physiological changes occurring in the cell regardless of changes in cell size or cell carbon per liter of biovolume. Brevetoxin per unit of cell volume (fg μm⁻³) provides an integrated measure of how CO₂-limitation simultaneously affects the brevetoxin:C ratio and the C:volume ratio. Finally brevetoxin per cell is determined by the combined effects of the latter two changes plus variations in volume per cell. When the PbTx concentration in the semi-continuous CO₂-limited cultures of strains CCMP 2228 and CCMP 2229 were normalized to cell carbon and biovolume, they showed a pattern similar to that observed previously for N- and P-limited cultures. Values for PbTx μm⁻³ began to increase on ~day 10 as growth began to slow and reached a peak around day 15-17. It then declined toward control values toward the end of the experiment (Figs 3.1-3.2 G&H). In the stationary phase of the batch culture experiments, the %C allocated to PbTxs showed a similar pattern as above for clone CCMP 2229 (Fig. 3.5H), but exhibited divergent behavior for PbTx per unit of cell volume (Fig. 3.5G) and per cell (Fig. 3.5E) because of changes in C:volume ratios (Table 3.1) and volume per cell (Fig. 3.5D) in the stationary phase cultures.
The increases in cellular brevetoxins under CO₂ limitation was compared with those observed under equivalent growth rate limitation in our previous experiments (Hardison et al. 2012, Hardison et al. submitted) (Fig. 3.8). Consistent with the CNB hypothesis, all strains increased in toxicity as growth slowed but to varying degrees. Most toxin increases under CO₂-limitation for these three strains were less than their corresponding increases under nutrient limitation at the same decreased growth rates. These results again are consistent with the CNB hypothesis as brevetoxins contain carbon, but not N or P. The increased toxicity of *Karenia brevis* strains under CO₂ and nutrient limitation varied among the strains, with the majority of CO₂ limited cells being the less toxic than nutrient limited ones (Fig. 3.8). For strains CCMP 2228 and CCMP 2229 the mean PbTx per cell was either the same or increased by 1.65-fold under CO₂ limitation compared to increases of 2- to 4.5-fold under nutrient limitation (Table 3.1, Fig. 3.8A). Strain SP3 did not follow this trend and had a higher per cell toxicity under semi-continuous CO₂ limitation than under N-limitation, yet it had equivalent brevetoxin concentrations under severe CO₂ limitation in the stationary phase of the batch culture experiment (Fig. 3.8A). When cell toxins were evaluated on a cell volume basis (fg µm⁻³), all three strains had 1.3- to 2-fold less brevetoxin concentrations under CO₂ limitation than for similar decreased growth rates under nutrient limitation (Fig. 3.8B). However, strain CCMP 2229 had higher brevetoxins per unit of cell volume during stationary phase in the CO₂–limited batch culture experiment due to a 49% decrease in volume per cell.
Toxin as a percent of total cell carbon was the most straightforward of all the cell toxin normalizations, but it can only be presented for the present experiments and previous P-limited ones because of a lack of accurate cell carbon data in our former N-limited experiments (Fig. 3.8C). Specifically, though the C-normalized data were previously measured for N-limitation of strain CCMP 2228 (Hardison et al. 2012), a comparison of the data from the previous nutrient sufficient controls and those conducted in the current study indicate that carbon per cell was underestimated in the original study (Hardison et al. submitted). The %C-PbTx measured in this study was lower in both strains CCMP 2228 and CCMP 2229 under CO₂ limitation than under P-limitation for the same low culture dilution rate (0.1 d⁻¹) indicating a decrease in the fraction of cellular C that was diverted into brevetoxins. The effect of N- or P-limitation was not examined in previous studies for strain SP3, so similar comparisons cannot be made for this strain. However, this strain showed similar relative increases in %C-PbTx values as strain CCMP 2228 under CO₂ limitation, and slightly lower increases than in strain CCMP 2229 (Fig. 3.8C).

Need to incorporate how the percent increase in biomass caused by the drop in pH over the next 100 years into the paragraph discussing this topic.

**DISCUSSION**

This study documented the effect of CO₂ limitation of growth rate on cellular brevetoxin levels in *Karenia brevis* to determine if the cellular response conforms to the CNB hypothesis. The CNB hypothesis states that as plant or algal growth slows under...
nutrient limited conditions, an imbalance occurs between carbon fixation and the supply of the limiting nutrients (Bryant et al. 1983, Bryant et al. 1985). The algae or plants then divert some of the excess fixed carbon into defensive compounds such as toxins to help alleviate this imbalance. This diversion serves two functions: 1) it protects the photosynthetic apparatus from over reduction and resulting oxidative stress until a balance between C-fixation and growth can be achieved and 2) it decreases grazing mortality rates (Hong et al. 2012, Waggett et al. 2012) to bring them more into line with the lower nutrient-limited rates of cellular growth and reproduction. Studies of N- and P-limitation of *Karenia brevis* have shown large increases in cellular brevetoxin:C ratios in accordance to the CNB hypothesis (Hardison et al. 2012, Hardison et al. submitted). However, for CO₂ limitation of *Karenia brevis* there is no excess fixed carbon to divert into toxins, but there still is a need for an increase in grazer protection. A corollary of the CNB hypothesis states that if the growth limiting nutrient is also contained in the toxin, then that toxin production would be limited in magnitude (Granéli & Flynn 2006). This indeed is the case for the production of brevetoxins since these toxins contain 67% C by weight and contain no N or P. Therefore, according to the CNB hypothesis, growth limitation by low CO₂ availability should also limit the amount of brevetoxin increase. Our results show that *Karenia brevis* does in fact increase its cellular brevetoxins as a percent of carbon as growth slows even under CO₂-limited conditions. However, the increase as a percent of fixed carbon (%C-PbTx) is lower by 20-40% when compared with cellular toxin increases under P-limitation (Fig. 3.8C). Thus our results conform to the predictions of the CNB hypothesis. The results also indicate that even under
severe CO₂-limitations there has been strong selection for investment in C-containing toxins as growth slows.

Because of the low CO₂ binding affinity of the carbon fixing enzyme Rubisco most cells employ carbon concentrating mechanisms (CCMs) to pump CO₂ into the cell (Falkowski & Raven 2007b). Previous studies have shown that the growth rate of various phytoplankton species is limited by CO₂ over a wide range of pH values (8.3-10) and associated large range of CO₂ concentrations (Hansen 2002). This large range of growth limiting CO₂ values was attributed to variations in the ability of different species to concentrate CO₂ intracellularly via various carbon concentrating mechanisms (CCMs). The data from this study suggests that *Karenia brevis* has a relatively efficient CCM as it is able to grow optimally at CO₂ concentrations down to 2.4 µmol L⁻¹ (pH 8.7) and growth rate ceases at CO₂ levels of ~ 1 µmol L⁻¹ (pH 9.0) (Figs. 3.4C-3.6C; Table 3.2). This ability to grow at low CO₂ concentrations likely contributes to its ability to form high biomass blooms (Figs. 3.1A,B,C-3.6A,B,C). While comparable data from the literature is limited, there is data on a co-occurring red tide dinoflagellate *K. mikimotoi*. The growth limiting pH values for this dinoflagellate are the same as we found for *K. brevis*: growth was limited by CO₂ at pH >8.7 and growth ceased at pH 9.0 (Hansen 2002).

The CCMs are fueled by photosynthetically produced ATP although the exact nature of the CCM is not known in most cases, including in *K. brevis* (Giordano et al. 2005). Our results show additional evidence for a robust CCM in *Karenia brevis*. First, we showed that as cells grew into CO₂ limitation they increased their chl *a:C* ratios relative to those observed
under similar N- or P-limitation of growth rate. This relative increase indicated an up-regulation of the cells’ photosynthetic apparatus, apparently to provide the additional ATP required by the CCMs to supply sufficient CO₂ inside the cells and more importantly within the chloroplast stroma, the site of CO₂-fixation by Rubisco. Second, we observed an increase in N:C ratios in CO₂-limited cells relative to values observed under P-limitation of growth rate, consistent in an increased investment of N into proteins within the photosynthetic apparatus and the CCM. These experiments also showed differences among strains with SP3 requiring higher chl a:C and N:C ratios to achieve similar CO₂ limited growth rates than strains CCMP 2228 and 2229.

Implications for studying toxicity of *K. brevis* in cultures and field studies. The results of our experiments have major implications for the measurement of the toxin content and the effect of nutrient limitation on toxin production in other toxic dinoflagellates and other toxic algal species. Typically, the effects of nutrient limitation on toxin production are measured in high density cultures because of the necessity of maintaining sufficiently high cell concentrations to accurately count by microscope. These dense cultures have the advantage that smaller aliquots are required to accurately measure algal numbers, biomass, growth rates, and toxin concentrations. Our results, demonstrate that in many cases these cultures are likely to be CO₂-limited, particularly in species with inefficient CCMs or lacking CCMs altogether. This CO₂-limitation can greatly reduce or abrogate nutrient responses which might otherwise be apparent in non CO₂-limited cultures. Nutrient effect experiments should be conducted in low density, non CO₂-limited cultures (Hardison et al. 2012,
Hardison et al. submitted), and high density culture experiments should be reserved for examining the effects of CO₂-limitation.

Our experiments also demonstrate the need for coastal managers and scientists to collect accurate pH measurements during oceanographic cruises studying Karenia brevis blooms. Those measurements can be converted to CO₂ concentrations and used to estimate how often and under what environmental conditions blooms are CO₂-limited. These data are crucial for predicting bloom toxicity and understanding when blooms are likely to have the greatest adverse effects on ecosystem services and public health.

The results of our study indicate that when Karenia brevis blooms reach sufficiently high cell densities (~5 to 10 million cells L⁻¹, depending on the strain), the growth demand for CO₂ exceeds the relatively slow rate at which atmospheric CO₂ can diffuse into water, and growth becomes CO₂-limited (Figs 3.1-3.6A)(Reinfelder 2011). High density Karenia brevis blooms can reach cell concentrations of up to 20 million cells L⁻¹ (Tester et al. 2008) along the west Florida shelf where they have caused significant adverse human health, ecological and economic impacts (Hoagland et al. 2009, Fleming et al. 2011). The growth of most high density K. brevis blooms are likely to become either nutrient (Hardison et al. 2012, Hardison et al. submitted) or CO₂-limited because of the high biomass demand for growth limiting resources. The finding that K. brevis cells accumulate less PbTx under CO₂-limitation than when growth slows under equivalent N- or P-limitation has important implications for predicating the toxicity of blooms and their adverse impacts. The results of this and previous studies predict that on a per cell basis, the increase in toxicity induced by
N-limitation would be reduced by 1.3- to 2-fold under equivalent CO₂-limitation of growth rate, and by 2- to 5-fold when P is the limiting nutrient. Thus, whether a bloom becomes CO₂- or nutrient-limited has the potential to significantly influence the toxicity of high density blooms.

Atmospheric CO₂ concentrations have increased by ~40% from the beginning of the industrial era to the present due to the burning of fossil fuels and land use changes (Sunda & Cai 2012). If current industrial and agricultural activities continue, CO₂ levels will increase another 2-fold by the end of the century (Feely et al. 2009). Equilibration of surface ocean waters with this higher atmospheric CO₂ results in proportional increases in surface water CO₂ concentrations and associated increases in total inorganic carbon (TIC). Based on our results, these higher TIC concentrations should increase the toxicity of K. brevis blooms because of two interacting effects. First, elevated ocean TIC concentrations should allow Karenia brevis blooms to achieve higher bloom biomasses, which in itself will increase the toxicity of the bloom. A second consequence of anthropogenic increases in ocean CO₂ concentrations is the increased likelihood that the growth of Karenia blooms becomes nutrient-limited rather CO₂-limited. If blooms become nutrient limited under these higher CO₂ conditions, there would be an increase in brevetoxin concentrations for a given bloom density resulting in more toxic and severe blooms. Together such CO₂ driven increases in bloom biomass and in toxin per unit of biomass should substantially increase bloom toxicity and resulting adverse impacts on human health, coastal ecosystems, and coastal economies. These effects are likely already occurring to some extent given the current 40% higher levels
of atmospheric CO₂ due to man’s activities compared to preindustrial times and will only worsen in the future with continuing anthropogenic increases in atmospheric CO₂.
Table 3.1. Average values ± standard deviations for specific growth rate (d\(^{-1}\)), volume per cell (\(\mu\text{m}^3\ \text{cell}^{-1}\)), chlorophyll \(a\) normalized on a \(\text{fg} \ \mu\text{m}^3\) and \(\text{mmol} \ \text{chl} \ \text{a} \ \text{mol} \ C^{-1}\) basis, total brevetoxins normalized as \(\text{fg} \ \mu\text{m}^3\), pg cell\(^{-1}\), and as a percent of total cellular C (%C-PbTx), and average brevetoxin production rates ([mmol PbTx C / mol C]/d) for CO\(_2\) replete and CO\(_2\)-limited experiments using three strains of \textit{Karenia brevis} (CCMP 2228, CCMP 2229 and SP3).

<table>
<thead>
<tr>
<th>Nutrient replete</th>
<th>CCMP 2228</th>
<th>CCMP 2229</th>
<th>SP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate (d(^{-1}))</td>
<td>0.504±0.003</td>
<td>0.468±0.002</td>
<td>0.368±0.003</td>
</tr>
<tr>
<td>Volume per cell ((\mu\text{m}^3\ \text{cell}^{-1}))</td>
<td>4231±304</td>
<td>4654±239</td>
<td>5443±178</td>
</tr>
<tr>
<td>Chlorophyll (a) ((\text{fg} \ \mu\text{m}^3))</td>
<td>1.74±0.06</td>
<td>1.64±0.11</td>
<td>1.28±0.079</td>
</tr>
<tr>
<td>Chlorophyll (a) ((\text{mmol} \ \text{chl} \ \text{a} \ \text{mol} \ C^{-1}))</td>
<td>0.196±0.029</td>
<td>0.164±0.013</td>
<td>0.125±0.006</td>
</tr>
<tr>
<td>Total brevetoxin ((\text{fg} \ \mu\text{m}^3))</td>
<td>2.73±0.29</td>
<td>1.61±0.32</td>
<td>3.22±0.52</td>
</tr>
<tr>
<td>Total brevetoxin (pg cell(^{-1}))</td>
<td>11.4±0.96</td>
<td>7.21±1.29</td>
<td>17.4±2.58</td>
</tr>
<tr>
<td>Total brevetoxin (% C-PbTx)</td>
<td>1.93±0.42</td>
<td>0.96±0.14</td>
<td>1.57±0.2</td>
</tr>
<tr>
<td>PbTx ([mmol PbTx C / mol C]/d)</td>
<td>9.73±2.12</td>
<td>4.49±0.66</td>
<td>5.78±0.74</td>
</tr>
<tr>
<td>PbTx production rate C per cell volume (mol C / L_{cells})</td>
<td>8.30±1.50</td>
<td>9.42±1.26</td>
<td>11.4±0.81</td>
</tr>
</tbody>
</table>
Table 3.1 Continued

**CO₂ limited**

<table>
<thead>
<tr>
<th></th>
<th>CCMP 2228</th>
<th>CCMP 2229</th>
<th>SP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate ($d^{-1}$)</td>
<td>0.091±0.006</td>
<td>0.088±0.002</td>
<td>0.099±0.004</td>
</tr>
<tr>
<td>Volume per cell ($µm³$ cell$^{-1}$)</td>
<td>2858±287</td>
<td>4320±223</td>
<td>4949±301</td>
</tr>
<tr>
<td>Chlorophyll $a$ (fg $µm³$)</td>
<td>0.880±0.137</td>
<td>0.88±0.05</td>
<td>1.39±0.11</td>
</tr>
<tr>
<td></td>
<td>(mmol chl $a$ mol $C^{-1}$)</td>
<td>0.173±0.033</td>
<td>0.122±0.029</td>
</tr>
<tr>
<td>Total brevetoxin (fg $µm³$)</td>
<td>3.26±0.59</td>
<td>2.76±0.57</td>
<td>4.89±1.16</td>
</tr>
<tr>
<td></td>
<td>(pg cell$^{-1}$)</td>
<td>9.36±2.38</td>
<td>11.9±1.83</td>
</tr>
<tr>
<td></td>
<td>(%C-PbTx)</td>
<td>3.10±0.66</td>
<td>2.24±0.84</td>
</tr>
<tr>
<td>PbTx ([mmol PbTx C / mol C]/d)</td>
<td>2.82±0.63</td>
<td>1.97±0.74</td>
<td>2.33±0.33</td>
</tr>
<tr>
<td>C per cell volume (mol C / L$_{cells}$)</td>
<td>6.78±1.54</td>
<td>7.72±1.87</td>
<td>11.4±0.73</td>
</tr>
</tbody>
</table>

**Stationary Experiments**

<table>
<thead>
<tr>
<th></th>
<th>CCMP 2228</th>
<th>CCMP 2229</th>
<th>SP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate ($d^{-1}$)</td>
<td>0.550±0.004</td>
<td>0.524±0.003</td>
<td>0.303±0.004</td>
</tr>
<tr>
<td>Volume per cell ($µm³$ cell$^{-1}$)</td>
<td>3710±383</td>
<td>4441±201</td>
<td>5075±389</td>
</tr>
</tbody>
</table>
Table 3.1 Continued

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll $a$</td>
<td>1.50±0.18</td>
<td>1.47±0.13</td>
<td>0.912±.077</td>
</tr>
<tr>
<td>(fg μm$^{-3}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol chl $a$ mol C$^{-1}$)</td>
<td>0.132±0.013</td>
<td>0.129±0.007</td>
<td>0.073±0.008</td>
</tr>
<tr>
<td>Total brevetoxin</td>
<td>2.53±0.38</td>
<td>3.31±0.43</td>
<td>2.56±0.35</td>
</tr>
<tr>
<td>(fg μm$^{-3}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pg cell$^{-1}$)</td>
<td>8.84±1.44</td>
<td>14.9±2.05</td>
<td>12.2±1.77</td>
</tr>
<tr>
<td>(%C-PbTx)</td>
<td>1.21±0.13</td>
<td>1.43±0.17</td>
<td>1.033±0.18</td>
</tr>
<tr>
<td>PbTx</td>
<td>6.65±0.72</td>
<td>7.49±0.89</td>
<td>5.78±0.74</td>
</tr>
<tr>
<td>([mmol PbTx C / mol C]/d)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>production rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C per cell volume</td>
<td>11.79±1.01</td>
<td>13.03±1.39</td>
<td>13.91±0.79</td>
</tr>
<tr>
<td>(mol C / L$_{cells}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CO$_2$ stationary phase growth

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth rate</td>
<td>-0.0009±0.0021</td>
<td>-.0202±0.0022</td>
<td>0.0172±0.009</td>
</tr>
<tr>
<td>(d$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume per cell</td>
<td>2350±146</td>
<td>2263±497</td>
<td>3568±320</td>
</tr>
<tr>
<td>(μm$^3$ cell$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorophyll $a$</td>
<td>0.689±0.201</td>
<td>0.572±0.239</td>
<td>1.06±0.073</td>
</tr>
<tr>
<td>(fg μm$^{-3}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(mmol chl $a$ mol C$^{-1}$)</td>
<td>0.063±0.023</td>
<td>0.046±0.026</td>
<td>0.081±0.006</td>
</tr>
<tr>
<td>Total brevetoxin</td>
<td>5.25±1.33</td>
<td>6.54±1.40</td>
<td>4.03±0.84</td>
</tr>
<tr>
<td>(fg μm$^{-3}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(pg cell$^{-1}$)</td>
<td>12.2±2.75</td>
<td>14.74±3.24</td>
<td>14.2±2.32</td>
</tr>
<tr>
<td>(%C-PbTx)</td>
<td>2.12±0.43</td>
<td>2.35±0.80</td>
<td>1.54±0.32</td>
</tr>
<tr>
<td>C per cell volume</td>
<td>12.79±1.57</td>
<td>14.63±3.84</td>
<td>11.4±0.73</td>
</tr>
<tr>
<td>(mol C / L$_{cells}$)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3.2. Average pH and corresponding calculated seawater CO₂ (µmol L⁻¹) concentrations for three *Karenia brevis* strains once the semi-continuous (µ = 0.1 d⁻¹) and batch cultures (µ = 0 d⁻¹) had become CO₂-limited. The CO₂ values were calculated from the pH values as described in Sunda and Cai (2012). Also shown are the maximum pH and corresponding minimum seawater CO₂ concentrations observed for each strain of *Karenia brevis* during either the semi-continuous or batch culture experiments. Standard deviations were calculated from triplicate pH measurements done when each sample was taken.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Semi-continuous experiments</th>
<th></th>
<th>Stationary experiments</th>
<th>Minimum CO₂ and maximum pH achieved</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO₂-replete CO₂-limited, 0.1 d⁻¹</td>
<td></td>
<td>CO₂-replete CO₂-limited, 0.0 d⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CO₂ (µmol L⁻¹) pH</td>
<td>CO₂ (µmol L⁻¹) pH</td>
<td>CO₂ (µmol L⁻¹) pH</td>
<td>CO₂ (µmol L⁻¹) pH</td>
</tr>
<tr>
<td>CCMP 2228</td>
<td>11.5±3.6 8.22±0.13 1.27±0.40 8.89±0.08</td>
<td>12.3±3.4 8.19±0.12 1.00±0.28 8.96±0.08</td>
<td>0.987±0.133 8.95±0.04</td>
<td></td>
</tr>
<tr>
<td>CCMP 2229</td>
<td>11.2±3.6 8.23±0.11 1.30±0.34 8.89±0.07</td>
<td>9.56±4.64 8.31±0.19 1.29±0.54 8.90±0.10</td>
<td>1.04±0.16 8.94±0.04</td>
<td></td>
</tr>
<tr>
<td>SP3</td>
<td>12.1±3.1 8.20±0.09 1.20±0.42 8.91±0.09</td>
<td>11.2±2.6 8.22±0.08 0.903±0.299 9.01±0.06</td>
<td>0.867±0.127 8.99±0.04</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.3. Average values ± standard deviations for cellular N:C ratios (mol mol⁻¹) for CO₂-replete and CO₂-limited semi-continuous and stationary phase batch cultures of isolates CCMP 2228, CCMP 2229 and SP3. The values used to calculate the averages were only those collected after the cultures had become CO₂-limited after approximately day 10 of each experiment.

<table>
<thead>
<tr>
<th>N:C (mol mol⁻¹)</th>
<th>CCMP 2228</th>
<th>CCMP 2229</th>
<th>SP3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Semi-continuous cultures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂-replete</td>
<td>0.124±0.008</td>
<td>0.119±0.009</td>
<td>0.122±0.005</td>
</tr>
<tr>
<td>CO₂-limited</td>
<td>0.134±0.006</td>
<td>0.131±0.007</td>
<td>0.138±0.007</td>
</tr>
<tr>
<td><strong>Batch culture experiment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CO₂-replete semi-continuous</td>
<td>0.117±0.005</td>
<td>0.109±0.012</td>
<td>0.114±0.009</td>
</tr>
<tr>
<td>CO₂-limited stationary phase</td>
<td>0.120±0.015</td>
<td>0.117±0.015</td>
<td>0.138±0.019</td>
</tr>
</tbody>
</table>
Figure 3.1. Time-dependent results for semi-continuous batch culture of *Karenia brevis* strain CCMP 2228 grown under nutrient/CO₂ replete (closed circles) and CO₂ limited (open squares) conditions: (A) Growth curves of total biovolume ($\mu L_{\text{cells}} L_{\text{culture}}^{-1}$) vs. time ($d^{-1}$), (B) Curves for the natural log (ln) total biovolume vs. time correcting for dilution (Sunda et al. 2007), (C) Measured culture pH values vs. time ($d^{-1}$), (D) Mean volume per cell ($\mu m^3$ cell$^{-1}$), (E) Total brevetoxins (pg cell$^{-1}$), (F) Chlorophyll $a$ normalized to cell carbon (mmol mol$^{-1}$), (G) Total brevetoxins normalized to cell volume (fg $\mu m^{-3}$), (H) Brevetoxins as a percent of cellular carbon (%C-PbTx). Error bars represent the standard deviation of three replicate measurements, except for F and H where the cell carbon values were measured in duplicate.
CCMP 2228

**A** Total biovolume ($\mu L_{\text{cells}} L_{\text{culture}}^{-1}$)

**B** Ln biovolume ($\ln L_{\text{cells}} L_{\text{culture}}^{-1}$)

- **CO$_2$ replete**
  - $\mu_{\text{CO}_2 \text{ replete}} = 0.504 \pm 0.003$ d$^{-1}$
  - $r^2 = 0.999$

- **CO$_2$ limited**
  - $\mu_{\text{CO}_2 \text{ limited}} = 0.091 \pm 0.006$ d$^{-1}$
  - $r^2 = 0.989$

**C** pH

**D** Volume per cell ($\mu m^3$)

**E** Total Brevetoxins (pg cell$^{-1}$)

**F** chl $\alpha$ : C (mmol mol$^{-1}$)

**G** Total brevetoxins (fg $\mu m^{-3}$)

**H** %C-PbTx

---

*Graphs and data analysis notes.*
Figure 3.2. Time-dependent results for semi-continuous batch culture of *Karenia brevis* strain CCMP 2229 grown under nutrient/CO$_2$ replete (closed circles) and CO$_2$ limited (open squares) conditions: (A) Growth curves of total biovolume ($\mu$L$_{\text{cells}}$ L$_{\text{culture}}^{-1}$) vs. time (d$^{-1}$), (B) Curves for the natural log (ln) total biovolume vs. time correcting for dilution (Sunda et al. 2007), (C) Measured culture pH values vs. time (d$^{-1}$), (D) Mean volume per cell ($\mu$m$^3$ cell$^{-1}$), (E) Total brevetoxins (pg cell$^{-1}$), (F) Chlorophyll $a$ normalized to cell carbon (mmol mol$^{-1}$), (G) Total brevetoxins normalized to cell volume (fg $\mu$m$^{-3}$), (H) Brevetoxins as a percent of cellular carbon (%C-PbTx). Error bars represent the standard deviation of three replicate measurements, except for F and H where the cell carbon values were measured in duplicate.
CCMP 2229

A. Total biovolume (L_{cell} L_{culture}^{-1})

B. Ln biovolume (L_{cell} L_{culture}^{-1})

C. pH

D. Volume per cell (μm^3 cell^{-1})

E. Total brevetoxins (pg cell^{-1})

F. chl a : C (mmol mol^{-1})

G. Total brevetoxins (fg m^{-2})

H. % C-PbTx

---

Total biovolume:

\[ \text{Total biovolume} = \mu \times t \]

\[ \text{Ln biovolume} = \mu \times t \]

\[ \mu_{\text{CO}_2 \text{ replete}} = 0.468 \pm 0.002 \ d^{-1} \]

\[ \mu_{\text{CO}_2 \text{ limited}} = 0.088 \pm 0.002 \ d^{-1} \]

\[ r^2 = 0.999 \]

\[ r^2 = 0.970 \]

---

Volume per cell:

\[ \text{Volume per cell} = \text{Volume per cell} \]

---

Total brevetoxins:

\[ \text{Total brevetoxins} = \text{Total brevetoxins} \]

---

Chl a : C:

\[ \text{Chl a : C} = \text{Chl a : C} \]

---

Total brevetoxins:

\[ \text{Total brevetoxins} = \text{Total brevetoxins} \]

---

% C-PbTx:

\[ \% \text{C-PbTx} = \% \text{C-PbTx} \]

---

Day 0 10 20 30 40
Figure 3.3. Time-dependent results for semi-continuous batch culture of *Karenia brevis* strain SP3 grown under nutrient/CO₂ replete (closed circles) and CO₂ limited (open squares) conditions: (A) Growth curves of total biovolume (µL cells L⁻¹ culture) vs. time (d⁻¹), (B) Curves for the natural log (ln) total biovolume vs. time correcting for dilution (Sunda et al. 2007), (C) Measured culture pH values vs. time (d⁻¹), (D) Mean volume per cell (µm³ cell⁻¹), (E) Total brevetoxins (pg cell⁻¹), (F) Chlorophyll *a* normalized to cell carbon (mmol mol⁻¹), (G) Total brevetoxins normalized to cell volume (fg µm⁻³), (H) Brevetoxins as a percent of cellular carbon (%C-PbTx). Error bars represent the standard deviation of three replicate measurements, except for F and H where the cell carbon values were measured in duplicate.
Total biovolume ($L_{cell} L_{culture}^{-1}$)

$pH$

Ln biovolume ($L_{cell} L_{culture}^{-1}$)

Volume per cell ($\mu m^3 cell^{-1}$)

Total brevetoxins (pg cell$^{-1}$)

chl $a : C$ (mmol mol$^{-1}$)

Total brevetoxins (fg $m^{-3}$)

%C-PbTx

Day

$\mu_{CO_2 replete} = 0.368 \pm 0.003 d^{-1}$

$r^2 = 0.999$

$\mu_{CO_2 limited} = 0.099 \pm 0.004 d^{-1}$

$r^2 = 0.989$

SP3

$\mu_{CO_2 replete} = 0.989r d^{-1}$

$\mu_{CO_2 limited} = 0.099 \pm 0.004 d^{-1}$

$r^2 = 0.989$
Figure 3.4. Time-dependent results for semi-continuous batch cultures of the CO₂ replete (closed circles) *Karenia brevis* strain CCMP 2228 and a batch culture of this strain which was allowed to grow into CO₂ limitation in stationary phase (open squares): (A) Growth curves of total cell volume (µL_{cells} L_{culture}^{-1}) vs. time (d^{-1}), (B) Curves for the natural log (ln) total cell volume vs. time correcting for dilution (Sunda et al. 2007), (C) Measured culture pH vs. time (d^{-1}), (D) Mean volume per cell (µm³ cell⁻¹), (E) Total brevetoxin per cell (pg cell⁻¹), (F) Chlorophyll *a* normalized to cell carbon (mmol mol⁻¹), (G) Total brevetoxins normalized to cell volume (fg µm⁻³), (H) Brevetoxins as a percent of cellular carbon (%C-PbTx). Error bars represent the standard deviation of three replicate measurements, except for F and H where the cell carbon values were measured in duplicate.
2228-CO$_2$ stationary

A. Total biovolume (L$_{cells}$/L$_{culture}$)

B. Ln biovolume (L$_{cells}$/L$_{culture}$)

C. pH

D. Volume per cell ($\mu$m$^3$/cell$^{-1}$)

E. Total brevetoxin (pg cell$^{-1}$)

F. Chl $a$ : C (mmol mol$^{-1}$)

G. Total brevetoxin (fg $m^{-3}$)

H. %C-PbTx

Day 0 5 10 15 20 25 30
Figure 3.5. Time-dependent results for semi-continuous batch cultures of the CO$_2$ replete *Karenia brevis* strain CCMP 2229 (closed circles) and a batch culture of this strain which was allowed to grow into CO$_2$ limitation in stationary phase (open squares): (A) Growth curves of total biovolume ($\mu$L cells L$_{culture}^{-1}$) vs. time (d$^{-1}$), (B) Curves for the natural log (ln) total biovolume vs. time correcting for dilution (Sunda et al. 2007), (C) Measured culture pH vs. time (d$^{-1}$), (D) Mean volume per cell ($\mu$m$^3$ cell$^{-1}$), (E) Total brevetoxins per cell (pg cell$^{-1}$), (F Chlorophyll $a$ normalized to cell carbon (mmol mol$^{-1}$), (G) Total brevetoxins normalized to cell volume (fg $\mu$m$^{-3}$), (H) Brevetoxins as a percent of cellular carbon (%C-PbTx). Error bars represent the standard deviation of three replicate measurements, except for F and H where the cell carbon values were measured in duplicate.
2299-CO$_2$ stationary

- **Total biovolume** ($\mu$L cells L$_{culture}^{-1}$)
- **Ln biovolume** ($\mu$L cells L$_{culture}^{-1}$)
- **pH**
- **Volume per cell** ($\mu$m$^3$ cell$^{-1}$)
- **Total brevetoxin (pg cell$^{-1}$)**
- **Chl a : C (mmol mol$^{-1}$)**
- **%C-PbTx**

**Legend:**
- **• CO$_2$ replete**
- **- CO$_2$ limited**

**Graphs**

- **Graph A:** Total biovolume over time (Day 0 to 50).
- **Graph B:** Ln biovolume over time (Day 0 to 50).
- **Graph C:** pH over time (Day 0 to 50).
- **Graph D:** Volume per cell over time (Day 0 to 50).
- **Graph E:** Total brevetoxin over time (Day 0 to 50).
- **Graph F:** Chl a : C over time (Day 0 to 50).
- **Graph G:** %C-PbTx over time (Day 0 to 50).

**Equations:**
- $\mu_{CO_2 \text{ replete}} = 0.524 \pm 0.033 \text{ d}^{-1}$
- $r^2 = 0.999$
- $\mu_{CO_2 \text{ limited}} = -0.0202 \pm 0.0022 \text{ d}^{-1}$
- $r^2 = 0.926$

**Table:**

<table>
<thead>
<tr>
<th>CO$_2$ status</th>
<th>CO$_2$ replete</th>
<th>CO$_2$ limited</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>8.2</td>
<td>8.4</td>
</tr>
<tr>
<td>8.6</td>
<td>8.8</td>
<td>9.0</td>
</tr>
<tr>
<td>9.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note:** The table shows the pH levels at different CO$_2$ status conditions.
Figure 3.6. Time-dependent results for semi-continuous batch culture of the CO₂ replete of *Karenia brevis* strain SP3 (closed circles) and a batch culture of this strain which was allowed to grow into CO₂ limitation in stationary phase (open squares): (A) Growth curves of total biovolume (µL cells L⁻¹ culture⁻¹) vs. time (d⁻¹), (B) Curves for the natural log (ln) total biovolume vs. time correcting for dilution (Sunda et al. 2007), (C) Measured culture pH vs. time (d⁻¹), (D) Mean volume per cell (µm³ cell⁻¹), (E) Total brevetoxins per cell (pg cell⁻¹), (F) Chlorophyll *a* normalized to cell carbon (mmol mol⁻¹), (G) Brevetoxins normalized to cell volume (fg µm⁻³), (H) Brevetoxins as a percent of cellular carbon (%C-PbTx). Error bars represent the standard deviation of three replicate measurements batch culture of this strain which was allowed to grow into CO₂ limitation stationary phase.
SP3-CO$_2$ stationary

![Graphs showing various measurements over time](image)

- Total biovolume ($L_{cells} L_{culture}^{-1}$)
- Ln biovolume ($L_{cells} L_{culture}^{-1}$)
- pH
- Volume per cell ($m^3 cell^{-1}$)
- Total brevetoxin (pg cell$^{-1}$)
- Chl $a$: C (mmol mol$^{-1}$)
- %C-PbTx

Day 0 5 10 15 20 25 30

**CO$_2$ replete**
- $\mu_{CO_2$ replete} = 0.303 \pm 0.004 d^{-1}$
- $r^2 = 0.999$

**CO$_2$ limited**
- $\mu_{CO_2$ limited} = 0.017 \pm 0.009 d^{-1}$
- $r^2 = 0.497$
Figure 3.7. Average values for (A) Chl $a$ normalized to cell C (mmol chl $a$ mol C$^{-1}$) vs. specific growth rate ($\mu$) and (B) nitrogen:carbon (N:C) ratios (mol mol$^{-1}$) vs. specific growth rate in three *Karenia brevis* strains. The data from CO$_2$-limited cultures experiments in this study are indicated by open circles (CCMP 2228), open squares (CCMP 2229) and open triangles (SP3) joined by solid lines. Data from a previous study in which the growth rate of *Karenia* was limited by phosphorus (Hardison et al. submitted) are indicated by closed circles (CCMP 2228) and squares (CCMP 2229) joined by dashed lines (Hardison et al. submitted).
growth rate ($\mu$ d$^{-1}$)

N:C (mol mol$^{-1}$)

Chl $a$ normalized to cell carbon (mol chl $a$ mol C$^{-1}$)

A

B

-CCMP 2228 CO$_2$
-CCMP 2229 CO$_2$
-SP3 CO$_2$
-CCMP 2228 P
-CCMP 2229 P

growth rate (d$^{-1}$)
Figure 3.8. A comparison of the toxin response of three strains of *Karenia brevis* collated from four separate nutrient- and CO$_2$-limited growth studies. Control values across all three limitations were averaged together and are represented by the black bars. The N-limited (diagonal hatch/grey), P-limited (white), CO$_2$ limited 0.1 d$^{-1}$ (cross hatch/grey), and CO$_2$ limited stationary phase (grey) values were calculated by averaging values after the reduction of growth rate occurred following approximately day 10 in each of the studies. The data were normalized as: (A) Total brevetoxins per cell (pg cell$^{-1}$), (B) Brevetoxins normalized to cell volume (fg µm$^{-3}$), and (C) Brevetoxins as a percent of total cellular carbon (%C-PbTx).
Total brevetoxin (pg cell$^{-1}$)

A

control

N-limited 0.1 d$^{-1}$

P-limited 0.1 d$^{-1}$

CO$_2$ limited 0.1 d$^{-1}$

CO$_2$ limited 0.0 d$^{-1}$

Total brevetoxin (fg um$^{-3}$)

B

control

N-limited 0.1 d$^{-1}$

P-limited 0.1 d$^{-1}$

CO$_2$ limited 0.1 d$^{-1}$

CO$_2$ limited 0.0 d$^{-1}$

%C-PbTx

C

CCMP 2228

CCMP 2229

SP3
SUMMARY

The primary goal of this dissertation project was to test whether cellular brevetoxins (PbTxs) increase in response to N-, P- and CO₂-limitation of growth rate as predicted by the carbon:nutrient balance (CNB) hypothesis. This hypothesis states that as growth slows due to a limiting nutrient that algal species will divert an increased amount of their fixed carbon into defensive compounds or structures. A corollary to this hypothesis is if the defensive compound or toxin contains the nutrient element, then the cellular increase in that compound or toxin would be limited. The findings from this study confirm the predictions of the CNB hypothesis. They showed that growth limitation by the nutrient elements N and P causes *Karenia brevis* to divert a greater percentage of fixed carbon into brevetoxins, thereby increasing brevetoxin:cell carbon ratios. This strategy simultaneously helps protect the photosynthetic apparatus from over reduction during the unbalanced growth brought on by N- and P-limitation and provides enhanced grazing defense. The latter should increase net survival rates under reduced rates of cellular growth and reproduction during nutrient limitation. Limitation of growth rate by N and P caused similar average 2.1- to 2.3-fold increases in cellular brevetoxins per unit of cell volume. Brevetoxins contain 67% carbon by weight, so according to the CNB hypothesis, CO₂-limitation should cause a lower increase in cellular brevetoxin to cell volume ratios than equivalent growth limitation by N or P. This indeed was observed under CO₂ limitation of growth rate there was an average 1.5-fold increase in brevetoxin:cell volume ratios, compared to the average 2.2-fold increase observed under N- and P-limitation. Under CO₂-limitation, there was no “excess fixed carbon” to be
diverted into toxin production so the entire effect here was likely due to a preferential allocation of fixed carbon into brevetoxins to increase grazing defenses.

The data from this study also showed that how the brevetoxin values are normalized can lead to different conclusions about how *Karenia brevis* responds to nutrient limitation. Currently, most researchers and managers normalize toxin levels on a pg cell\(^{-1}\) basis. This study showed that toxin normalization on a per cell basis can be misleading. For example, N- and P-limitation studies showed similar increases in cellular PbTxs on a cell volume basis, but showed a two-fold larger increase in PbTx per cell under P-limitation compared to N-limitation. This apparent difference in toxicity response was caused by the N-limited cells getting smaller and P-limited cells increasing in size, resulting in a two-fold higher average volume per cell in the P-limited cultures than in the N-limited ones. Thus, on a per cell basis the P-limited cells appeared to be twice as toxic as the N-limited cells. Overall, toxin normalization to cell carbon (e.g., as a percent of cellular carbon [%C-PbTx]) is the best normalization of brevetoxins. It describes the percent allocation of cell carbon to brevetoxins regardless of changes in volume per cell or cell C to volume ratios.

Experimental design is a critically important part of any study. Over the course of this dissertation, there were several important experimental design modifications that helped us obtain repeatable results not observed in previous studies. All *Karenia brevis* cultures were acclimated to maximum growth under nutrient and CO\(_2\) replete conditions for approximately four to six weeks. Over this acclimation period, cells were not allowed to reach high enough concentrations to deplete nutrients or CO\(_2\) to levels that limit growth rates.
These cultures were diluted with fresh media before such limitation occurred which allowed for continuous maximum exponential growth rates that were higher than in all previous culture studies with the same strains. Our cultures were not grown in the commonly used L1 or f/2 media which have major nutrient concentrations far exceeding those we employed. Instead the media we used was a custom mix that included a trace metal buffer system that did not allow for precipitation of metals as typically is observed with L1 and f/2 media.

Likewise, we maintained our cultures at low cell densities in the N- and P- limitation studies to prevent CO₂ limitation of growth rate. As seen in Chapter 3, CO₂ limitation can affect results by increasing cellular brevetoxins. The use of these improved experimental designs allowed us to observe the nutrient limitation effects on cellular brevetoxins that have eluded others in the past.

The results of the study also have significant ramifications for resource managers and public health officials that have the task of tracking and monitoring *Karenia brevis* blooms and their adverse environmental, human health, and economic impacts. Currently, when *Karenia brevis* blooms occur, shellfish beds are closed when cell concentrations exceed 5000 cells L⁻¹. The inherent assumption behind this approach is that the toxicity of each cell is the same. The nutrient-limitation experiments conducted in this study demonstrated that cell concentrations alone cannot provide an accurate prediction of bloom toxicity and that the toxicity of individual blooms may vary considerably even if cell concentrations are the same. This means that a threshold level of 5,000 cells L⁻¹ may not always provide adequate protection against adverse impacts of brevetoxins. These results suggest greater emphasis
should be placed on getting direct toxin measurements in addition to the cell counts. Coastal managers and public health officials who have access to ambient cellular nutrient data may be able to use that information to predict the severity of a bloom and what resources may be needed to cope with the subsequent public health and environmental damage. Specifically, N:C or P:C ratios in *K. brevis* cells can be used to assess N- or P-limitation of growth to predict when cellular brevetoxins are likely to increase. The CO$_2$ limitation study has highlighted at what pH and CO$_2$ concentration a high density bloom’s growth should become limited in high nutrient situations. This study provided insight into the effect of increasing CO$_2$ concentrations in surface seawater linked to ongoing anthropogenic increases in atmospheric CO$_2$ on *K. brevis* bloom toxicity. Such increases in CO$_2$ are likely to increase the toxicity of *K. brevis* blooms by supplying additional carbon to support bloom growth and by increasing the likelihood that bloom growth becomes limited by nutrients (N or P) rather than by CO$_2$. The current findings should help coastal managers and public health officials to ensure public safety and protect ecosystem health by providing better prediction of *K. brevis* bloom toxicity and adverse impacts.
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