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An investigation of the mechanisms promoting toxic dinoflagellate blooms in estuarine

ecosystems

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Abstract of the Dissertation

An investigation of the mechanisms promoting toxic dinoflagellate blooms in estuarine

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Dinoflagellates can cause harmful algal blooms (HABs) associated with human health syndromes such as paralytic shellfish poisoning (PSP) and diarrhetic shellfish poisoning (DSP) and these HABs are a growing concern in many coastal regions. My dissertation explored the factors promoting toxic dinoflagellate blooms in estuarine ecosystems. Northport Bay, NY, which has experienced chronic PSP-induced shellfish closures due to the toxic dinoflagellate *Alexandrium fundyense* and blooms of the DSP-producer, *Dinophysis acuminata*, was used as a natural system to explore these factors while multiple strains of *A. fundyense* and a newly established *D. acuminata* culture isolated from North America were investigated in controlled, laboratory experiments.

Culture experiments, field experiments, and field observations demonstrated that North American strains of *Alexandrium* were capable of allelopathically inhibiting competing autotrophic nanoflagellates and diatoms. This inhibition contributed to elevated organic matter

production, bacterial respiration, and pCO_2 levels during *Alexandrium* blooms. Levels of pCO_2 recorded in Northport Bay (~1700µatm) were highest in regions with the greatest *Alexandrium* abundances. Culture and field experiments demonstrated that elevated pCO_2 (~1700µatm) enhanced *Alexandrium* growth rates and, on occasion, toxicity, suggesting that blooms may be promoted by coastal ocean acidification.

Dinophysis acuminata blooms exceeding one million cells L^{-1} were documented for the first time anywhere during my dissertation, and in several cases DSP toxins in shellfish exceeded the USFDA action level (160 ng g^{-1} of shellfish tissue). A four year field investigation of *D. acuminata* blooms revealed that abundances were significantly correlated with multiple nutrients. Field and culture experiments demonstrated that both inorganic and organic nutrients were capable of significantly enhancing *Dinophysis* growth and toxicity. Hence, this dissertation provides the first evidence that blooms of this ubiquitous, toxic dinoflagellate can be directly promoted by nutrient loading.

This dissertation demonstrated that multiple biological and chemical factors can act in combination to promote dinoflagellate growth and toxicity which, in turn, can affect human health and local economies. Hence, these findings contribute toward the information needed to develop management practices that mitigate blooms and protect human health. While factors such as competition, nutrients, and acidification were investigated here, additional factors that affect dinoflagellate blooms warrant future exploration.

Dedication Page

I dedicate this to my parents and my husband. Thank you for always supporting my endeavors.

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Chapter 1

Introduction

Coastal marine ecosystems are among the most ecologically and socioeconomically productive on the planet, providing an estimated US$25 trillion, or about 77% of the global total, in ecosystem goods and services annually (Martínez et al., 2007). About 40% of the global population lives within 100km of the coast (Martínez et al., 2007) and nearly 75% of the US population lives within 75km of the coastline, making these regions subject to a suite of anthropogenically induced stresses (de Jonge et al., 2002; Valiela, 2006) which in turn can lead to ecological perturbations such as harmful algal blooms and hypoxia (Cloern, 2001; Anderson et al., 2008; Heisler et al., 2008). Anthropogenic nutrient and organic matter loading, increases in CO_2 levels and subsequent decreases in pH are all factors associated with cultural eutrophication (Nixon, 1995; Borges and Gypens, 2010; Cai et al., 2011) and have been cited as factors influencing harmful algal blooms (HABs) around the world (Hallegraeff, 1993; Anderson et al., 2008; Fu et al., 2008; Heisler et al., 2008; Fu et al., 2010; Hallegraeff, 2010; Fu et al., 2012), many of which can disrupt important ecosystem services and coastal industries (Cosper et al., 1987; Hoagland et al., 2002; Bricelj and MacQuarrie, 2007), as well as negatively affect human health (Anderson et al., 2012).

The spatial and temporal expansion and increased intensity of HABs is a globally recognized phenomenon (Hallegraeff, 1993; Glibert et al., 2005). HABs associated with human health syndromes, for example, paralytic shellfish poisoning (PSP) and diarrhetic shellfish poisoning (DSP), are a growing human health and economic concern in many coastal regions (Anderson et al., 2008; Anderson et al., 2012). These dinoflagellate-related HABs are often associated with substantial economic losses due to the closure of shellfish beds containing toxic shellfish (Hoagland et al., 2002; Koukaras and Nikolaidis, 2004; Jin and Hoagland, 2008; Jin et al., 2008). HABs undergo several developmental phases including initiation, growth and

maintenance, and termination (Steidinger and Garcés, 2006). Documenting the factors affecting the growth and maintenance period is of critical importance as this is the time period during which vegetative toxin-forming cells can accumulate in shellfish. Any factor acting to extend this developmental phase can have subsequent effects on human health and coastal economies. Moreover, the need for species-specific, even strain-specific, information has become increasingly important as it has become clear that a one-explanation-fits-all bloom populations scenario does not exist (Burkholder and Glibert, 2009; Pitcher, 2012). Given the human health threats that toxin producing blooms pose and the global increase in these events (Hallegraeff, 1993; Van Dolah, 2000), more strain-specific documentation and research is needed to understand what promotes and aids in the maintenance of these blooms.

Blooms of the dinoflagellate *Alexandrium* are common to coastal regions around the world and are particularly harmful because they produce saxitoxins, the suite of toxins that cause the potentially fatal human health syndrome, paralytic shellfish poisoning (PSP; Anderson, 1994; Anderson, 1997; Van Dolah, 2000; Glibert et al., 2005). *Alexandrium* blooms are especially common along the northeast coast of the United States where they occur as both large-scale coastal events (Anderson, 1997; Anderson et al., 2005a; Anderson et al., 2005b; Townsend et al., 2005) as well as regional events in estuaries and coastal embayments (Anderson and Morel, 1979; Anderson, 1997; Hattenrath et al., 2010). *Alexandrium* blooms on the east coast of North America exhibit a north to south PSP toxicity gradient with northern strains (e.g. Canada, Maine, Massachusetts) predominantly synthesizing the more potent carbamate toxins and the southern strains (e.g. Connecticut, New York) containing a higher ratio of the less potent N-sulfocarbamoyl toxins (Maranda et al., 1985; Anderson et al., 1990; Anderson et al., 1994; Bricelj and Shumway, 1998). The presence of *A. fundyense* on Long Island was first

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documented during the early 1980's (Anderson et al., 1982; Schrey et al., 1984). At that time, moderate densities of *A. fundyense* ($> 10^2$ cell L^{-1}) were found on the north shore of Long Island in Northport Bay and Mattituck Inlet (Schrey et al., 1984); these blooms, however, were not associated with PSP events (e.g. toxic shellfish or human illness; Anderson et al., 1982; Schrey et al., 1984). Since 2006, however, Northport Bay, NY, has hosted chronic *A. fundyense* blooms with shellfish bed closures occurring for six out of the last seven years (2006 - 2012; Hattenrath et al., 2010). While some aspects of the ecology of *Alexandrium* blooms in Europe, Australia, and Asia have been established, far less is known regarding the ecology of *Alexandrium* blooms in North America, particularly in estuarine ecosystems. While the effects of inorganic nutrients on *Alexandrium* densities and toxicity have been studied in NY (Hattenrath et al., 2010), the effects of changing pCO_2 concentrations resulting from cultural eutrophication (Borges and Gypens, 2010; Cai et al., 2011), have not been assessed. In addition, other factors that can potentially affect the growth and maintenance of these blooms in eutrophied estuaries, such as community interactions (i.e. allelopathy; Tillmann and John, 2002; Fistarol et al., 2004a; Fistarol et al., 2004b), have never been investigated in North America.

In contrast to PSP, diarrhetic shellfish poisoning (DSP) is globally less common with recurring cases primarily occurring in Europe and Southeast Asia (Hallegraeff, 1993; Van Dolah, 2000). Both *Prorocentrum lima* and other bloom-forming dinoflagellates of the *Dinophysis* genus have been implicated in DSP events and closures around the world (Hallegraeff, 1993; Van Dolah, 2000; Reguera et al., 2012). These dinoflagellates synthesize the causative toxins of DSP, namely okadaic acid (OA), and associated congeners, dinophysistoxins (DTXs; Lee et al., 1989). Also found associated with DSP toxins are another group of toxins known as pectenotoxins (PTXs), which are potentially hepatotoxic to, and promote tumor formation in,

mammals when injected intraperitoneally (Lee et al., 1989; Burgess and Shaw, 2001). While PSP closures are a common occurrence in North America, (Anderson, 1997; Todd, 1997; Anderson et al., 2005a; Anderson et al., 2008; Jester et al., 2009; Hattenrath et al., 2010), there are relatively few reports of DSP closures (Quilliam et al., 1991; Subba Rao et al., 1993; Todd, 1997; Tango et al., 2004; Deeds et al., 2010). On the east coast of North America most reports of DSP-producing blooms have been attributed to the benthic dinoflagellate *Prorocentrum lima* (Marr et al., 1992; Morton et al., 1999; Maranda et al., 2007) with a handful of reports associated with toxin-producing blooms of the *Dinophysis* genus (Maranda and Shimizu, 1987; Cembella, 1989; Subba Rao et al., 1993; Tango et al., 2004). The rarity of DSP closures due to *Dinophysis* blooms in North America is despite the recent isolation of toxin-producing *Dinophysis* clones from the Northeast coast of North America (Hackett et al., 2009; Fux et al., 2011). While over a decade of monitoring on Long Island (NY, USA) showed that twelve species of *Dinophysis* were present across multiple harbors, cells densities were generally low ($<10^4$ cells L^{-1}) and toxic shellfish were not observed in these regions (Freudenthal and Jijina, 1988). During a recent investigation of *Alexandrium* in Northport Bay, NY (Hattenrath et al., 2010), *Dinophysis* densities were one to two orders of magnitude greater than those reported by Freudenthal and Jijina (1988) over 25 years ago. Given this information a re-evaluation of DSP and factors promoting DSP blooms in North American coastal waters is needed.

Nutritional Ecology of Harmful Algae

Harmful algae, dinoflagellates in particular, are known to possess a diverse array of nutritional strategies (Jacobson and Anderson, 1996; Jacobson, 1999; Stoecker, 1999; Jeong et al., 2005; Glibert and Legrand, 2006; Stoecker et al., 2006; Burkholder et al., 2008), including phototrophy, heterotrophy (osmotrophy (uptake of dissolved organic substances) or phagotrophy

(ingestion of particles or prey items)), and mixotrophy when a combination of these strategies is employed. The nutritional strategy of dinoflagellates may change depending on ambient inorganic nutrient concentrations and light levels and it has been hypothesized that limitations of light or nutrients may cause a switch from phototrophy to mixotrophy to supplement cellular nutrition (Burkholder et al., 2008, and references therein), with these triggers often being species-specific (Stoecker et al., 2006). Originally, it was further hypothesized that mixotrophs should possess lower growth rates than organisms having strictly a heterotrophic or phototrophic strategy due to the energy requirements to maintain the cellular machinery to undertake both nutritional modes (Raven, 1997; Burkholder et al., 2008). However, mixotrophy has been shown to significantly increase the growth rates of several HABs in comparison to strict phototrophy (Jeong et al., 2005; Stoecker et al., 2006; Burkholder et al., 2008, and references therein). Moreover, it has recently been proposed that mixotrophy may not be exclusive to nutrient poor habitats but may be more prevalent in eutrophied estuaries than originally thought due to low light regimes and unbalanced N:P ratios (Burkholder et al., 2008). Overall, mixotrophy may play a large part in the growth and maintenance of HABs (Anderson et al., 2002; Burkholder et al., 2008) as well as the succession of the phytoplankton community (Anderson et al., 2002; Jeong et al., 2005). Given the important role that mixotrophy plays in HAB ecology more strain-specific research is needed, particularly within areas that are heavily influenced by anthropogenic nutrient loading such the East Coast of North America.

Anthropogenic loading of nutrients and organic matter is thought to play a central role in the outbreak of multiple HABs (Paerl, 1988; Hallegraeff, 1993; Anderson et al., 2002; Glibert et al., 2005; Anderson et al., 2008; Heisler et al., 2008). In estuaries, dissolved organic nitrogen (DON) and particulate organic nitrogen (PON) comprise a relatively small portion of the total

nitrogen pool in comparison with dissolved inorganic nitrogen (DIN; ammonium and nitrate +

nitrite) which accounts for a large majority of the total pool (Berman and Bronk, 2003). The

composition of the nitrogen pool can deviate from that normally found in estuaries particularly if

influenced by strong point sources of nutrients such as a sewage treatment plant (STP). Due to

the use of nitrification-denitrification processes in STPs, DON is often the main component of

STP effluent, in some cases comprising up to 98% of the dissolved N fraction while DON in the

effluent of conventional STPs is only 65% of dissolved N (Pehlivanoglu-Mantas and Sedlak,

2006, 2008; Bronk et al., 2010). Deviations in the composition of the nitrogen pool have been

observed in estuaries influenced by sewage treatment plants, with average DON concentrations

comprising 33% of the total nitrogen pool (personal observation, T. Hattenrath-Lehmann);

however it is recognized that these deviations in DON can also be due to the *in situ* formation of

DON from phytoplankton production, sloppy feeding by zooplankton, and the viral lysis of

phytoplankton and bacteria (Nagata and Kirchman, 1992; Bronk et al., 1994; Gobler et al., 1997;

Berman and Bronk, 2003) with some of these processes capable of being stimulated by increased

anthropogenic nutrient loading. Furthermore, the DON found in STP effluent often contains a

small fraction of amino acids (Bronk et al., 2010) and humic substances that are often found to

stimulate HAB species (Pehlivanoglu-Mantas and Sedlak, 2006, 2008). Multiple culture and

field investigations have reported the stimulation of several HAB species by different dissolved

organic substrates including urea, amino acids, peptides, humic acids and uncharacterized

organic substances (Mulholland et al., 2002; Dyhrman and Anderson, 2003; Glibert et al., 2005;

Glibert et al., 2006; Glibert and Legrand, 2006; Anderson et al., 2008; Heisler et al., 2008).

Additionally, Tang et al. (2010) surveyed 41 different HAB strains and found that 96% of them

require vitamin B_{12}, another organic substrate that may potentially be an important component of

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DON in STP effluent given the bacterial component in these waters. The mixotrophic tendencies of HABs is not surprising given that the presence of pathways to incorporate N from other sources beyond DIN would afford them a competitive advantage over other phytoplankton (Stoecker et al., 2006; Anderson et al., 2008). While *Dinophysis* is a well-known phagotroph (Park et al., 2006; Reguera et al., 2012), very little is known regarding the effects of either inorganic nutrients or organic matter on *Dinophysis* growth and toxicity.

The nutritional ecology of *Dinophysis* is poorly understood. This is due, in part, to the inability to culture this species until recently (Park et al., 2006). Culturing this species requires the addition of a ciliate prey, *Mesodinium* (=*Myrionecta*) that must in turn be grown on cryptophytes; and thus far culture experiments examining the effects of nutrients (inorganic or organic) on this genus have not been performed. Furthermore, field investigations of *Dinophysis* and nutrients performed to date have come to contrasting conclusions. Some correlative field studies have found no relationship between *Dinophysis* densities and nutrient concentrations (Delmas et al., 1992; Giacobbe et al., 1995; Koukaras and Nikolaidis, 2004). Contrastingly, a study in South Africa using ^{15}N-labeled compounds found that *D. acuminata* has a high affinity for recycled N (ammonium) therefore giving it a competitive advantage in highly stratified, nitrate deplete waters (Seeyave et al., 2009). The only study investigating the effects of nutrients on non-N containing okadaic acid congeners in *Dinophysis* found contrasting results from the two *Dinophysis* species investigated (Johansson et al., 1996). While *D. acuminata* had higher okadaic acid concentrations under N-deficient conditions, levels were highest in *D. acuta* during nutrient sufficient conditions (+N+P; Johansson et al., 1996). However, an investigation using cultures of the benthic DSP-producing dinoflagellate, *Prorocentrum lima*, found that either N- or P- limitation increased okadaic acid concentrations (Vanucci et al., 2010). Given the limited and

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conflicting nature of results obtained to date, more research is needed to understand the effects of nutrients on the growth and toxicity of *Dinophysis*.

Elevated CO_2, nutrient loading, and their effects on HABs

Marine phytoplankton play a critical role in regulating global CO_2 concentrations (Beardall et al., 2009; Anderson et al., 2012). Since the industrial revolution anthropogenic emissions have increased atmospheric CO_2 concentrations from ~280ppm to present day concentrations of ~397ppm (average for May 2012, measured at the Mauna Loa Observatory by the Earth Systems Research Laboratory NOAA). Increased atmospheric CO_2 concentrations have increased atmospheric temperatures (Doney, 2006; Beardall et al., 2009), and have caused subsequent changes in surface ocean chemistry: increasing pCO_2, decreasing pH, and decreasing carbonate concentrations which can effect marine calcifiers (e.g. coccolithophores) and in turn global carbon cycles (Takahashi, 2004; Fabry, 2008). Additionally, it has recently been recognized that eutrophication that results from anthropogenic nutrient loading can contribute to the acidification of coastal systems (Borges and Gypens, 2010; Cai et al., 2011). While atmospheric CO_2 levels are predicted to rise somewhere between 750 to 1000 ppm by 2100 (Beardall et al., 2009; Cai et al., 2011), many estuaries are already experiencing levels >1000ppm (Feely et al., 2008; Talmage and Gobler, 2009; Cai et al., 2011; Barton et al., 2012, T. Hattenrath-Lehmann, pers. obs.). Other estuarine locations have reported pH levels as low as 7.4 (Cai et al., 2011; Hofmann et al., 2011). These high CO_2 and low pH levels have reportedly decreased nitrification rates (Beman et al., 2011), changed hydrolytic enzyme activity (Yamada and Suzumura, 2010), as well as changed trace metal chemistry (Millero et al., 2009), all of which can alter global nutrient cycles and in turn affect the phytoplankton community. Given

the important role that marine phytoplankton play in the global carbon cycle more research on the effects of ocean acidification on phytoplankton is needed.

During the past decade there have been several studies that investigated the effects of climate change (increased temperature, increase pCO_2 and decreased pH) on the composition of natural phytoplankton communities (Egge et al., 2009; Feng et al., 2009) as well as single phytoplankton species during culture experiments (Riebesell et al., 2000; Feng et al., 2008; Fu et al., 2008; Iglesias-Rodriguez et al., 2008; Langer et al., 2009; Fu et al., 2010; Wang et al., 2010; Sun et al., 2011; Lefebvre et al., 2012). Multiple studies (Riebesell et al., 2000; Feng et al., 2008; Iglesias-Rodriguez et al., 2008; Langer et al., 2009) have found that elevated pCO_2 can yield increases or decreases in growth and calcification rates for multiple strains of the coccolithophore, *Emiliania huxleyi*, emphasizing the importance of strain-specific information. Natural phytoplankton community experiments have shown marked changes in community composition with diatom or flagellate dominance depending on nutrient availability, CO_2 concentrations, temperatures, or the interaction between the latter two (Egge et al., 2009; Feng et al., 2009). While some long-term oceanic data sets have recorded a shift from diatom to flagellate dominance with increasing sea surface temperatures (Wasmund et al., 1998; Edwards et al., 2001), more recently Hinder et al. (2012) reported the opposite with increased abundance of the toxin forming HAB *Pseudo-nitzschia* with these dominance shifts occurring in the last 10 years. In agreement with this finding, increasing pCO_2 concentrations have been shown to increase both *Pseudo-nitzschia* growth rates and concentrations of its toxin, domoic acid (Sun et al., 2011). Other marine HABs, such as *Karlodinium veneficum* and *Heterosigma akashiwo* have displayed significantly faster growth rates under elevated levels of pCO_2, while the dinoflagellate *Prorocentrum minimum* was not affected (Fu et al., 2008; Fu et al., 2010). Clearly, more

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research on the effects of CO_2 on HAB taxa is needed given the wide range of effects that has already been observed for this group. In addition, changes in pH could subsequently effect changes in intracellular pH of phytoplankton (Suffrian et al., 2011) which can alter toxin biosynthesis by changing enzyme activity (Fu et al., 2012) as had already been observed for other enzymes (Yamada and Suzumura, 2010). Moreover, changes in intracellular pH could potentially cause conversions in saxitoxin congeners with low pH environments converting less potent N-sulfocarbamoyl toxins to the more potent carbamate toxins, as has been demonstrated with weak acid hydrolysis (Laycock et al., 1995). Given that coastal systems are already affected by high CO_2 as a result of cultural eutrophication (Nixon, 1995; Heisler et al., 2008) it is important to assess the effects of changing pCO_2 concentrations on *Alexandrium* growth and toxicity.

Allelopathy

The ability of *Alexandrium* blooms to persist and attain high cell densities has been attributed to a number of factors including cyst bed distribution and cyst abundance (Anderson, 1997; Anderson et al., 2005a; Anderson et al., 2005b; Anderson et al., 2005c; Anderson et al., 2008), deterrence of zooplankton grazing (Teegarden, 1999; Teegarden et al., 2008), and anthropogenic nutrient loading (Hattenrath et al., 2010). Another factor that is likely important for the development and persistence of *Alexandrium* blooms is the production of allelochemicals. Potent allelochemicals produced by some *Alexandrium* species and strains are capable of inhibiting the growth and/or lysing a diversity of cultured phytoplankton, and PSP toxins are not the chemical that elicits these effects (Tillmann and John, 2002; Fistarol et al., 2004b; Tillmann et al., 2009; Yang et al., 2010). Investigations of the allelochemical potency of *Alexandrium* spp. have almost exclusively focused on strains from Europe, Asia, South America and New Zealand

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(Arzul et al., 1999; Tillmann and John, 2002; Fistarol et al., 2004a; Fistarol et al., 2004b; Tillmann et al., 2007; Tillmann et al., 2008; Tillmann et al., 2009; Yang et al., 2010) with only one study examining two strains of the North American ribotype (Tillmann and John, 2002). Furthermore, a majority of studies have explored the allelopathic nature of *Alexandrium* using bi-algal cultures (Arzul et al., 1999; Tillmann and John, 2002; Fistarol et al., 2004b; Tillmann et al., 2007; Tillmann et al., 2008; Tillmann et al., 2009; Yang et al., 2010) with only two studies investigating the effects of allelopathy on natural phytoplankton assemblages in Europe (Fistarol et al., 2004a; Fistarol et al., 2004b). Moreover, of all the studies conducted on *Alexandrium*, very few have examined the temporal dynamics of *Alexandrium* blooms in parallel with the phytoplankton community composition (Anderson et al., 1983; Penna et al., 2002). As such, the allelopathic effects of *Alexandrium* on phytoplankton in the field during bloom events and the allelochemical potency of *Alexandrium* strains from North America have not been well characterized.

The overarching goal of my doctoral dissertation was to understand the processes promoting the increased occurrence of toxigenic dinoflagellates in estuarine ecosystems. This research specifically focused on the estuaries of NY and investigated PSP-causing blooms of *Alexandrium fundyense* and DSP-causing blooms of *Dinophysis*. Regarding *Alexandrium*, there is a chapter focused on establishing the allelopathic effects of North Atlantic strains of *Alexandrium* on competing phytoplankton, as well as a chapter assessing the effects of varying levels of pCO$_2$ on *Alexandrium* growth and toxicity. Regarding *Dinophysis*, I first characterized blooms in New York estuaries including species identification and the temporal dynamics of cells and toxins in both cells and shellfish. Next, I established the relative importance of inorganic nutrients, organic nutrients, and trophic interactions in promoting *Dinophysis* blooms

and toxicity. In all cases, the research involved field and laboratory investigations and combined

observational and experimental approaches.

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Chapter 2

Allelopathic inhibition of competing phytoplankton by North American strains of the toxic dinoflagellate, *Alexandrium fundyense*: evidence from field experiments, laboratory experiments, and bloom events

Hattenrath-Lehmann, T.K., Gobler, C.J., 2011. Allelopathic inhibition of competing phytoplankton by North American strains of the toxic dinoflagellate, *Alexandrium fundyense*: Evidence from field experiments, laboratory experiments, and bloom events. Harmful Algae 11, 106-116.

Abstract

The potential role of allelopathy in bloom formation by the paralytic shellfish poisoning (PSP) dinoflagellate, *Alexandrium fundyense,* was examined using five strains isolated from across the latitudinal PSP-toxicity gradient found along the North American East Coast. We specifically present bi-algal laboratory experiments, field experiments using cultured *A. fundyense* and natural phytoplankton communities, and the temporal dynamics of plankton assemblages during *A. fundyense* blooms within Northport Bay, NY, USA. Culture experiments demonstrated that all *Alexandrium* strains (from NY, CT, ME, and Canada) were capable of inhibiting the growth of the cryptophyte, *Rhodomonas salina*, as well the diatoms, *Thalassiosira pseudonana* and *Thalassiosira weissflogii*. This allelopathic effect was density dependent for both donor and target species as well as strain specific with the NY strain having the largest allelopathic effect (up to 100% reduction) on *R. salina*, followed by the ME, Canadian and CT strains. During field experiments all five strains caused significant decreases in autotrophic nanoflagellate and diatom abundances and significant increases in dinoflagellate densities. Consistent with these experimental results, *Alexandrium* bloom events were accompanied by significant declines in autotrophic nanoflagellate and diatom populations. Finally, density dependent inhibition of another harmful alga, the pelagophyte *Aureococcus anophagefferens*, was observed when *Alexandrium* filtrate was added to water from Quantuck Bay, NY, during a brown tide bloom. Collectively, these results suggest that allelopathic inhibition of competing phytoplankton promotes *Alexandrium* blooms in North America.

Introduction

Blooms of the dinoflagellate *Alexandrium* are common to coastal regions around the world and are particularly harmful because they produce saxitoxins, the suite of toxins that cause the potentially fatal human health syndrome, paralytic shellfish poisoning (PSP; Anderson, 1994; 1997; Glibert et al., 2005). *Alexandrium* blooms along the northeast coast of the United States occur as large-scale coastal events (Anderson, 1997; Anderson et al., 2005a,b; Townsend et al., 2005) as well as regional events in estuaries and coastal embayments (Anderson and Morel, 1979; Anderson, 1997; Hattenrath et al., 2010) and are often associated with substantial economic losses due to the closure of shellfish beds (Anderson et al., 2000; Jin and Hoagland, 2008; Jin et al., 2008). North American blooms exhibit a well-established north to south PSP toxicity gradient with northern strains (e.g. Canada, Maine, Massachusetts) predominantly synthesizing the more potent carbamate toxins and the southern strains (e.g. Connecticut, New York) containing a higher ratio of the less potent N-sulfocarbamoyl toxins (Maranda et al., 1985; Anderson et al., 1990; Anderson et al., 1994; Bricelj & Shumway 1998).

The ability of *Alexandrium* blooms to persist and attain high cell densities has been attributed to a number of factors including cyst bed distribution and cyst abundance (Anderson, 1997; Anderson et al., 2005a, b, d; Anderson et al., 2008), deterrence of zooplankton grazing (Teegarden, 1999; Teegarden et al., 2008), and anthropogenic nutrient loading (Hattenrath et al., 2010). Another factor that is likely important for the development and persistence of *Alexandrium* blooms is the production of allelochemicals. Potent allelochemicals produced by *Alexandrium* are capable of inhibiting the growth and/or lysing a diversity of cultured phytoplankton, and PSP toxins are not the chemical that elicits these effects (Tillmann and John, 2002; Fistarol et al., 2004b; Tillmann and Hansen, 2009; Tillmann et al., 2009; Yang et al., 2010). Investigations of the allelochemical potency of *Alexandrium* spp. have almost exclusively

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focused on strains from Europe, Asia, South America and New Zealand (Arzul et al., 1999; Tillmann and John, 2002; Fistarol et al., 2004a, b; Tillmann et al., 2007, 2008, 2009; Yang et al., 2010) with only one study examining two strains of the North American ribotype (Tillmann and John, 2002). Furthermore, a majority of studies have explored the allelopathy of *Alexandrium* using bi-algal cultures (Arzul et al., 1999; Tillmann and John, 2002; Fistarol et al., 2004b; Tillmann et al., 2007, 2008, 2009; Yang et al., 2010) with only two investigating the effects of allelopathy on natural phytoplankton assemblages in Europe (Fistarol et al., 2004a, b). Moreover, of all the studies conducted on *Alexandrium* in general, few studies have examined the temporal dynamics of *Alexandrium* blooms in parallel with the phytoplankton community composition (Anderson et al., 1983; Penna et al., 2002). As such, the allelopathic effects of *Alexandrium* on phytoplankton in the field during bloom events have not been well characterized.

Here we report on the role of allelochemicals in *Alexandrium* bloom formation using multiple strains of *Alexandrium* spanning the latitudinal PSP toxicity gradient found along the North American East Coast. We present experiments using natural phytoplankton communities from Northport Bay, an area that has experienced chronic shellfish bed closures due to PSP (Hattenrath et al., 2010; pers. obs.), as well as controlled laboratory experiments with multiple species of diatoms and dinoflagellates as well as the widely used target alga, *Rhodomonas salina*. We also report on the natural dynamics of *Alexandrium* and the phytoplankton community during multiple bloom events that support the hypothesis that allelopathic inhibition of competing phytoplankton occurs during blooms.

Materials and Methods

Cultures and culturing conditions

Five *Alexandrium fundyense* strains spanning the latitudinal PSP toxicity gradient found along the North American East Coast were used as donor species during our investigation of allelopathy. Two high toxicity strains (with regard to saxitoxin production), one from the Bay of Fundy (CCMP 2304; herein BoF) and the other from the Gulf of Maine (CCMP 1719, synonymous with GTCA28; herein GoM) were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; Maine, USA). Two low toxicity strains, ATNPD7 and NPB8 (herein NY), both isolated from Northport Bay, NY were generously provided by Dr. Donald M Anderson's lab and Dr. Hans Dam's lab (isolation by Hayley Skelton), respectively. Finally, a non-toxic strain (GTCN-16; herein CT) isolated in Mumford Cove, CT was also obtained from the Dam lab. The cryptophyte, *Rhodomonas salina* (CCMP 1319; isolated from CT waters) which has often been used as a model target species to test the potency of allelochemicals (Fistarol et al., 2004b; Tillmann et al., 2007, 2008, 2009; Ma et al., 2009) was used as our target species in laboratory experiments. Other target species investigated included the diatoms, *Thalassiosira pseudonana* (CCMP 1335; isolated from NY waters) and *Thalassiosira weissflogii* (CCMP 1336; isolated from NY waters), and the dinoflagellates, *Prorocentrum minimum* (CCMP 696; isolated from NY waters) and *Heterocapsa arctica* (MS5, isolated from NY by Dr. YZ Tang; species identity confirmed via sequencing of the large subunit of the ribosome; Tang et al., 2010).

Algal cultures were grown in sterile *f*/2 medium (Guillard and Ryther, 1962) with a salinity of 32 PSU, made with boiled and 0.2 μm-filtered seawater, at 18°C in an incubator with a 12:12 h light:dark cycle, illuminated by a bank of fluorescent lights that provided a light

intensity of ~100 µmol quanta m^{-2} s^{-1} to cultures. Antibiotics (stock solution, Thermo Scientific HyClone Penicillin (10,000U/mL) Streptomycin (10,000µg/mL) in 0.85% NaCl) were added to all cultures at a final concentration of 1% by volume to discourage bacterial contamination. Cultures used in field experiments were maintained as described above but were gradually adjusted to a salinity of 25 PSU to match the salinity of the field study site, Northport Bay.

Laboratory experiments using algal monocultures

Whole cell addition experiments- To assess the allelopathic properties of *Alexandrium* strains from the North American East Coast, laboratory experiments were conducted by creating a mixture of a single donor species and a single target species. Donor species included the above described strains of *Alexandrium* from NY (NPB8), CT, GoM and BoF, whereas our target species was the widely used model organism, *Rhodomonas salina* (CCMP 1319; Fistarol et al., 2004b; Tillmann et al., 2007, 2008, 2009; Ma et al., 2009). Dose-response experiments were conducted by: 1) keeping the cell densities of donor species (*Alexandrium* (NY, CT, GoM and BoF) = 400 cells ml^{-1}) constant and varying densities of the target organism (*Rhodomonas*= 100, 1,000, 7,000, 10,000, 14,500 and 29,000 cells ml^{-1}), and 2) varying the cell density of donor species (*Alexandrium* (NY)= 5, 10, 50, 60, 80, 100, 200 and 400 cells ml^{-1}) and keeping the target organism density fixed (*Rhodomonas*= 7,000 cells ml^{-1}). All experiments were conducted in sterile, autoclaved 250ml Erlenmeyer flasks. Stock cultures of target and donor species in exponential growth phase were mixed to appropriate experimental densities using sterile, 0.2µm filtered *f*/2 media to a total volume of 50ml with 1% antibiotic solution. All experiments were incubated as described above for 72 hours. At the end of each experiment aliquots were preserved with Lugol's iodine and cells were enumerated with either a 0.1ml or 1ml Sedgewick-Rafter counting chamber using a compound microscope. Nutrient samples were obtained from

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experiments and all experiments had nitrate concentrations >40μM by the end of the experiments, significantly above the half-saturation constant of diatoms, dinoflagellates, and flagellates (Smayda, 1997). Furthermore, the difference in pH values between controls and treatments was always <0.3 units, and always within the range typically found in target algal cultures, suggesting that the observed effects were not due to differences in pH.

Filtrate experiments- The effects of filtrate from the NY strain (NPB8) and CT strain of *Alexandrium* were assessed using environmentally realistic densities of several target organisms: the diatoms, *Thalassiosira pseudonana* (CCMP 1335; 4,000 cells ml^{-1}) and *Thalassiosira weissflogii* (CCMP 1336; 4,000 cells ml^{-1}), and the dinoflagellates, *Prorocentrum minimum* (CCMP 696; 1,000 cells ml^{-1}) and *Heterocapsa arctica* (MS5; 1,000 cells ml^{-1}). *Alexandrium* cultures in exponential growth phase were diluted to concentrations of 400 cells ml^{-1} with *f*/2 and cell-free *Alexandrium* medium was obtained by gentle filtration (<5psi) through sterile 0.2 μm Millipore Steritop filters. Experiments were conducted in 7ml borosilicate vials adding 2.5ml of either *f*/2 (control), NY filtrate (final concentration=200 cells ml^{-1}), or CT filtrate (final concentration=200 cells ml^{-1}) to 2.5ml of the above mentioned target organisms (final densities of target organisms are noted above). Experimental cultures were supplemented with *f*/2 to ensure any treatment effect was due to allelochemicals and not nutrient limitation. Vials were incubated for 72 hours as described above and at the end of the experiment Lugol's iodine was added to each vial and cells were enumerated.

Natural phytoplankton community experiments

To assess the potential role of allelopathy in promoting *Alexandrium* blooms several field experiments were conducted over a two-year period (2009 and 2010) in which cultured *Alexandrium* (whole cells and filtrate) was added to a natural phytoplankton assemblage from

Northport Bay, NY, an embayment that has experienced intense *Alexandrium* blooms annually since 2006 (Hattenrath et al., 2010; pers. obs.). While filtrate additions provided a single dose of allelochemicals, the whole cell additions provided a constant source of allelochemicals throughout experiments. Finally, to assess the ability of *Alexandrium* to inhibit another harmful alga, the pelagophyte *Aureococcus anophagefferens*, *Alexandrium* filtrate was added to a natural phytoplankton community from Quantuck Bay, NY, an embayment that has experienced harmful brown tides since 1985 (Gobler et al., 2011). This experiment was particularly relevant since Quantuck Bay experienced its first ever PSP closure in May of 2011 (K.N. Chytalo, NYS Department of Environmental Conservation, pers. comm.) and our experiments were conducted the following month (June 2011) as the PSP event subsided and a brown tide initiated.

Filtrate experiments- During the spring of 2009 (28 Apr, 5 May, 12 May, 19 May) filtrate experiments were conducted in triplicate, 330ml bottles filled with 50% whole water from Northport Bay, NY, and 50% filtrate (0.2µm) of two origins. A control was established whereby half of the bottle was filled with 0.2µm filtered Northport Bay water, and for the treatment, half of the bottle was 0.2µm filtered *Alexandrium* culture, specifically NY strain ATNPD7. For all experiments final *Alexandrium* densities exceeded 180 cells ml^{-1} and averaged 300 ± 90 cells ml^{-1} (Table 1). Both filtered Northport Bay water and cell-free *Alexandrium* medium was obtained by gentle filtration (<5psi) through a sterile 0.2 µm Millipore Steritop filter. To ensure that the effects seen by the addition of *Alexandrium* filtrate were due to allelochemicals and not nutrients, saturating concentrations of N (100µM), P (6.25µM), and Si (20µM) as well as vitamins and trace metals (both at *f*/2 concentrations) were added to both control and treatment bottles (nitrate concentrations in all bottles were >40µM at the end of each experiment conducted). All experimental bottles were incubated at ambient light and temperature for 48 hours in flow

through chambers in Shinnecock Bay at the Stony Brook Southampton Marine Science Center (Gobler et al., 2004). At the end of each experiment samples were preserved in Lugol's iodine (2% final concentration) and enumerated using a 1ml Sedgewick-Rafter slide on a compound light microscope. At least 200 cells per slide were enumerated and cells larger than 10μm were identified to at least genus level and grouped as dinoflagellates, diatoms, and autotrophic nanoflagellates. In cases when 200 cells could not be quantified, samples were settled in settling chambers and enumerated using an inverted microscope. Chlorophyll *a* was measured using glass fiber filters (GF/F) and 20μm polycarbonate filters and processed according to Parsons et al. (1984). Changes in chlorophyll *a* were converted to net growth rates that were calculated using the following formula: $\mu = [\ln(B_t/B_o)]/t$, where μ is the net growth rate, B_t is the amount of biomass (chl *a*) present at the end of the experiments, B_o represents the amount of biomass at the beginning of experiments, and *t* is the duration of the experiment in days. Growth rates (herein chl*a*) or cell densities are presented as percent change relative to the control using the equation: (final treatment cell densities or chl*a* – final mean control cell densities or chl*a*)/ (final mean control cell densities or chl*a*) x 100.

Whole cell addition experiments- A series of whole cell *Alexandrium* addition experiments were conducted during the spring of 2010: 5 Apr, 12 Apr, 20 Apr, 27 Apr, 4 May, 18 May. Triplicate, 330ml bottles were filled with 50% whole water from Northport Bay, NY, and a control was established whereby the other half of the bottle was filled with *f*/2 medium. In addition, four treatments were established where half of the bottle was filled with an *Alexandrium* culture using strains from NY (NPB8), CT, GoM, and BoF described above (Table 1). To every experimental bottle, nutrients were added at *f*/2 levels (+ Si) to ensure nutrient replete conditions. Nitrate concentrations at the end of all experiments conducted were >40μM

indicating that the effects seen were not due to nutrient limitation (Smayda, 1997). Bottles were incubated at ambient light and temperature for a 48 hour period in a temperature control room. At the end of the experiment aliquots were preserved in Lugol's iodine as described above. Here, total non-*Alexandrium* dinoflagellates were enumerated since the *Alexandrium* added could not be distinguished from the natural field population. Cell densities of autotrophic nanoflagellates, diatoms and non-*Alexandrium* dinoflagellates are presented here as mean percent change relative to the control as described above.

Filtrate experiments during a brown tide bloom - During June of 2011 (14 June, 22 June, 29 June) filtrate experiments were conducted in triplicate, 330ml bottles filled with 50% whole water from a brown tide in Quantuck Bay, NY (*A. anophagefferens* cell densities $> 10^5$ cells ml^{-1}) and 50% filtrate (0.2µm) of two origins. A control was established whereby half of the bottle was filled with 0.2µm filtered Quantuck Bay water and, for the treatment, half of the bottle was 0.2µm filtered *Alexandrium* culture (NY strain NPB8; Table 1). Both filtered Quantuck Bay water and cell-free *Alexandrium* medium were prepared as above. Nutrients were added at *f*/2 levels (+ Si) to all bottles ensuring nutrient replete conditions. All experimental bottles were incubated as above. At the end of each experiment samples were preserved in 1% glutaraldehyde and *A. anophagefferens* abundances were quantified using a monoclonal antibody technique (Caron et al. 2003) as detailed in Harke et al. (2011). *A. anophagefferens* densities are reported as percent change relative to the control during the incubation as described above.

Field sampling and analyses

During 2009 and 2010, field samples were collected on a weekly to twice-weekly basis from March through June. Samples were collected in Northport Harbor at 40.8916°N, 73.3572°W (site 2 in Hattenrath et al., 2010), which is within the southeastern portion of the

Northport-Huntington Bay complex, located on the north shore of Long Island, NY, USA. Northport Bay water was filtered for nutrient analysis using precombusted (4 hr @ 450°C) glass fiber filters (GF/F, 0.7 µm pore size) and frozen in acid washed scintillation vials. Filtrate was analyzed colorimetrically for nitrate, phosphate, ammonium, and silicate (Jones, 1984; Parsons et al., 1984) using a spectrophotometeric microplate reader. To determine the size distribution of phytoplankton biomass, chlorophyll *a* was fractionated using GF/F (nominal pore size 0.7 µm) and polycarbonate filters (2 µm & 20 µm) and measured using standard fluorometric techniques described in Parsons et al. (1984). Whole water samples were preserved in Lugol's iodine. Aliquots were settled in counting chambers and plankton were identified and enumerated using an inverted light microscope (Hasle, 1978). Cells larger than 10 µm were identified to at least genus level and grouped as autotrophic nanoflagellates, dinoflagellates, and diatoms. *Alexandrium fundyense* cell densities were enumerated using a highly sensitive molecular technique developed by Anderson et al. (2005c) and is discussed at length in Hattenrath et al. (2010).

Statistics

Student t-tests or ANOVAs were used to compare the cell densities or growth rates (chl *a*) of the control and treatments using Sigma Plot 11.0. If data failed normality tests the appropriate non-parametric tests were used. All data from laboratory and field experiments are presented in graphic form as mean percent change relative to the control of cell densities for target species/groups and of growth rates for chlorophyll *a* and were calculated using the aforementioned equations.

Results

Laboratory experiments using algal monocultures

      Whole cell addition experiments- All four of the east coast North American *Alexandrium* strains (NY, CT, GoM, and BoF) had allelopathic effects on the target organism, *Rhodomonas salina* (Fig. 1). When *Alexandrium* strains were fixed at 400 cells ml^{-1}, the NY strain exhibited the largest allelopathic effect causing large and significant decreases (98 to 100%) for all densities of *R. salina* (1,000 to 29,000 cells ml^{-1}) tested relative to the control (p<0.01 for each; Fig. 1). The GoM strain significantly reduced 1,000 and 7,000 *R. salina* cells ml^{-1} relative to the control by 95% and 85%, respectively (p<0.001 for each), but exhibited no effect on larger cell densities of 10,000 and 14,500 *R. salina* cells ml^{-1} (Fig. 1). The BoF and CT strains reduced 100 *R. salina* cells ml^{-1} (45% (p<0.01) and 10% reduction, respectively; Fig. 1), but did not alter higher *R. salina* densities. Finally, the NY strain was able to significantly reduce (98-99%; p<0.01) 7,000 *R. salina* cells ml^{-1} compared to the control when *Alexandrium* densities were as low as 100 cells ml^{-1} but was incapable of doing so when *Alexandrium* densities were below this level (Fig. 2). When percent changes in *Rhodomonas* densities relative to the control were ≤-45% (all treatments that were significantly different from the control) growth occurred in the control but not in the treatment, whereas experiments with a percent change of 0 through -45% had some growth in treatments, albeit less than that of the control (growth inhibition; Fig. 1, 2).

      Filtrate experiments- The addition of cell-free filtrate from the NY *Alexandrium* strain and the non-PSP producing CT strain caused both decreases and increases in cell densities of target organisms (Fig. 3). The addition of filtrate from both the NY and CT strains resulted in significant reductions (36% and 34%, respectively) in *Thalassiosira pseudonana* compared to the control (p<0.01; Fig. 3). The addition of filtrate from the CT *Alexandrium* strain caused a

significant reduction (18%; p<0.05) in *T. weissflogii* densities whereas the NY strain did not (Fig. 3). The addition of both the NY and CT strain filtrate caused significant increases (395% and 1350%, respectively) in densities of the dinoflagellate, *Heterocapsa arctica* (p<0.01; Fig. 3) but did not alter *Prorocentrum minimum* densities. Reductions in absolute cell densities compared to the control occurred for *T. pseudonana* treated with *Alexandrium* filtrate whereas *T. weissflogii* experienced significantly reduced growth compared to control treatments.

Natural phytoplankton community experiments

Filtrate experiments- The addition of cell-free culture filtrate from a NY *Alexandrium* isolate (ATNPD7) caused significant alterations in Northport Bay's phytoplankton community in all four of the experiments conducted during the spring of 2009 (Fig. 4). The addition of *Alexandrium* filtrate caused 14 to 83% decreases in autotrophic nanoflagellates compared to the control with significant decreases occurring in three of the four experiments conducted (all except 19 May; p<0.05; Fig. 4). During an experiment conducted in late April (28 April) the addition of *Alexandrium* filtrate caused a significant increase (30%; p<0.01; Fig. 4) in diatom concentrations compared to the control, a trend driven almost exclusively by a significant increase (60%) in *Guinardia* spp. densities (p<0.001; data not shown). In all other experiments, the addition of *Alexandrium* filtrate decreased diatom concentrations up to 27% compared to the control with two of these three decreases being significant (5 May and 12 May; p<0.01; Fig. 4); *Thalassiosira*, *Thalassionema*, and *Skeletonema* spp. were the most frequently inhibited genera of diatom. The addition of *Alexandrium* filtrate also caused significant alterations in the dinoflagellate community in all four experiments (p<0.05 for all; Fig. 4). During an experiment conducted in late April (28 April) dinoflagellate concentrations decreased significantly (99%; p<0.001; Fig. 4) with the addition of *Alexandrium* filtrate, a result caused by a significant decline

(100%) in *Prorocentrum* spp. densities (p<0.001; Fig. 4). For all other experiments conducted during the spring of 2009 the addition of *Alexandrium* filtrate significantly increased dinoflagellate concentrations (17-68%; p<0.05 for all; Fig. 4). Finally, the <20μm chlorophyll *a* size fraction significantly decreased by 10 to 95% in all four experiments conducted (p<0.05 for each; Fig. 4).

The addition of culture filtrate from a NY *Alexandrium* isolate (NPB8) caused significant changes in densities of the harmful pelagophyte, *Aureococcus anophagefferens*, in all experiments conducted during a brown tide bloom in June of 2011 (Fig. 5). During an experiment on 22 June, the addition of filtrate from a culture with 120 ± 14 *Alexandrium* cells mL^{-1} yielded a significant increase (62%; p<0.01; Fig. 5) in *Aureococcus* densities compared to the control. In contrast, during the two other experiments (14 June and 29 June) the addition of *Alexandrium* filtrate originating from cultures with a higher cell densities (503 ± 60 cells mL^{-1} and 278 ± 25 cells mL^{-1}, respectively) caused significant declines (44 - 92%; p<0.01; Fig. 5) in *Aureococcus* cell densities compared to the control. For all experiments, changes in *Aureococcus* densities were significantly correlated with *Alexandrium* densities used to make the cell-free filtrate (r^2=0.96; p<0.0001; Fig. 5) demonstrating a density-dependent inhibition of *Aureococcus* by *Alexandrium*.

Whole cell addition experiments- Whole cell additions of four strains of *Alexandrium* caused significant alterations in Northport Bay's phytoplankton community during the spring of 2010 (Fig. 6, 7). The addition of NY and CT strain *Alexandrium* cells resulted in significant decreases (53 to 100%; p<0.01 for each strain) in autotrophic nanoflagellates with the exception of one experiment (18 May) where the addition of the NY strain resulted in a 10% increase (Fig. 6). Similarly, these strains caused diatom densities to decrease significantly (21 to 93%; p<0.01)

with only two exceptions: the addition of the CT *Alexandrium* strain resulted in a non-significant

decrease on 20 April and the addition of the NY *Alexandrium* strain resulted in a non-significant

(23%) increase on 27 April (Fig. 6). The most commonly inhibited diatom genera were

Thalassiosira, *Thalassionema*, *Guinardia*, *Chaetoceros* and *Skeletonema* spp. In contrast, during

four of the six experiments conducted in the spring of 2010, the addition of *Alexandrium* resulted

in significant increases (47 to 1550%; $p<0.01$) in non-*Alexandrium* dinoflagellates with a few

exceptions: the addition of the NY and CT *Alexandrium* strains resulted in significant decreases

(18 to 75%; $p<0.01$) on 27 April and 4 May both due to decreases in *Heterocapsa spp.* (Fig. 6).

Finally, during an experiment conducted on 18 May the addition of three of the four

Alexandrium strains tested (all but the NY strain) significantly decreased (32 to 67%; $p<0.01$)

autotrophic nanoflagellate densities compared to the control, with the GoM strain causing a

significantly greater decrease (67%) than all other strains tested ($p<0.05$, Tukey; Fig. 7). All

four *Alexandrium* strains caused the abundance of diatoms to decrease significantly (46 to 67%;

$p<0.001$; Fig. 7). Paralleling other field experiments conducted during 2010, the addition of each

of the four *Alexandrium* strains resulted in significantly greater (266 to 683%; $p<0.001$)

abundances of non-*Alexandrium* dinoflagellates (Fig. 7).

Natural phytoplankton community dynamics

Spring 2009- During May and early June, an *Alexandrium* bloom developed and

persisted for five weeks during which peak cell densities reached ~9,000 cells L^{-1} (27 May; Fig.

8a). During the peak of the *Alexandrium* bloom nanophytoplankton (2-20 μm) chlorophyll *a*

concentrations were lower (mean ± SE, 19-27 May; $3.0 ± 1.9$μg L^{-1}) compared to after the peak

of the bloom (2-12 June; $11.8 ± 7.4$ μg L^{-1}; Fig. 8a, Table 2). Once *Alexandrium* densities

exceeded 1,300 cells L^{-1} (8 May; Fig. 8a), diatom concentrations decreased by more than an

order of magnitude from ~1,700 cells ml^{-1} (8 May) to 100 cells ml^{-1} (12 May) and remained

below 200 cells ml^{-1} and comprised less than 10% of the phytoplankton community (Fig. 8a) for

the duration of the *Alexandrium* bloom (12 May to 2 June). Upon the demise of the *Alexandrium*

bloom, diatom concentrations increased to concentrations similar to those before the

Alexandrium bloom (Fig. 8a). Autotrophic nanoflagellates were comprised mainly of

haptophytes and overall contributed a large percentage of the total phytoplankton community

composition during the spring months (22 to 94% of cells) as well as during the *Alexandrium*

bloom (31 to 86%; 1 May- 5 June; Fig. 8a). During the peak of the *Alexandrium* bloom (27

May) autotrophic nanoflagellate concentrations decreased ~ 50% (from ~2,100 cells ml^{-1} on 22

May to ~1,100 cells ml^{-1} on 27 May; Fig. 8a). Silicate concentrations were well above 6μM

during the spring months and remained above 15μM during the *Alexandrium* bloom (Table 2).

Dissolved inorganic nitrogen (DIN) and phosphate concentrations did not fall below 5μM and

0.2μM, respectively, for the duration of the sampling period (Table 2).

   Spring 2010 - The peak of the 2010 *Alexandrium* bloom (~90,000 cells L^{-1}; 14 May; Fig.

8b) was an order of magnitude larger than the 2009 bloom (Fig. 8a). Nanophytoplankton

biomass was significantly lower during the peak of the *Alexandrium* bloom (4-18 May; 1.8 ±

0.4 μg chlorophyll *a* L^{-1}; p<0.05) compared to before (25 March to 30 April) and after (21 May

to 18 June) the *Alexandrium* bloom (6.2 ± 1.0 μg chlorophyll *a* L^{-1}; Table 2). During the peak of

the *Alexandrium* bloom (4-18 May), diatom concentrations decreased significantly from >8,000

cells ml^{-1} before the bloom to <1,000 cells ml^{-1} (Mann-Whitney Rank Sum test, p=0.01; Fig 8b).

Upon the demise of the *Alexandrium* bloom, diatom abundances increased significantly to levels

similar to those found before the bloom (~7,000 cells ml^{-1}; Mann-Whitney Rank Sum test,

p=0.01; Fig. 8b). Autotrophic nanoflagellates were abundant in April (~8,000 cells ml^{-1}; 16

April) but vanished during the peak of the *Alexandrium* bloom (14 May; Fig. 8b). Upon the demise of the bloom, the autotrophic nanoflagellates reestablished densities (~6,000 cells ml^{-1}) found prior to the *Alexandrium* bloom (Fig. 8b). Silicate concentrations were above 12μM from March through June and above 25μM during the *Alexandrium* bloom (Table 2). DIN and phosphate concentrations remained above 5μM and 0.1μM, respectively, March through June (Table 2).

Discussion

This is the first comprehensive study of the allelochemical potency of North American strains of *Alexandrium fundyense*. *Alexandrium* cultures from across the East Coast of North America were capable of inhibiting the growth of autotrophic nanoflagellates, HAB forming pelagophytes and diatoms as well as promoting the growth of dinoflagellates in laboratory and field experiments. The ability of both whole cells and filtrate from cultures to elicit these effects demonstrated that extracellular allelochemicals were the active agents in these experiments. In a manner consistent with field and laboratory experiments, *Alexandrium* blooms in Northport, NY, were coincident with significant reductions in the densities of autotrophic nanoflagellates and diatoms. These findings collectively suggest that allelopathy plays an important role in *Alexandrium* establishing dominance within the phytoplankton community during bloom events.

The allelopathic effects of North American strains of *Alexandrium* were density dependent for both target and donor phytoplankton as well as strain dependent. Laboratory experiments demonstrated that all *Alexandrium* strains (NY, CT, GoM and BoF) were capable of inhibiting the growth of *R. salina* at environmentally realistic densities. The NY strain had the largest allelopathic effect on *R. salina*, being capable of significantly reducing (up to 100%) 1,000 to 29,000 cells ml^{-1} even when the donor cell densities (NY *Alexandrium* strain) were as

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low as 100 cells ml^{-1}. The GoM and BoF strains were less potent, significantly reducing only lower densities of *R. salina* (7,000 and 100 cells ml^{-1}, respectively) whereas CT strains had negligible effects on *Rhodomonas* densities (Fig. 1, 2). These findings are consistent with prior studies that have concluded that the allelopathic effects of harmful algae are cell density dependent and strain specific (Tillmann et al., 2007, 2008; Poulson et al., 2010; Tang and Gobler, 2010). For example, Tillmann et al.'s (2007 and 2008) investigation of the allelopathic effects of multiple *Alexandrium* species and strains (densities ranging from 50 to 8,000 cells ml^{-1}) on *R. salina* (KAC 30; densities ranging from 9,300 to 20,000 cells ml^{-1}) demonstrated that cultures ranged from strong allelopathic potency (99% reduction) to absolutely no effect on *R. salina*.

Differences in allelopathic potency of the four different *Alexandrium* strains on the target organism *Rhodomonas* may have been due to geographic differences or strain variability. Tillmann et al. (2009) demonstrated that clonal isolates from distinct geographic locations display large intra-population variability in both PSP production and allelochemical potency, with these two factors generally being unrelated. Such clonal variability could have been responsible for the weaker allelopathic effect of the BoF and CT strains examined in this study on *R. salina*. It is notable, however, that the two different NY strains investigated were both strongly allelopathic to the natural phytoplankton community. The only other study that has examined the allelopathic capabilities of northeastern US coast strains (NY and MA) found that a NY strain had a low and a MA strain had a strong allelopathic effect on *O. marina* (10 and 95% reduction in cell density in 3 hrs; Tillmann and John, 2002) affirming the variability in potency among strains from this region. Regardless, this study supports the hypothesis that saxitoxin is not the agent responsible for allelopathy in *Alexandrium* (Tillmann et al., 2009). Although

Alexandrium strains from the BoF and GoM are known to produce proportionally more of the potent saxitoxin congeners and therefore have a higher toxin content than strains from NY (Maranda et al., 1985; Anderson et al., 1990; Anderson et al., 1994; Bricelj & Shumway 1998), NY strains were actually more allelopathic to *R. salina* than these two strains. In addition, the CT strain, which does not produce saxitoxin, had strong allelopathic effects on diatoms and wild autotrophic nanoflagellate populations.

Patterns of allelochemical potency of North American strains of *Alexandrium* against *R. salina* differed from the patterns that emerged from experiments with other phytoplankton and during field experiments. For example, while the BoF and CT *Alexandrium* strains only weakly affected densities of *R. salina* in laboratory experiments, they significantly and strongly altered densities of autotrophic nanoflagellates, diatoms, and dinoflagellates during field experiments. Furthermore, culture filtrate of the CT strain also proved to have a strong allelopathic effect on several species of diatoms. Conversely, while the NY strain was allelochemically the strongest against *R. salina* in laboratory experiments, it was the weakest against autotrophic nanoflagellate populations in the field experiment that compared strains, perhaps suggesting that *R. salina* CCMP 1319 was not representative of the autotrophic nanoflagellate populations observed in the field. Finally, changes in target cell abundances were observed at *Alexandrium* cell densities as low as 10^5 cells L^{-1} in culture but as low as 10^4 cells L^{-1} during blooms indicating the existence of clonal variability in allelo-potency or a loss of allelo-potency over time in culture (Martins et al 2004). Collectively, all of these findings suggest that different *Alexandrium* strains produce multiple allelochemicals that target and differentially impact different phytoplankton (Ma et al., 2009; Poulson et al., 2010; Prince et al., 2010).

The allelopathic effects of North American *Alexandrium* strains have the ability to distinctly shape phytoplankton community structure. In field and laboratory experiments, nearly all *Alexandrium* strains displayed the ability to significantly reduce the densities of diatoms and autotrophic nanoflagellates, and often significantly increased the densities of other dinoflagellates, a finding consistent with Fistarol et al. (2004b). In addition, field experiments using *Alexandrium* filtrate demonstrated that the inhibition of a HAB forming pelagophyte, *Aureococcus anophagefferens*, was donor density dependent. Beyond demonstrating that these phytoplankton are differentially vulnerable to allelochemicals from *Alexandrium*, these findings also support a mechanism by which *Alexandrium* blooms may be promoted. Since *Alexandrium* is a slow-growing alga ($\mu_{max} \sim 0.5$ d^{-1}; personal observation) compared to diatoms and flagellates (Smayda, 1997) it must rely on mechanisms besides rapid growth to form blooms. Allelopathic effects on competitors together with grazer deterrence (Teegarden, 1999; Teegarden et al., 2008) could promote *Alexandrium* blooms through positive feedback (Sunda et al., 2006) whereby higher *Alexandrium* cell densities yield fewer competitors and predators that in turn facilitates higher cell densities. Since the allelopathic effects of *Alexandrium* on co-occurring algae are density dependent, allelopathy is more likely to contribute to bloom maintenance when cell densities are high, rather than bloom initiation when cell densities are low (Jonsson et al., 2009). Similarly, allelopathy is more likely to affect plankton dynamics during larger (dense) blooms ($\sim \geq 10^4$ cells L^{-1}) than smaller (less dense) bloom events. Consistent with this hypothesis, during the 2009 Northport, NY, USA, bloom, cell densities of *Alexandrium* were $\sim 10^4$ cells L^{-1} at the peak of the bloom and autotrophic nanoflagellate densities declined by only 50% while dinoflagellate densities did not change. In contrast, the 2010 bloom densities were $\sim 10^5$ cells L^{-1} and autotrophic nanoflagellates completely disappeared from the water column while

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dinoflagellates became the dominant (93%) component of the phytoplankton community. Despite the lower *Alexandrium* densities during the 2009 bloom, allelopathy did seem responsible, at least in part, for the changes seen in community structure, with densities of autotrophic nanoflagellates and diatoms decreasing during the *Alexandrium* bloom and increasing shortly after its demise. The existence of these allelopathic effects of *Alexandrium* on the rest of the phytoplankton community during the 2009 bloom even at low *Alexandrium* densities ($\sim 10^4$ cells L^{-1}) is further substantiated by the ability of whole cell *Alexandrium* additions of 10^4 cells L^{-1} to cause significant decreases in autotrophic nanoflagellate and diatom populations during field experiments.

While *Alexandrium* suppressed densities of nanoflagellates and diatoms in field and laboratory experiments, cultures also significantly enhanced the abundance of dinoflagellates, many of which, like *Alexandrium*, were often HAB species such as *Heterocapsa* sp. Phytoplankton commonly secrete extracellular organic compounds as they grow (Mague et al., 1980; Biddanda and Benner, 1997) and the whole and filtered components of *Alexandrium* cultures were likely to contain a milieu of organic compounds beyond allelochemicals. Given the well-known osmotrophic abilities and mixotrophic tendencies of dinoflagellates (Smayda, 1997; Anderson et al., 2008), these algae may benefit nutritionally from extracellular compounds released by *Alexandrium* while being relatively immune to allelochemicals that seem to specifically target diatoms and other flagellates. This process would provide a mechanism beyond nutrient dynamics and stratification to account for the post-spring diatom bloom dominance of dinoflagellates in temperate ecosystems (Sverdrup, 1953; Barlow et al., 1993; Behrenfeld, 2010).

Past studies have demonstrated the ability of *Alexandrium* to cause significant changes in the community structure of natural phytoplankton communities by conducting field manipulations using cultured *Alexandrium* (Fistarol et al., 2004 a, b; the present study). Our study, however, also provides evidence for allelopathy in action during bloom events in Northport Bay, NY. During *Alexandrium* blooms, significant decreases in diatoms, autotrophic nanoflagellates and the nanophytoplankton (2-20μm) size fraction of chlorophyll *a* occurred compared to before and after the bloom, while nutrient concentrations remained above levels known to be limiting to most phytoplankton (Egge and Aksnes, 1992; Smayda, 1997). Similar patterns among the plankton communities were observed in this system in prior years (2007 and 2008; Hattenrath et al., 2010; pers. obs.) as well as in other studies (Anderson et al., 1983; Penna et al., 2002). In some cases, precipitous decreases in abundances of the affected phytoplankton were seen during the peaks of the *Alexandrium* blooms. Such dramatic declines could be due to density dependent allelopathy and/or a winnowing of phenotypic diversity as blooms mature with strongly allelopathic strains present at the pinnacle of the bloom (Tillmann et al., 2009). We further acknowledge that there are multiple, complex and co-occurring processes affecting plankton community diversity during HABs including the differential susceptibility of plankton to zooplankton grazing, algicidal bacteria, and nutrient limitation (Caron et al., 2004; Frazier et al., 2007; Anderson et al., 2008). The coherence of our culture and field experiments and our field observations (all displayed repression of nanoflagellates and diatoms and promotion of dinoflagellates by elevated *Alexandrium* densities), however, provides the strongest evidence to date of the active role allelopathy plays in the occurrence of toxic *Alexandrium* blooms.

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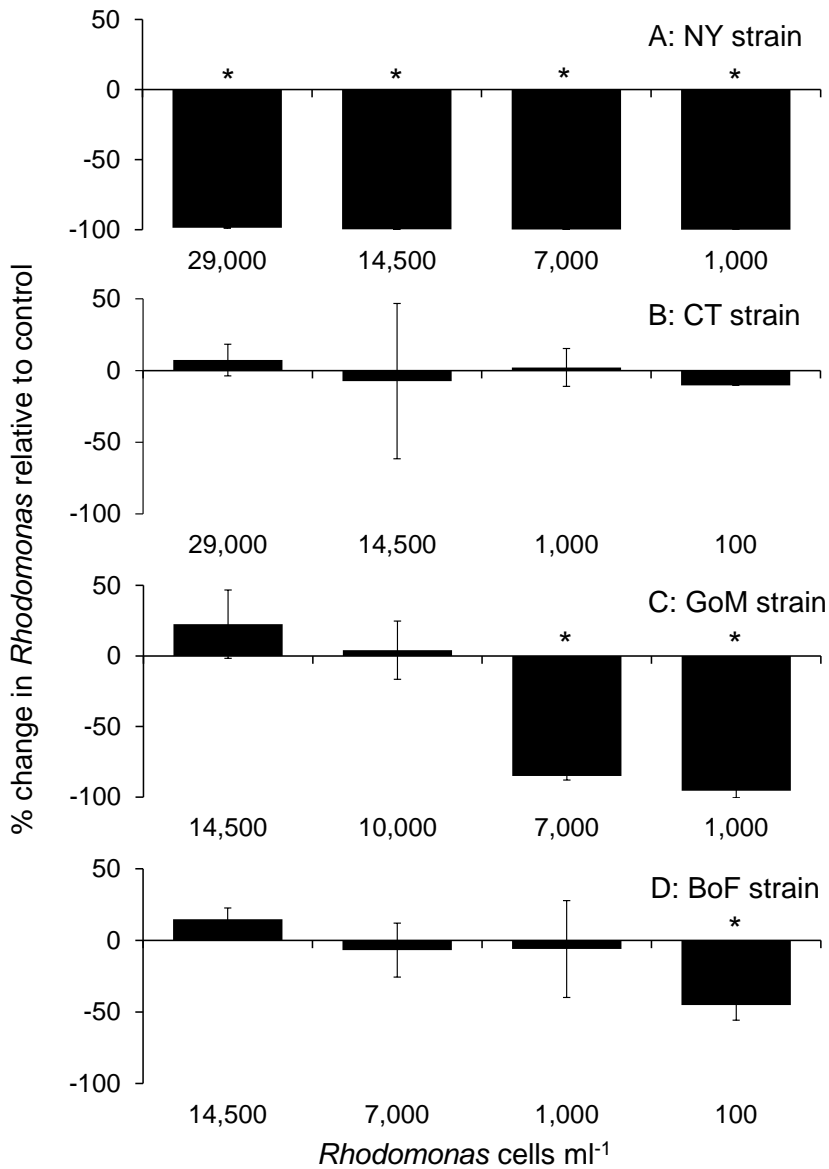
Figure 1. The lytic effects of four strains of *Alexandrium* (400 cells ml^{-1}) (A) New York isolate (NY strain), (B) a non-PSP producing isolate from Connecticut (CT strain), (C) an isolate from the Gulf of Maine (GoM strain), and (D) an isolate from the Bay of Fundy (BoF) on varying concentrations of the target organism *Rhodomonas salina* (CCMP 1319). Bars represent the percent (%) change in *Rhodomonas* relative to the control of triplicate measurements. Error bars represent SD. Asterisks indicate significant differences between the treatment and the control.
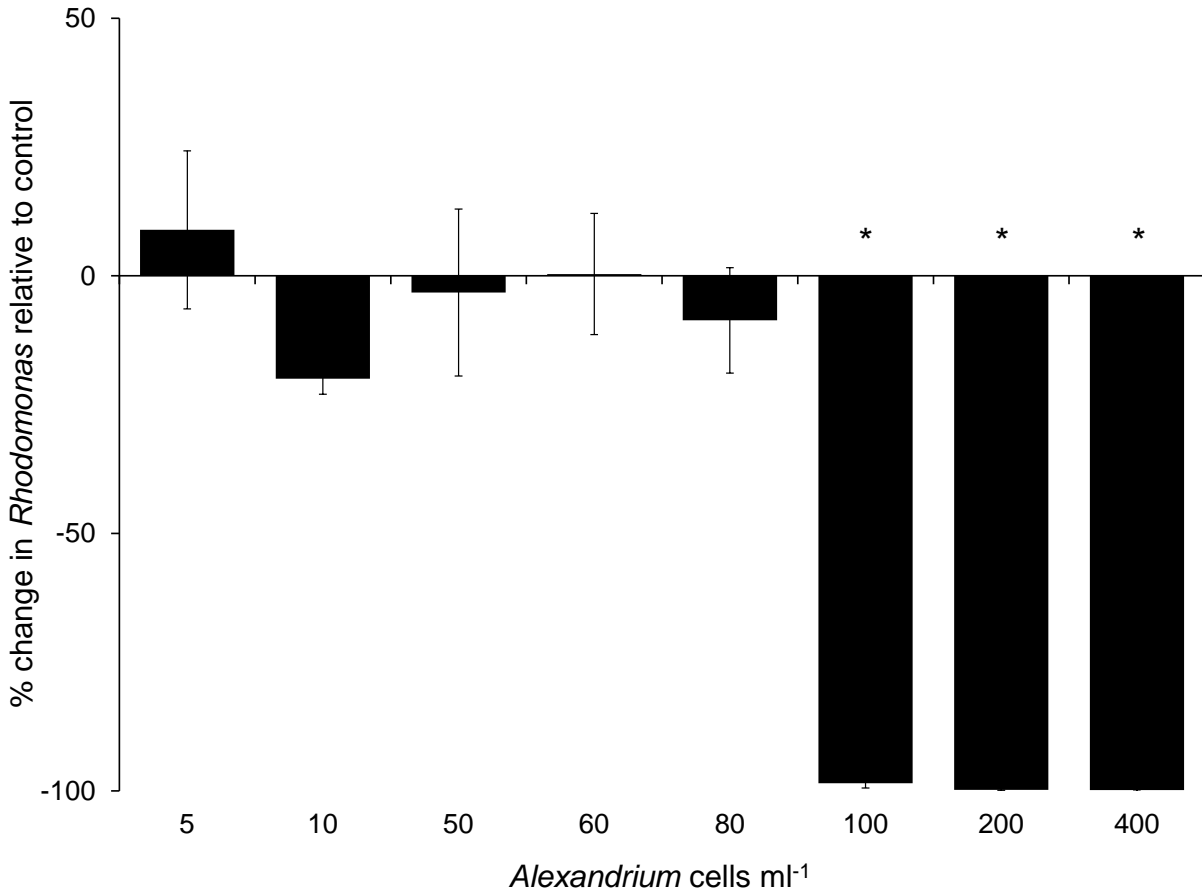
Figure 2. The lytic effects of varying concentrations of the New York *Alexandrium* isolate on the target organism *Rhodomonas salina* (CCMP 1319; 7,000 cells ml^{-1}). Bars represent the percent (%) change in *Rhodomonas* relative to the control of triplicate measurements. Error bars represent SD. Asterisks indicate significant differences between the treatment and the control.
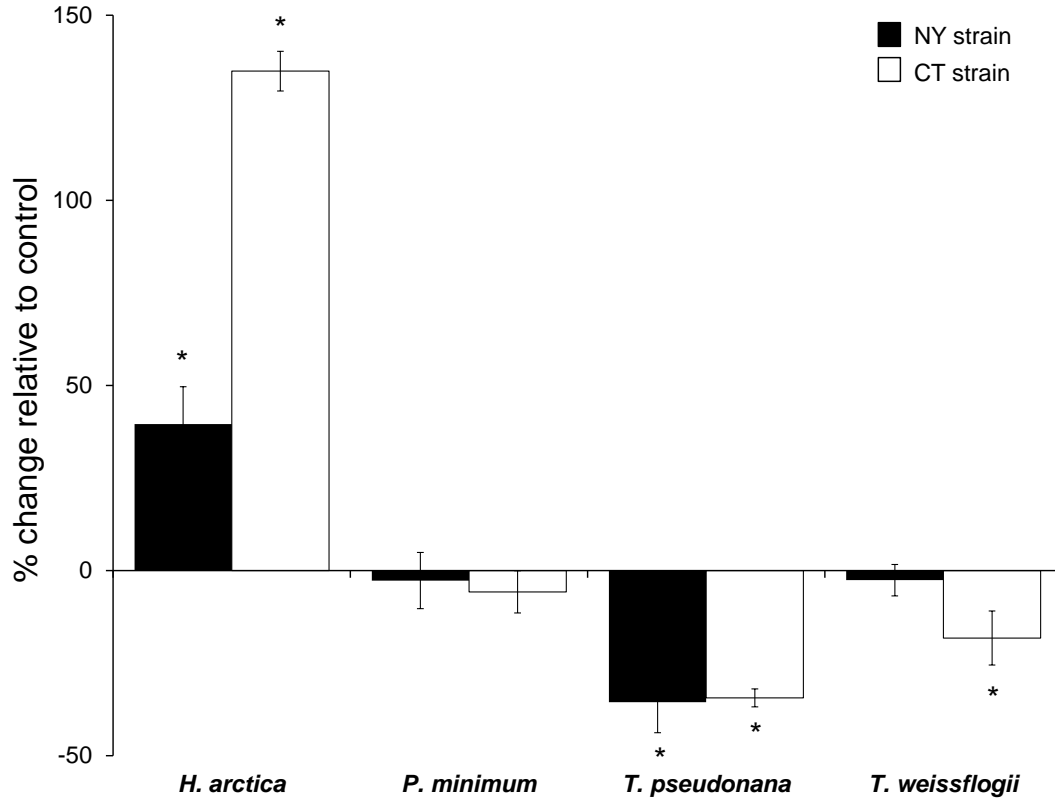
Figure 3. The effects of culture filtrate from *Alexandrium* isolates (NY strain=black bars, CT strain= white bars) on the target algae: *Heterocapsa arctica* (MS5; 1,000 cells ml^{-1}), *Prorocentrum minimum* (CCMP 696; 1,000 cells ml^{-1}), *Thalassiosira pseudonana* (CCMP 1335; 4,000 cells ml^{-1}) and *Thalassiosira weissflogii* (CCMP 1336; 4,000 cells ml^{-1}). Bars represent the percent (%) change in target organism relative to the control of triplicate measurements. Error bars represent SD. Asterisks indicate significant differences between the treatment and the control. Note: Percent change in *Heterocapsa arctica* has been divided by 10 to scale with other species in this figure.
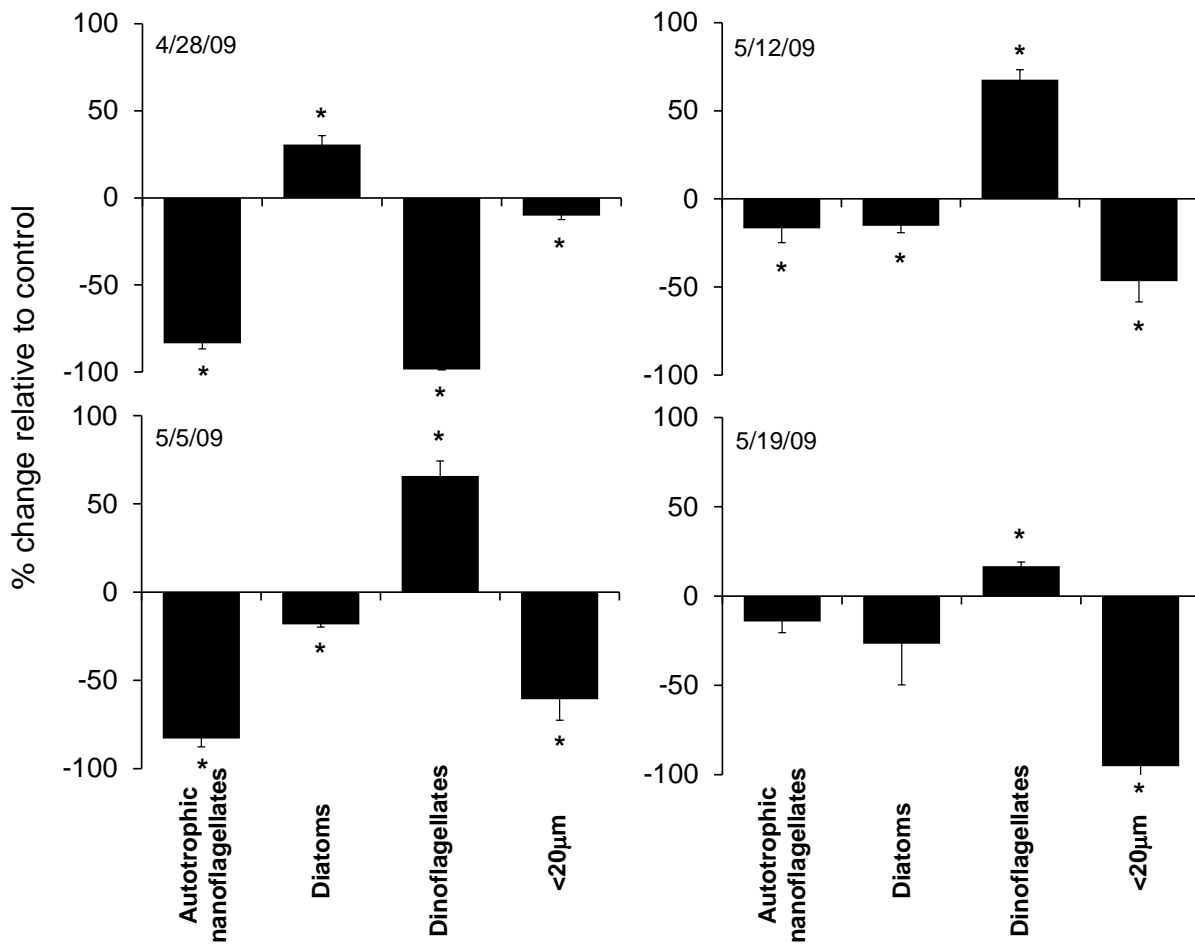
Figure 4. The effects of the addition of culture filtrate from a NY isolate of *Alexandrium* (ATNPD7) to natural phytoplankton communities from Northport Bay, NY during experiments conducted during spring 2009. Bars represent the percent (%) change of autotrophic nanoflagellates, diatoms, dinoflagellates, and <20μm chlorophyll *a* size fraction relative to the control of triplicate measurements. Error bars represent SD. Asterisks indicate significant differences between the treatment and the control.
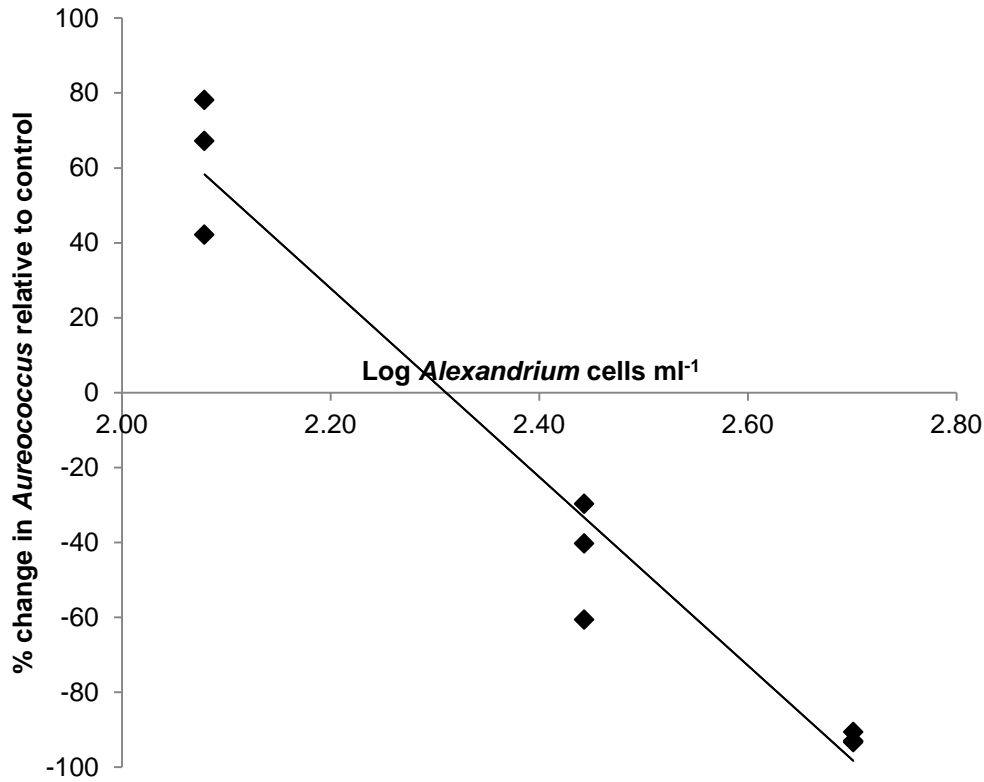
Figure 5. Percent change in *Aureococcus anophagefferens* densities in replicated experimental bottles treated with *Alexandrium* culture filtrate (NY isolate NPB8) relative to a control plotted as a function of log *Alexandrium* densities present in the filtrate used for each experiment. Experiments were conducted using water from Quantuck Bay, NY, during a brown tide bloom ($>10^5$ cells ml^{-1}) in June of 2011. Formula of the regression is $y = -251x + 5810$ ($R^2 = 0.96$).
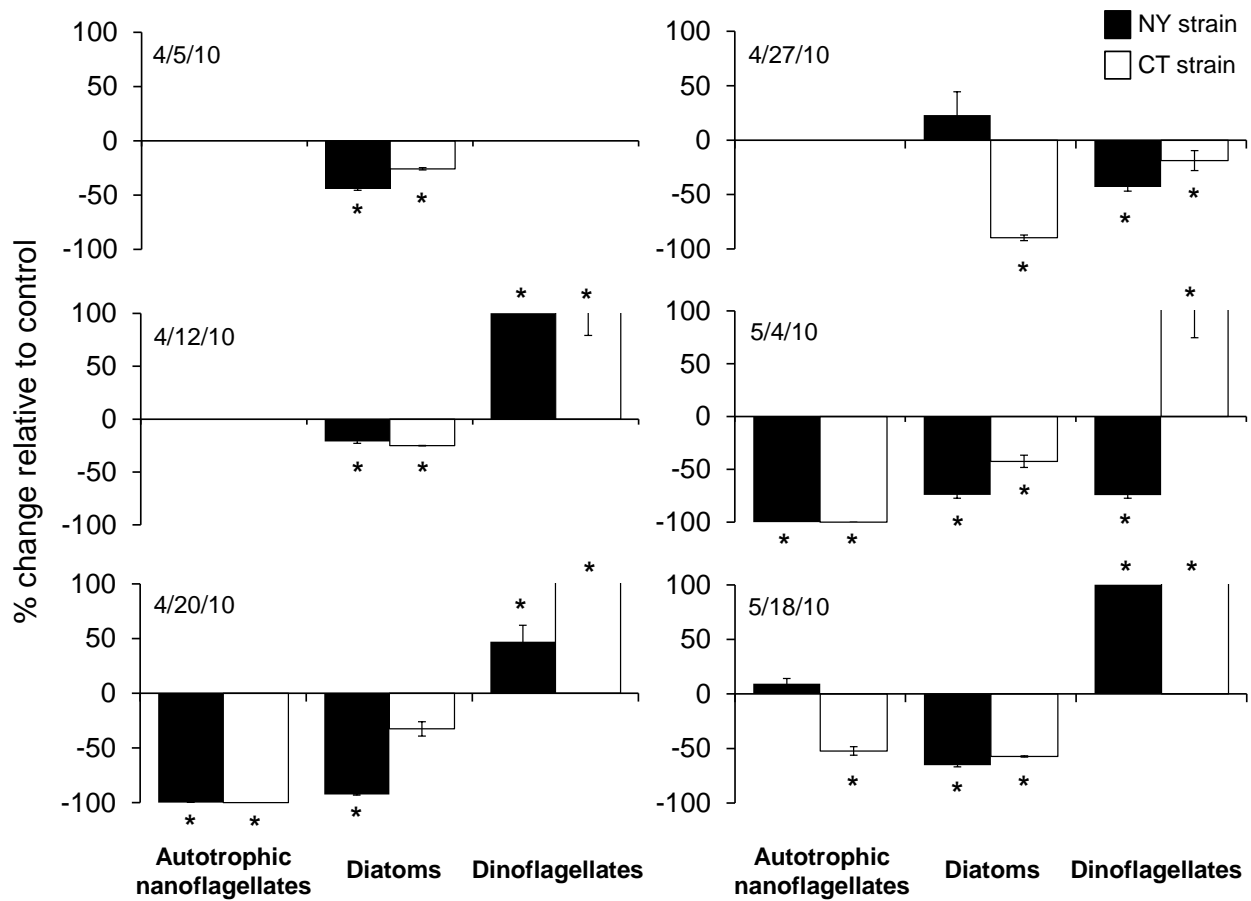
Figure 6. The effects of *Alexandrium* cells (NY strain (NPB8)= black bars, CT strain=white bars) on natural phytoplankton communities from Northport Bay, NY during experiments conducted during spring 2010. Bars represent the percent (%) change of autotrophic nanoflagellates, diatoms, and non- *Alexandrium* dinoflagellates relative to the control of triplicate measurements. Error bars represent SD. Asterisks indicate significant differences between the treatment and the control.
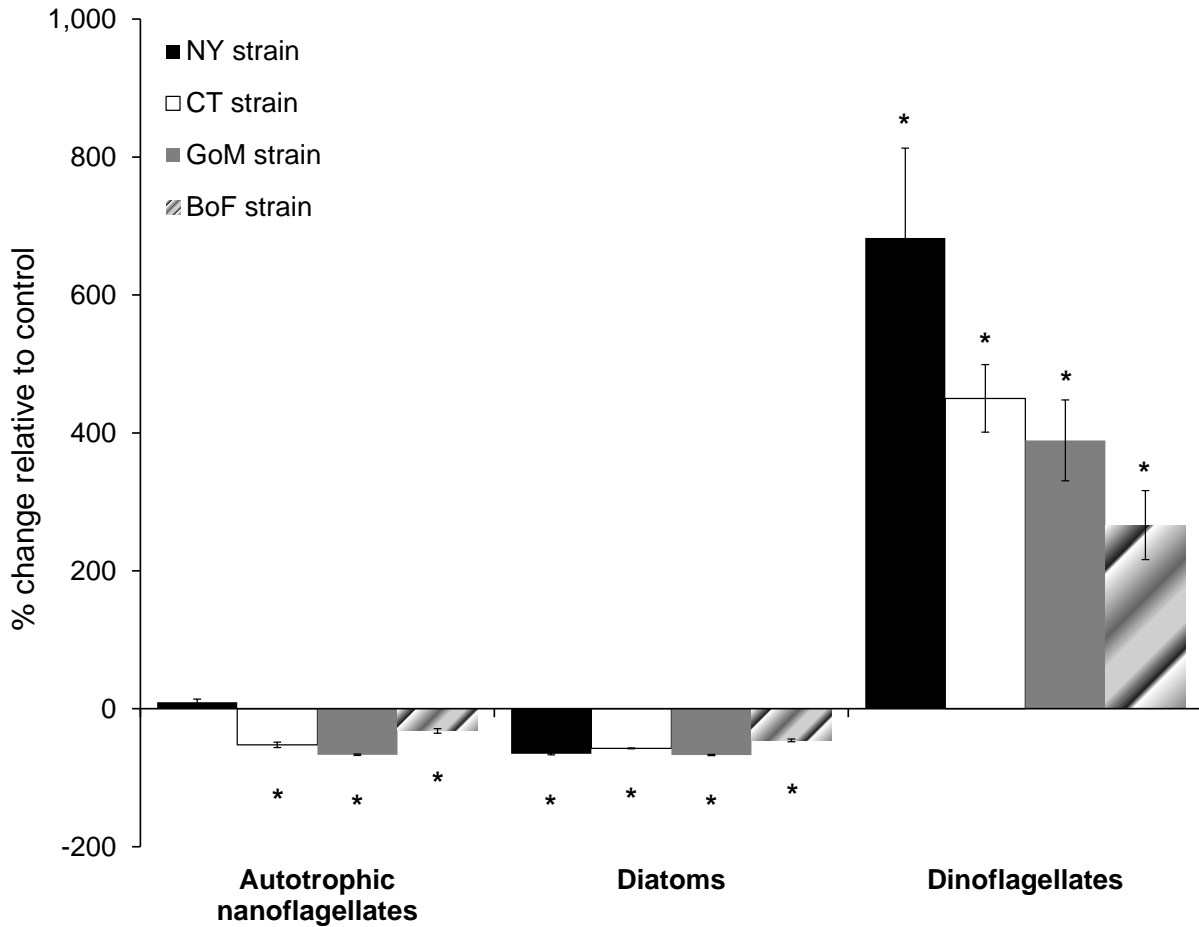
Figure 7. The effects of *Alexandrium* cells (NY strain=black bars, CT strain=white bars, Gulf of Maine strain=grey bars, Bay of Fundy strain=striped bars) on a natural phytoplankton community from Northport Bay, NY, during an experiment conducted on 18 May 2010. Bars represent the percent (%) change of autotrophic nanoflagellates, diatoms, and non- *Alexandrium* dinoflagellates relative to the control of triplicate measurements. Error bars represent SD. Asterisks indicate significant differences between the treatment and the control.
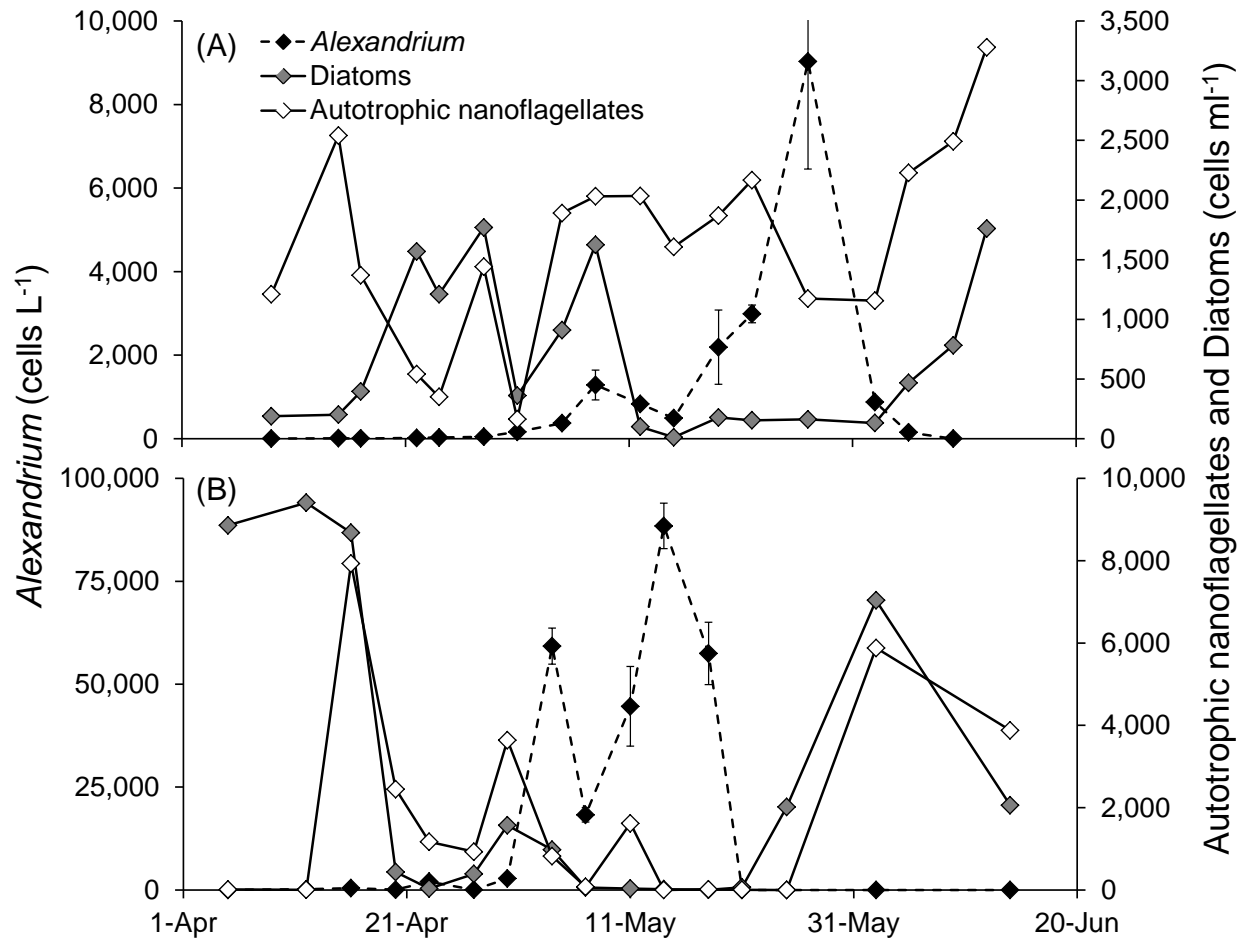
Figure 8. Dynamics of *Alexandrium fundyense* (cells L⁻¹), autotrophic nanoflagellate, and diatom densities (cells ml⁻¹) in Northport Harbor, NY during: A) spring 2009 and B) spring 2010. Points for *A. fundyense* are means of duplicate counts while error bars represent SD. Points for autotrophic nanoflagellates and diatoms represent single counts.

Table 1. The final concentrations of five strains of *Alexandrium* (cells ml⁻¹) added as cell-free filtrate (2009, 2011) and whole cell additions (2010) to experimental bottles containing a natural phytoplankton community from Northport Bay, NY (2009, 2010) and Quantuck Bay, NY (2011). Strains are abbreviated as follows: NY= New York; CT= a non-PSP producing isolate from Connecticut; GoM= Gulf of Maine; and BoF= Bay of Fundy. Asterisk denotes that two different New York isolates, both isolated from Northport Bay, were used for experiments; ATNPD7 in 2009 and NPB8 in 2010 and 2011.

	Final concentration of *Alexandrium* cells ml⁻¹				
	Date	NY*	CT	GoM	BoF
cell-free filtrate	4/28/2009	349			
	5/5/2009	392			
	5/12/2009	263			
	5/19/2009	188			
	6/14/2011	503			
	6/22/2011	120			
	6/29/2011	278			
whole cell additions	4/5/2010	123	183		
	4/12/2010	90	168		
	4/20/2010	330	265		
	4/27/2010	350	140		
	5/4/2010	273	38		
	5/18/2010	268	20	95	55

Table 2. Mean size fractionated (2-20μm) chlorophyll *a* (μg L^{-1}) and inorganic nutrient concentrations (μM) in Northport Harbor, NY during spring 2009 and 2010. Means of triplicate measurements are shown and values in parentheses represent standard deviations.

2009					
Date	2- 20μm	Phosphate	Silicate	Ammonium	Nitrate
9-Apr	1.34 (0.13)	0.97 (0.07)	21.2 (1.25)	0.89 (0.12)	20.1 (0.34)
15-Apr	3.29 (0.14)	1.13 (0.20)	24.3 (0.69)	1.37 (0.08)	23.5 (0.81)
17-Apr	1.36 (0.20)	0.94 (0.08)	17.8 (1.25)	0.60 (0.05)	17.5 (0.35)
22-Apr	2.51 (1.29)	0.85 (0.11)	6.59 (0.52)	0.86 (0.06)	9.65 (1.67)
24-Apr	5.01 (0.86)	0.49 (0.13)	26.7 (0.96)	0.86 (0.04)	18.9 (8.54)
28-Apr	3.74 (2.01)	0.79 (0.20)	17.0 (0.49)	1.38 (0.28)	4.30 (0.36)
1-May	1.48 (0.11)	0.92 (0.22)	15.3 (2.09)	5.70 (0.13)	6.85 (1.19)
5-May	4.47 (0.31)	0.91 (0.14)	19.3 (2.61)	6.43 (0.55)	13.7 (0.39)
8-May	3.86 (0.17)	0.63 (0.13)	20.1 (0.27)	1.95 (0.02)	11.2 (0.21)
12-May	2.51 (0.92)	0.65 (0.22)	21.7 (0.71)	4.64 (0.23)	12.8 (3.03)
15-May	2.54 (0.58)	0.36 (0.07)	22.1 (0.31)	7.02 (1.58)	10.5 (2.37)
19-May	6.83 (1.32)	0.32 (0.04)	19.8 (0.21)	7.41 (1.00)	21.9 (4.53)
22-May	1.07 (0.15)	0.45 (0.03)	25.0 (0.02)	12.8 (2.98)	18.7 (2.94)
27-May	1.23 (0.29)	0.40 (0.04)	22.7 (0.19)	2.12 (0.44)	7.39 (0.41)
2-Jun	2.25 (0.18)	0.92 (0.17)	25.0 (2.80)	6.87 (0.65)	11.3 (0.34)
5-Jun	11.9 (0.65)	0.51 (0.06)	25.7 (0.63)	10.8 (0.58)	16.4 (2.83)
9-Jun	0.18 (0.37)	0.16 (0.02)	20.6 (4.39)	2.90 (0.16)	12.3 (1.68)
12-Jun	32.7 (2.04)	0.23 (0.01)	9.38 (1.26)	1.52 (0.16)	2.93 (0.07)

2010					
Date	2- 20μm	Phosphate	Silicate	Ammonium	Nitrate
1-Apr	3.41 (0.32)	0.36 (0.04)	28.8 (0.29)	0.92 (0.11)	28.8 (0.77)
5-Apr	7.63 (3.12)	0.13 (0.04)	15.2 (1.88)	0.09 (0)	4.75 (1.56)
12-Apr	7.99 (3.38)	0.15 (0.03)	22.8 (0.25)	0 (0)	12.9 (1.39)
16-Apr	4.07 (0.26)	0.16 (0.02)	15.4 (0.48)	0 (0.03)	5.39 (0.63)
20-Apr	1.09 (0.44)	0.80 (0.03)	30.9 (0.52)	3.50 (0.10)	25.0 (1.73)
23-Apr	4.25 (0.54)	0.52 (0.03)	28.8 (0.44)	0.37 (0.09)	17.3 (0.25)
27-Apr	11.2 (2.88)	0.21 (0.01)	39.1 (1.76)	0.64 (0.05)	37.6 (1.98)
30-Apr	9.17 (1.30)	0.13 (0.01)	31.7 (1.53)	0.26 (0)	10.8 (0.49)
4-May	1.57 (0.17)	0.41 (0.03)	29.1 (0.73)	3.04 (0.26)	16.6 (1.02)
7-May	1.33 (0.22)	0.87 (0.05)	42.5 (0.14)	5.73 (0.17)	55.4 (2.31)
11-May	2.45 (0.24)	0.90 (0.05)	33.1 (0.37)	5.32 (0.99)	29.1 (0.20)
14-May	2.75 (0.29)	0.47 (0.09)	32.6 (0.64)	2.78 (0.04)	22.4 (1.03)
18-May	0.87 (0.10)	0.74 (0.04)	42.5 (3.25)	7.90 (0.13)	39.8 (1.81)
21-May	1.09 (0.12)	0.65 (0.15)	33.1 (0.48)	8.92 (0.66)	24.3 (0.10)
25-May	4.57 (0.43)	0.27 (0.06)	28.2 (0.27)	0.93 (0.15)	18.0 (0.27)
2-Jun	10.7 (0.36)	0.16 (0.03)	12.8 (0.32)	0.88 (0)	12.5 (1.02)
14-Jun	13.1 (1.07)	0.93 (0.06)	23.2 (0.27)	4.39 (0.38)	14.1 (0.80)
18-Jun	4.23 (0.33)	1.26 (0.04)	32.0 (0.56)	5.56 (0.70)	17.9 (1.08)

Chapter 3

The effects of elevated CO$_2$ on the growth and toxicity of field populations and cultures of

the saxitoxin-producing dinoflagellate, *Alexandrium fundyense*

Abstract

The effects of coastal acidification on the growth and toxicity of the saxitoxin-producing dinoflagellate, *Alexandrium fundyense*, were examined in ecosystem and culture studies. In culture experiments, *Alexandrium* strains isolated from Northport Bay NY, USA, and the Bay of Fundy, Canada, grew significantly faster (16 -190%; $p<0.05$) when exposed to elevated levels of pCO_2 (~ 800- 1900µatm) compared to lower levels (~390µatm). The total toxin content (fg STX eq. cell^{-1}) of the Northport Bay strain increased (71 – 81%) significantly when exposed to elevated pCO_2 levels while no significant changes in toxin content were observed in the Bay of Fundy strain. During *Alexandrium* blooms in Northport Bay, pCO_2 concentrations increased over the course of a bloom to more than 1700µatm and were highest in regions with the greatest *Alexandrium* abundances. Field experiments performed during blooms using the range of pCO_2 levels recorded in Northport Bay (390 – 1500 µatm) demonstrated that *Alexandrium* densities were significantly and consistently enhanced by higher levels of pCO_2. The co-occurrence of *Alexandrium* blooms and elevated pCO_2 represents a previously unrecognized environmental threat to coastal ecosystems. The ability of elevated pCO_2 to enhance the growth and toxicity of *Alexandrium* indicates that acidification promoted by eutrophication or climate change can intensify these, and perhaps other, harmful algal blooms.

Introduction

It has recently been recognized that eutrophication resulting from anthropogenic nutrient loading can contribute to the acidification of coastal systems (Borges and Gypens, 2010; Cai et al., 2011; Melzner et al., 2013). While atmospheric CO_2 levels are estimated to rise beyond 800 ppm by 2100 (I.P.C.C., 2007), many estuaries are already experiencing CO_2 levels exceeding these projected climate change scenarios (Talmage and Gobler, 2009; Cai et al., 2011; Hofmann et al., 2011; Barton et al., 2012; Melzner et al., 2013). These high CO_2 and low pH conditions can change nitrification rates (Beman et al., 2011; Fulweiler et al., 2011), hydrolytic enzyme activity (Yamada and Suzumura, 2010; Maas et al., 2013), and alter trace metal chemistry (Millero et al., 2009; Hoffmann et al., 2012) all of which can alter nutrient cycles and in turn affect algal communities. Given the important role that marine phytoplankton play in food webs and carbon cycling, further research on the effects of ocean acidification on phytoplankton is needed.

During the past decade there have been multiple studies investigating the effects of ocean acidification (increased pCO_2 and decreased pH) on individual phytoplankton species as well as the composition of natural phytoplankton communities (Riebesell et al., 2000; Lefebvre et al., 2012; Nielsen et al., 2012, and references therein). One group of phytoplankton that may be strongly affected by acidification is harmful algae. Among *Pseudo-nitzschia* spp., increasing pCO_2 concentrations can increase cellular growth rates and concentrations of its toxin, domoic acid (Sun et al., 2011; Tatters et al., 2012). Other marine HABs, such as *Karlodinium veneficum* and *Heterosigma akashiwo* have displayed significantly faster growth rates under elevated levels of pCO_2 (Fu et al., 2008; Fu et al., 2010). Contrastingly, using acid additions to manipulate pH, other studies have reported that multiple coastal phytoplankton strains (including *P. minimum*

and *K. veneficum*) are unaffected by large changes in pH (7.0 to 8.4; Berge et al., 2010). Clearly, more research on the effects of CO_2 on HAB taxa is needed given the wide range of effects that has already been observed for this group.

One group of harmful algae that seems particularly sensitive to elevated pCO_2 concentrations is that comprised of the saxitoxin-producing dinoflagellate species in the genus *Alexandrium* (Flores-Moya et al., 2012; Fu et al., 2012; Kremp et al., 2012; Tatters et al., 2013a; Van de Waal et al., 2014). *Alexandrium* species from Europe (*A. minutum*, Flores-Moya et al., 2012; *A. ostenfeldii*, Kremp et al., 2012) and the west coast of North America (*A. catenella*; Fu et al., 2012, Tatters et al., 2013) have displayed strain-specific increases in growth and/or toxicity when exposed to elevated pCO_2. While *A. fundyense* strains from the east coast of North America have caused paralytic shellfish poisoning (PSP) for more than fifty years (Martin and Richard, 1996), the responses of this species to elevated pCO_2 are poorly known. Given that dinoflagellates possess form II RubisCO, which has a low affinity for CO_2 (Morse et al., 1995; Rost et al., 2006; Reinfelder, 2011) and is the key enzyme facilitating CO_2 fixation, *Alexandrium* and other dinoflagellates may flourish within a high CO_2 environment (Fu et al., 2012). Furthermore, high pCO_2 (low pH) environments may change cellular toxin levels of *Alexandrium* by altering biosynthesis rates (Fu et al., 2012) and/or causing pH-induced toxin conversions (Laycock et al., 1995). Hence, it is important to assess the effects of elevated CO_2 on the growth and toxicity of North American strains of *Alexandrium* given that many coastal systems within this region are currently experiencing levels of elevated pCO_2 (Talmage and Gobler, 2009) as a result of cultural eutrophication (Nixon, 1995; Heisler et al., 2008).

Here I report on the effects of elevated CO_2 on the growth and toxicity of the saxitoxin-producing dinoflagellate, *Alexandrium fundyense*. I present a series of culture experiments using

strains of *Alexandrium* (from NY, USA, and the Bay of Fundy, Canada) with differing toxin profiles to assess the effects of pCO_2 on the growth and toxicity of *Alexandrium*. In addition, I examined the temporal and spatial dynamics of *Alexandrium* densities, water chemistry, plankton communities, and pCO_2 concentrations in a coastal system. Finally, natural phytoplankton communities were subjected to varying levels of pCO_2 to assess changes in *Alexandrium* densities and toxicity as well as the total phytoplankton community during bloom events.

Materials and Methods

Culture experiments- Culture experiments were performed to assess the effects of different CO_2 levels on *Alexandrium* growth and toxicity (total toxin content and toxin profiles). Experiments were performed using two *Alexandrium* strains (clone NPB8 isolated from Northport Bay, NY, USA and clone CCMP 2304 isolated from the Bay of Fundy, Canada) with differing toxin profiles (Maranda et al., 1985; Anderson et al., 1990; Anderson et al., 1994), affording a comparison of changes in toxin composition due to changes in pCO_2. Stock cultures were maintained at 20°C using *f*/2 (-Si) media (Guillard and Ryther, 1962) made from filtered coastal Atlantic Ocean water (40.7969°N, 72.4606°W; salinity = 32-33) supplemented with 2% antibiotic solution (stock solution, Thermo Scientific HyClone Penicillin (10,000 U ml^{-1}) Streptomycin (10,000 µg ml^{-1}) in 0.85% NaCl) under 100 µmol quanta m^{-2} s^{-1}.

Experiments were designed to assess how current, eutrophication-induced coastal acidification may affect the development of *Alexandrium* blooms. To assess the effects of CO_2 on *Alexandrium* growth and toxicity, cultures were subjected to a control level of pCO_2 (390µatm; Mauna Loa Observatory by the Earth Systems Research Laboratory NOAA) as well as elevated levels observed in local coastal systems with *Alexandrium* blooms (800- 1900 µatm; this study) using a gas proportionator system (Cole Parmer® Flowmeter system, multitube

frame) that mixed ambient air with 5% CO_2 gas at a net flow rate of 300 ± 5 mL min⁻¹ (Talmage and Gobler, 2009). Experiments with each strain were repeated 3 - 4 times over the course of two years; within each experiment, treatments were run in triplicate or quadruplicate and incubated at 20°C under 100 µmol quanta m⁻² s⁻¹.

Experimental cultures were grown semi-continuously (Feng et al., 2008), being diluted to 400 cells mL⁻¹ every three days to maintain cells in exponential growth phase and to minimize pH fluctuations associated with the photosynthetic consumption of CO_2. Stock media (*f*/2 -Si) with 2% antibiotic solution was bubbled at the proper CO_2 level to ensure that, upon diluting cultures to starting densities for each time point, cells were inoculated into media set to the proper CO_2 and pH level. For each continuous culture transfer, culture aliquots were preserved in Lugol's iodine and quantified using a Multisizer 3 Coulter Counter (Beckman Coulter, USA) to determine the dilution needed for each experimental flask. Enumeration of cells via the Multisizer and a microscope differed by ≤5% and each method yielded a relative standard deviation of 5-10%. Cellular growth rates were calculated at each time point. Aliquots of culture were pelletized using centrifugation, 1500 xg for 11 minutes, and the supernatant aspirated without disturbing the pellet in preparation for extraction and HPLC-FLD analysis (see below).

Experiments were performed to match the duration of bloom events and thus lasted two to four weeks (Anderson, 1997; Hattenrath et al., 2010; Hattenrath-Lehmann and Gobler, 2011). Measurements of pH within cultures (Table 1) were made throughout each experiment using an Orion 3-star Plus electrode ($\pm$ 0.001) calibrated prior to each use using NBS traceable standards. Measurements using this pH meter were highly similar to and never significantly different from scale corrected (Dickson, 1993) spectrophotometric pH measurements made using *m*-cresol

purple as described by Dickson et al. (2007). Total dissolved inorganic carbon (DIC) concentrations in cultures were measured using an EGM-4 Environmental Gas Analyzer (PP Systems) system that quantifies DIC levels after separating the gas phase from seawater via acidification and using a Liqui-Cel Membrane (Membrana; Talmage and Gobler, 2009). This instrument provided a methodological precision better than ± 5% for replicated measurements of total dissolved inorganic carbon. The levels of DIC and pH within Dr. Andrew Dickson's (University of California San Diego, Scripps Institution of Oceanography) certified reference material (Batch 102 and 123) were measured during every analytical run as a quality assurance measure; analysis of samples proceeded only after complete recovery of those standards was obtained. pCO_2 levels were calculated using measured levels of DIC, pH (NBS scale), temperature, and salinity, as well as the first and second dissociation constants of carbonic acid in seawater according to Roy et al. (1993) using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/).

Toxin analysis- Cell pellets in pre-weighed tubes were resuspended in 500 µL or 1,000 µL of 0.05M acetic acid, weighed, and freeze-thawed three times to aid in cell rupture. Cell suspensions were then sonified (Branson, Model S-250D), on ice, using a microtip at 40% for one minute. Samples were centrifuged at 3,000 g for five minutes at room temperature and supernatants were passed through an Oasis HLB solid phase cartridge (Waters, 3cc, 60mg) to remove interfering compounds after the cartridge was equilibrated with 3 mL of methanol and 3 mL of Milli-Q water, following the manufacturer's instructions. The eluate was transferred to a filter unit (Amicon Ultra 0.5 10,000 MW, regenerated cellulose) and centrifuged for 15 minutes at 12,000 g. Samples were stored frozen at -20 °C prior to HPLC-FLD analysis at which time the extracts were thawed, mixed and analyzed by HPLC for saxitoxins using the three-step

isocratic elution method of Oshima (1995) with post-column derivatization, as modified in Anderson et al. (1994). Twelve congeners were quantified against reference standards (National Research Council, Canada): saxitoxin; neosaxitoxin; decarbamoyl saxitoxin; gonyautoxins 1, 2, 3, 4, 5 (or B1); decarbamoyl gonyautoxins 2, 3; toxins C1 and C2. All samples were analyzed by Juliette Smith (in the Anderson Lab at Woods Hole Oceanographic Institution). Toxicities (in fg STX equivalent cell^{-1}) were calculated from molar composition data using congener-specific conversion factors (mouse units/μmol toxin) published in Oshima (1995) and epimer pairs were then pooled. In several instances, non-detects were reported as DL/2 (i.e. half the method detection limit) to avoid artificial changes to toxin profiles where the lack of a congener's presence was due to lower detection limits. To qualify for this adjustment, data met the following criteria: 1) ≥ half of the replicates showed the congener present, and 2) the congener was present in other experiments and/or pellets of a high density culture of that same strain. Differences in growth rates and toxin levels among treatments within experiments were elucidated by means of a one-way ANOVA, using Sigma Stat software embedded within Sigma Plot 11.0. Data not meeting the assumptions of normality were log transformed.

Field study - Field samples were collected on a weekly basis from March through June during 2011 and 2012. Samples were collected from a site in Northport Harbor, NY, USA (40.8916°N, 73.3572°W; site 2, Fig. 1; Hattenrath et al., 2010), which is a shallow (2 - 4m), well mixed, eutrophic system within the southeastern portion of the Northport-Huntington Bay complex, located on the southern shore of Long Island Sound. Additionally, in 2012 a cruise was conducted across multiple sites (Fig.1) to assess the spatial extent of these blooms. Further samples were collected from Mattituck Creek, NY, USA (40.9942°N, 72.5381°W), a tributary 50

km east of Northport Bay that also tidally exchanges with Long Island Sound and experiences annual *Alexandrium* blooms.

At each site, concentrated water samples were made by sieving 2L of water through a 200 μm mesh (to eliminate large zooplankton) and then onto a 20 μm sieve that was backwashed into a 15mL centrifuge tube. *Alexandrium fundyense* densities were enumerated using a highly sensitive molecular probe procedure described by Anderson et al. (2005) and described in detail by Hattenrath et al. (2010). Briefly, aliquots of phytoplankton concentrates (formalin and then methanol preserved) were hybridized with an oligonucleotide probe specific for the NA1 North American (Group I) ribotype *Alexandrium fundyense/catenella/tamarense* with Cy3 dye conjugated to the 5' terminus (5'-/5Cy3/AGT GCA ACA CTC CCA CCA-3'). Cells were enumerated using a Nikon epifluorescence microscope with a Cy3™ filter set (Anderson et al., 2005).

Samples for chlorophyll *a* and bacterial enumeration were collected from Northport Harbor (Fig.1). For the determination of chlorophyll *a*, water was filtered in triplicate using glass fiber filters (GF/F; nominal pore size 0.7μm) and measured using standard fluorometric techniques described in Welschmeyer (1994). Whole water samples were preserved in 10% buffered formalin (0.5% v/v final), stored at -80°C, and analyzed flow cytometrically to quantify the abundance of heterotrophic bacteria. Samples were stained with SYBR Green I and heterotrophic bacteria were quantified using a FACScan (BD®) flow cytometer (Jochem, 2001). To quantify the pCO_2 concentrations present during *Alexandrium* blooms, two types of in situ measurements were made in Northport Bay. In 2011, pCO_2 levels were measured during the *Alexandrium* bloom in Northport Harbor via the stationary deployment of a HydroC™/CO2 probe (Contros, Kiel, Germany) that makes in situ measurements every 5 seconds using infrared

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technology. This instrument has been shown to provide measurements of CO_2 in multiple coastal systems consistent with levels determined from discrete measurements of dissolved inorganic carbon and pH using standard methods (ACT, 2010; Fiedler et al., 2012; Baumann et al., submitted). To groundtruth measurements made by the HydroC™/CO_2 probe during this study, total dissolved inorganic carbon (DIC) samples were collected from the same depth in the water column that the probe was deployed (0.5 m) using a Van Dorn bottle. Water was transferred without bubbling to a 300 mL borosilicate bottle and preserved using a saturated mercuric chloride solution added as 0.03% of the sample volume and kept at 4°C until analysis of pH and DIC and determination of carbonate chemistry as described above for laboratory experiments.

The spatial distribution of pCO$_2$, chlorophyll a, and salinity during *Alexandrium* blooms was assessed in May 2012 during a horizontal transect cruise through Northport Bay (Fig. 1). The HydroC™/CO_2 probe and a YSI 6920v2 sonde (YSI Inc., Yellow Springs, OH) equipped with salinity and chlorophyll a fluorescence sensors were affixed to a bracket mounted on the side (towards the stern) at a depth of 0.5m on a small vessel that proceeded below wake speed ($\sim$1 m s^{-1}) to minimize turbulent mixing around sensors. Prior to the cruise, the time signatures of the HydroC™/CO_2 probe and the YSI sonde were aligned with a GeoChron Blue GPS device (SparkFun™ Electronics, Boulder, CO) to link measurements in space and time. Maps of these measured parameters were generated using ARC GIS 10 (Esri, Redlands, CA).

Incubations of natural populations- To assess how short term changes in CO_2 levels that occurred during this study may affect the growth and toxin production of *Alexandrium fundyense* as well as competing phytoplankton, Northport Bay water was subjected to three levels of CO_2 ($\sim$390, $\sim$750, and $\sim$1500 µatm; 13 and 22 May 2011) under controlled laboratory conditions. An

additional experiment was conducted on 27 May 2011 using water from Mattituck Creek, NY, USA, (Fig 1). To reduce algal biomass levels and thus permit better control of carbonate chemistry and further algal growth, triplicate 2.5L bottles were filled with 1.25L whole seawater and 1.25L of 0.2 µm filtered seawater made via gravity filtration with a sterile, 0.2 µm capsule filter (Pall© Port Washington, NY). Bottles were amended with f/80 nutrients (with a N:Si ratio of 1:1) and incubated in front of a bank of fluorescent lights (100 µmol quanta m^{-2} s^{-1}) at the temperature of the bloom water (~16°C) for 4-6 days at the Stony Brook Southampton Marine Science Center. A gas proportionator system was used to deliver ambient air (390 µatm) and premixed CO_2 gas (750, 1500 µatm; Praxair) to seawater treatments at a net flow rate of 300 ± 5 mL min^{-1} which was continuously delivered to the bottom of the experimental bottles using airstones (Table 2; Rose et al., 2009). This delivery rate turned over the volume of experimental bottles >100 times daily, ensuring that desired CO_2 concentrations and pH levels were maintained (Talmage and Gobler, 2009). Multiple pH measurements were made throughout the experiment using both Oakton® (± 0.01) and Orion 3-star plus (± 0.001) electrodes calibrated prior to each use using NBS traceable standards (Table 2). pH measurements made via the Orion and Oakton® probes were highly correlated to each other ($r^2 = 0.99$) and highly similar to and not significantly different from scale corrected spectrophotometric pH measurements (Dickson, 1993; Dickson et al., 2007).

Upon termination of the experiment, *A. fundyense* cells were enumerated and cell pellets from 1L of water were collected, extracted and the toxin content quantified via HPLC, as described above. Size fractionated chlorophyll *a* (GF/F and 20µm polycarbonate filters, see Field study) and Lugol's iodine samples were preserved and analyzed to assess changes in the plankton community. Plankton cells larger than 10 µm were identified to at least genus level and

grouped as dinoflagellates and diatoms using a 1mL Sedgewick-Rafter slide under a compound microscope. Differences among treatments were assessed using a One-Way ANOVA using Sigma Stat software embedded within Sigma Plot 11.0.

Results

Culture experiments- Carbon dioxide concentrations altered the growth and toxicity of the Northport Bay and Bay of Fundy strains of *Alexandrium fundyense*. Both *Alexandrium* strains, isolated from Northport Bay (NPB8) and Bay of Fundy (CCMP2304), had significantly higher growth rates (16 -190%) when exposed to elevated levels of pCO_2 (~ 800- 1900µatm, Table 1) compared to the control (~390µatm; Fig. 2). These growth rate enhancements were statistically significant ($p<0.05$) in six of the seven experiments performed with one of four experiments with NPB8 being the single exception (Fig. 2). In addition, the total cellular toxicity (fg STX eq. cell^{-1}) of the Northport Bay strain was significantly higher (71-81%) in cultures exposed to elevated pCO_2 compared to the control ($p<0.05$; Exp. #2, 4; Fig. 3). While the toxicity of all individual derivatives increased, only GTX1, 4 increased significantly ($p<0.05$; Table 3). Patterns in toxin content were similar to those observed for cellular toxicity (Fig. 3). In general, variations in the percent molar toxin composition due to changes in pCO_2 were small (Exp#4, GTX1,4 and GTX5; Fig. 3). In contrast, the total cellular toxicity was not significantly altered by pCO_2 in the Bay of Fundy strain and was highly variable between experiments, with both small increases and decreases in the same individual toxin derivatives (Fig. 4; Table 4). Similar to the Northport Bay strain, the Bay of Fundy strain had negligible changes in percent molar toxin composition, suggesting for both these strains that changes in cellular toxicity were driven by changes in toxin content and not composition.

The temporal and spatial dynamics of pCO₂ during Alexandrium blooms- During

spring 2011, *Alexandrium* was detectable in the water column of Northport Bay from late March

through late May, with peak densities occurring on 9 May (25,300 cells L^{-1}) and a smaller

secondary peak (6,600 cells L^{-1}) on 16 May (Fig 5A). Total phytoplankton biomass was

significantly lower during the *Alexandrium* bloom (3- 24 May; 3.3 ± 0.9 μg chlorophyll *a* L^{-1})

compared to before (28 March –29 April) and after (1- 6 June) the bloom (11.5 ± 2.1 μg

chlorophyll *a* L^{-1}; Fig. 5A; $p<0.01$, Mann-Whitney Rank Sum test). Heterotrophic bacterial

abundances were higher (6.8 ± 0.9 x 10^6 cells mL^{-1}) during the bloom compared to before and

after (4.4 ± 1.0 x 10^6 cells mL^{-1}) but not significantly so (t-test, $p>0.05$; Fig. 5B). During the

Alexandrium bloom, autonomously recorded pCO₂ concentrations displayed daily fluctuations

but gradually increased from 235μatm (7 May) to 1799μatm (21 May; Fig. 5B). The first peak

of the *Alexandrium* bloom coincided with lower pCO₂ levels (9 May; 350 – 560μatm), while the

secondary peak (16 May) occurred during elevated pCO₂ levels (590 – 1000μatm; Fig. 5A,B).

The levels of pCO₂ measured by the probe were slightly lower (3 - 22%) than levels measured

via the discrete DIC samples, but concentrations measured using both of these methodologies

were highly correlated ($R=0.96$; $p=0.10$). Finally, pCO₂ levels determined within discrete

samples were inversely correlated with chlorophyll *a* concentrations ($R= -0.77$; $p=0.15$).

During spring 2012, *Alexandrium* was found in Northport Bay from mid-March to late

May with peak densities reaching 23,000 cells L^{-1} on 7 and 15 of May (Fig. 6A). Heterotrophic

bacterial abundances (peak= 5.6 x 10^6 cells mL^{-1}) gradually increased over the course of, and

peaked in unison with, the *Alexandrium* bloom (Fig. 6B). pCO₂ concentrations (as measured

from discrete DIC samples) measured before and during the peak of the *Alexandrium* bloom

were elevated and ranged from 896 to 1260 μatm (Fig. 6B). Similar to 2011, phytoplankton

biomass was lower during the peak of the *Alexandrium* bloom (30 April- 16 May; 4.3 ± 0.3 µg chlorophyll a L^{-1}) compared to before (15 March –24 April) and after (21- 29 May) the bloom (9.7 ± 1.9 µg chlorophyll a L^{-1}; Fig. 6A).

During the peak of the *Alexandrium* bloom (16 May 2012), a cruise was conducted to assess the spatial distribution of *Alexandrium* densities, pCO_2 concentrations, salinity, and chlorophyll a concentrations across Northport Bay (Fig. 7). *Alexandrium* densities ranged from $180 – 8,300$ cells L^{-1} with the highest densities occurring in Northport Harbor (site 2) and gradually decreasing towards Northport Bay (site 10; Fig. 7A). A transect from Northport Harbor into Northport Bay (and back) measured pCO_2 concentrations from $360 – 1230$µatm with the highest levels ($>1,000$µatm) of pCO_2 confined to the Northport Harbor region and lower levels towards the Bay (<500µatm; Fig. 7B). In contrast, salinity was lower in the Harbor region (~ 24) and increased (25.7) towards the Bay (Fig. 7C). Chlorophyll a concentrations ranged from 1- 19 µg L^{-1} and were generally lower in the Harbor (<9 µg L^{-1}) and higher in the Bay (Fig. 7D). Across the region, pCO_2 levels were inversely correlated with salinity ($R=-0.85$, $p<0.001$) and chlorophyll a concentrations ($R= -0.83$, $p<0.001$) while chlorophyll a was positively correlated with salinity ($R=0.86$, $p<0.001$). Similarly, *Alexandrium* densities were highly correlated with pCO_2 levels ($R=1.00$, $p=0.08$).

Incubations of natural populations - Altering levels of pCO_2 caused significant alterations in the phytoplankton communities in experiments conducted during *Alexandrium* blooms in Northport Bay and Mattituck Creek (Fig. 8). Compared to ambient pCO_2 levels, elevated pCO_2 concentrations significantly enhanced *Alexandrium* densities (10 - 123% and 27 - 155%, for ~ 750 and ~ 1500µatm, respectively; $p<0.01$) during all experiments conducted except for 27 May when the increase at ~ 1500µatm was statistically significant but the increase at

~750µatm was not (Fig. 8). The effect of elevated pCO_2 levels on the cellular toxicity of *Alexandrium*, however, was less consistent (Table 5). While the total cellular toxicity increased 35% under the highest pCO_2 level (1500µatm) during the first Northport Bay experiment (13 May), overall elevated pCO_2 levels resulted in both increases and decreases in the toxicity of individual toxin derivatives in all experiments (Table 5). Patterns in toxin content were similar to those observed for cellular toxicity. Additionally, variations in the percent molar toxin composition due to changes in pCO_2 were negligible (data not shown). Higher pCO_2 levels resulted in both increases and decreases (in some cases significant; $p<0.05$) in different components of the phytoplankton community (diatoms, dinoflagellates, chlorophyll *a* size fractions; Table 6). The most significant and consistent observation was that *Alexandrium* densities increased with higher pCO_2 concentrations.

Discussion

This is the first study to assess the effects of acidification on the growth and toxicity of North American strains of the saxitoxin-producing dinoflagellate, *Alexandrium fundyense.* The growth of two *Alexandrium* strains from North America as well as field populations from two New York estuaries were significantly enhanced by elevated pCO_2. While toxin concentrations of both culture and field populations varied at higher pCO_2 levels, there were significant increases in total cellular toxicity as well as analog-specific toxin equivalents (GTX1, 4) for the Northport Bay isolate. In an ecosystem setting, the levels of pCO_2 measured during blooms were within the range found to enhance *Alexandrium* growth experimentally, suggesting *Alexandrium* growth rates may be stimulated by elevated pCO_2 levels in situ. These findings provide new perspective regarding the causes and impacts of HABs caused by *Alexandrium* and perhaps other harmful algae.

Growth of <u>Alexandrium fundyense</u> under varying levels of CO_2

Elevated pCO_2 (low pH) levels have been shown to increase the growth rates of multiple HABs. Using acid additions to manipulate pH, Hwang and Lu (2000) found that a culture of *Alexandrium minutum* grew maximally at a pH of 7.5. Using similar methodology, Flores-Moya et al. (2012) found that cultures of *Alexandrium minutum* grown at pH of 7.5 at 25°C had significantly higher growth rates compared to those at pH 8 at 20°C. Kremp et al. (2012) reported a significant enhancement in *Alexandrium ostenfeldii* growth rates at elevated pCO_2 (750ppm) in one of eight strains examined. Recently, Tatters et al. (2013a) reported significantly higher growth rates in *Alexandrium catenella* when exposed to 750ppm compared to 380ppm. Similarly, the growth rate of other HABs such as *Pseudo-nitzschia multiseries* and *Pseudo-nitzschia fraudulenta* (diatoms), *Karlodinium veneficum* (dinoflagellate) and *Heterosigma akashiwo* (raphidophyte) increased significantly with elevated pCO_2 (Fu et al., 2008; Fu et al., 2010; Sun et al., 2011; Tatters et al., 2012). In contrast, higher pCO_2 levels had no effect on the growth rate of cultures of the dinoflagellate *Prorocentrum minimum* (Fu et al., 2008), and *K. veneficum* and *P. mimimum* cultured at pH 7.0 to 8.5 (achieved via acid additions) exhibited invariant growth rates (Berge et al., 2010). Whether due to strain- or species- specific differences (Burkholder and Glibert, 2009; Pitcher, 2012), or potential differences in experimental methodology (acid addition v bubbling CO_2), the above research suggests that increasing pCO_2 differentially affects HABs. Regardless of methodology, of the species and strains tested thus far, species within the genus *Alexandrium* (*A. fundyense***,** *A. minutum, A. ostenfeldii and A. catenella*) have consistently displayed enhanced growth rates when exposed to elevated levels of pCO_2 (low pH; Hwang and Lu, 2000; Flores-Moya et al., 2012; Kremp et al., 2012; Tatters et al., 2013a, this study) with the exception of a strain of *A. tamarense* (Alex 2), for

which growth rates decreased by up to 25% (Van de Waal et al., 2014). While some of these prior studies were short-term experiments (weeks), Tatters et al. (2013b) recently reported that the effects of elevated CO_2 on coastal phytoplankton strains observed after two weeks persisted after one year of maintenance under the same condition, suggesting these short term changes may be indicative of expected longer term alterations.

Dinoflagellates evolved ~350 million years ago when atmospheric CO_2 concentrations were high (~3000ppm; Beardall and Raven, 2004) and possess a low CO_2 affinity form of RubisCO (form II; Morse et al., 1995; Rost et al., 2006; Reinfelder, 2011). Some species possess carbon concentrating mechanisms (CCMs) including the ability to transport bicarbonate (HCO_3^-), and/or either extra- or intracellular carbonic anhydrase, which converts HCO_3^- to CO_2 (Reinfelder, 2011; Fu et al., 2012). Among the few marine dinoflagellates that have been assessed thus far, there is a wide range of CCM capabilities. *Heterocapsa oceanica* and *Amphidinium carterae* are highly dependent on free CO_2 given their limited capacity for bicarbonate uptake (Dason et al., 2004), whereas *Prorocentrum minimum*, *Heterocapsa triquetra*, and *Ceratium lineatum* possess HCO_3^- transport coupled with internal carbonic anhydrase capabilities (Rost et al., 2006). This may partly account for the invariant growth of *P. minimum* under a range of pCO$_2$ levels (Fu et al., 2008; Berge et al., 2010). While there are no studies regarding CCMs in *Alexandrium*, the positive growth response of strains within this genus suggest that if they do possess CCMs, they are not effective enough to prevent slowed growth under current pCO$_2$ levels.

While it has been suggested that diatoms may not benefit from increasing CO_2 levels given that they possess highly efficient CCMs, and that algae such as coccolithophores and dinoflagellates with less efficient CCMs and/or low CO_2 affinities may benefit from living in a

high CO_2 world (Reinfelder, 2011), exceptions to this dogma abound (Fu et al., 2008; Berge et al., 2010; Sun et al., 2011). Consistent with this, although *Alexandrium* benefited from higher levels of pCO_2 during field experiments, responses of diatom and dinoflagellate populations varied, perhaps due to differential CO_2 requirements (use of free CO_2 vs HCO_3^-) of individual species present during each experiment (Fu et al., 2012). Given that these experiments were conducted at different time points over the duration of *Alexandrium* blooms, the community structure of each experiment differed along with the effects of CO_2 on competing phytoplankton. Its seems likely that assessing impacts of varying CO_2 on natural plankton communities will require species or even strain specific evaluations and should account for concurrent changes in grazing pressure as well (Rose et al., 2009).

Toxicity of <u>Alexandrium fundyense</u> under varying levels of CO_2

Some harmful algae synthesize more toxin when exposed to elevated levels of pCO_2, perhaps as a means to divert excess carbon and maintain internal elemental balance (Fu et al., 2012). Fu et al. (2010) found that increasing pCO_2 increased cellular toxin production in the dinoflagellate, *Karlodinium veneficum*, with higher pCO_2 levels increasing the production of the more potent karlotoxin form, KmTx-1, while decreasing production rates of KmTx-2. Domoic acid quotas in the diatom, *Pseudo-nitzschia multiseries*, were significantly higher at elevated pCO_2 (730ppm) compared to the lowest pCO_2 level (220ppm; Sun et al., 2011), while toxin quotas for *Pseudo-nitzschia fraudulenta* increased at higher pCO_2 but not significantly (Tatters et al., 2012). Flores-Moya et al.'s (2012) assessment of pH effects on the toxicity of *Alexandrium minutum* were inconclusive, and Kremp et al. (2012) found that while total toxins in *Alexandrium ostenfeldii* were relatively unaffected by elevated pCO_2, the STX fraction significantly increased. Tatters et al. (2013a), however, found that the total toxicity of

Alexandrium catenella more than doubled when grown at 750ppm CO_2 compared to 380ppm. In addition to these differences among species of *Alexandrium,* our observations demonstrate that the effects of pCO_2 on the toxicity of *Alexandrium fundyense* are strain-specific, as cellular toxicity was significantly and consistently enhanced (70- 80%) at higher pCO_2 levels in the Northport Bay strain while the Bay of Fundy strain displayed more variability. While the most abundant toxin in the Northport Bay strain was the epimers C1,C2, the cellular toxicity was driven mainly by the more toxic derivative, GTX1,4, which was the only derivative whose toxicity significantly increased (almost doubled) with increasing pCO_2 (Table 3). Interestingly, Tatters et al. (2013a) also demonstrated that concentrations of GTX1,4 doubled in high pCO_2 treatments, and therefore this finding specifically warrants further investigation. In contrast, Van de Waal et al. (2014) found that increased pCO_2 levels decreased cellular PST (paralytic shellfish poisoning toxin) content and cellular toxicity in two strains (Alex 2 and 5) of *Alexandrium tamarense* from the North Sea. Changes in cellular toxicity for Alex2 were driven by toxin content while changes in Alex5 were driven by changes in toxin composition (i.e. a shift towards less toxic derivatives; Van de Waal et al., 2014). Given that these studies demonstrated vast differences in toxicity patterns among different strains of *Alexandrium*, more research is clearly warranted.

While the precise mechanism controlling the changes in the toxicity of HABs under varying levels of pCO_2 has not been identified, there are several plausible explanations. Drawing from terrestrial systems and observed increases in secondary metabolites with higher pCO_2 in plants, Fu et al. (2012) suggested that algal toxin synthesis could increase via the shunting of excess fixed carbon toward toxin synthesis. Changes in toxicity may also be related to changes in the intracellular pH of phytoplankton (Suffrian et al., 2011) which can alter toxin biosynthesis

by changing enzyme activity (Yamada and Suzumura, 2010; Fu et al., 2012). While changes in intracellular pH may also cause transformations of saxitoxin congeners with low pH environments converting less potent N-sulfocarbamoyl toxins to the more potent carbamate toxins, as has been demonstrated with weak acid hydrolysis (Laycock et al., 1995), this phenomenon was not observed during this study. Furthermore, while pCO_2 significantly increased the total cellular toxicity of the Northport Bay strain as well as individual derivatives of both strains of *Alexandrium*, the differences in toxicity seen in the same derivative (STX, GTX5) between the two strains under nutrient replete conditions are more suggestive of a genetically controlled modification of toxicity rather than a chemical one (i.e. hydrolysis). In *Alexandrium*, where the gene pathway responsible for saxitoxin biosynthesis has been characterized, including several putatively identified genes involved in the modification of the saxitoxin parent compound (Kellmann et al., 2008; Stüken et al., 2011; Neilan et al., 2013), how acidification affects toxicity at the transcriptional or post-translational (chemical) level has rarely been evaluated (Van de Waal et al., 2014). The mechanisms controlling changes in cellular toxicity under elevated pCO_2 clearly warrants further study for all toxin producing HABs.

During this study, *Alexandrium* blooms were observed to occur in regions with levels of pCO_2 not predicted for the open ocean until the next century (e.g. >1,000 µatm; I.P.C.C., 2007). Concentrations of pCO_2 progressively increased during the course of an *Alexandrium* bloom and were higher in regions with the highest *Alexandrium* densities. Furthermore, distinct and consistent changes in the microbial and phytoplankton community were observed, with *Alexandrium* blooms being associated with lower chlorophyll *a* and increases in bacterial abundances. The consistently lower chlorophyll *a* levels associated with the bloom may have been a consequence of allelochemical production which has been reported for *Alexandrium* spp.

(Tillmann et al., 2009) including North American strains of *A. fundyense* (Hattenrath-Lehmann and Gobler, 2011). Allelochemicals have been shown to inhibit or lyse co-occurring phytoplankton (Tillmann et al., 2009; Hattenrath-Lehmann and Gobler, 2011) and thus may result in the release of organic matter from allelopathically affected phytoplankton, enhanced bacterial respiration, and ultimately, increased pCO_2 concentrations (Agusti and Duarte, 2013). In this regard, *Alexandrium* may indirectly influence pCO_2 levels in its surrounding environment. Other HABs with allelopathic properties (Prince et al., 2008; Tang and Gobler, 2010) or associated with elevated bacterial and/or organic matter levels (Gobler and Sanudo-Wilhelmy, 2003; Gasol et al., 2005) may also have the potential to co-occur with elevated pCO_2 concentrations. Many studies have demonstrated that variation in pCO_2 is tightly coupled to temporal variation in primary and bacterial production (Frankignoulle et al., 1998; Algesten et al., 2004; Borges et al., 2008). We suggest that *Alexandrium*, and HABs in general, may indirectly contribute to changes in estuarine pCO_2 by causing alterations in organic matter cycling and bacterial production.

Further evidence of the association of *Alexandrium* blooms with elevated levels of pCO_2 came from the spatial survey which detected elevated *Alexandrium* densities and pCO_2 levels in the southern region of Northport Bay along with lower chlorophyll *a* concentrations and salinities. This spatial distribution of *Alexandrium* is consistent with prior surveys of this region and has been linked to nitrogen loading from wastewater (Hattenrath et al., 2010). The lower salinities found in Northport Harbor are likely associated with intense groundwater discharge in this region (Young et al., 2013) which has the potential to be a significant source of pCO_2 (Basterretxea et al., 2010). The elevated *Alexandrium* densities and pCO_2 concentrations in the Harbor as well as the salinity gradient between the Bay and Harbor are indicative of a long

residence time in the Harbor region which may create positive feedback with regard to pCO_2 concentrations within the system. Low flushing rates would retain nutrients (from point and non-point sources) and phytoplankton which would initially stimulate primary production and subsequently lower pCO_2 concentrations. However, without a removal mechanism (i.e. flushing) coupled with the constant input of nutrients from wastewater, stagnant algal productivity would ultimately increase the organic load to sediments and increase bacterial respiration, all of which would enhance pCO_2 levels in the Harbor and overall make Northport Harbor a net heterotrophic system (Frankignoulle et al., 1998; Algesten et al., 2004; Borges et al., 2008). Our experimental results demonstrate that these higher pCO_2 concentrations can promote the growth and toxicity of *Alexandrium* in this system.

A vast body of research has documented the potential for ocean acidification to negatively impact an array of ocean organisms (Doney et al., 2009; Baumann et al., 2012; Gazeau et al., 2013). While HABs are also known for their negative effects on marine life, only one study has assessed the impacts of acidification and HABs, reporting that the alga *Aureococcus anophagefferens* acted synergistically with acidification to cause near complete mortality in bivalve larvae (Talmage and Gobler, 2012). Given the co-occurrence of HABs and acidification reported here, and the likely co-occurrence in other coastal systems, a comprehensive assessment of the effects of concurrent acidification and HABs such as *Alexandrium* on marine animals is needed to more fully understand their ecosystem impacts.

Anthropogenic nutrient loading and coastal acidification are processes associated with cultural eutrophication (Nixon, 1995; Borges and Gypens, 2010; Cai et al., 2011) and are factors that promote many HABs around the world (Anderson et al., 2008; Heisler et al., 2008; Hallegraeff, 2010). While HABs may directly or indirectly exacerbate eutrophication-enhanced

acidification, acidification can in turn increase the growth and toxicity of HABs. Given the large scale ecosystem effects that these interactions could have, this is certainly an area of study that warrants further investigation, especially in coastal regions where acidification occurs seasonally (Cai et al., 2011) and is intensified at estuarine salinities (Hu and Cai, 2013; Melzner et al., 2013) where HABs are often a recurrent problem.

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Figure 1. Northeast US and the Long Island embayments Northport Bay and Mattituck Inlet. Black circles indicate sampling sites.
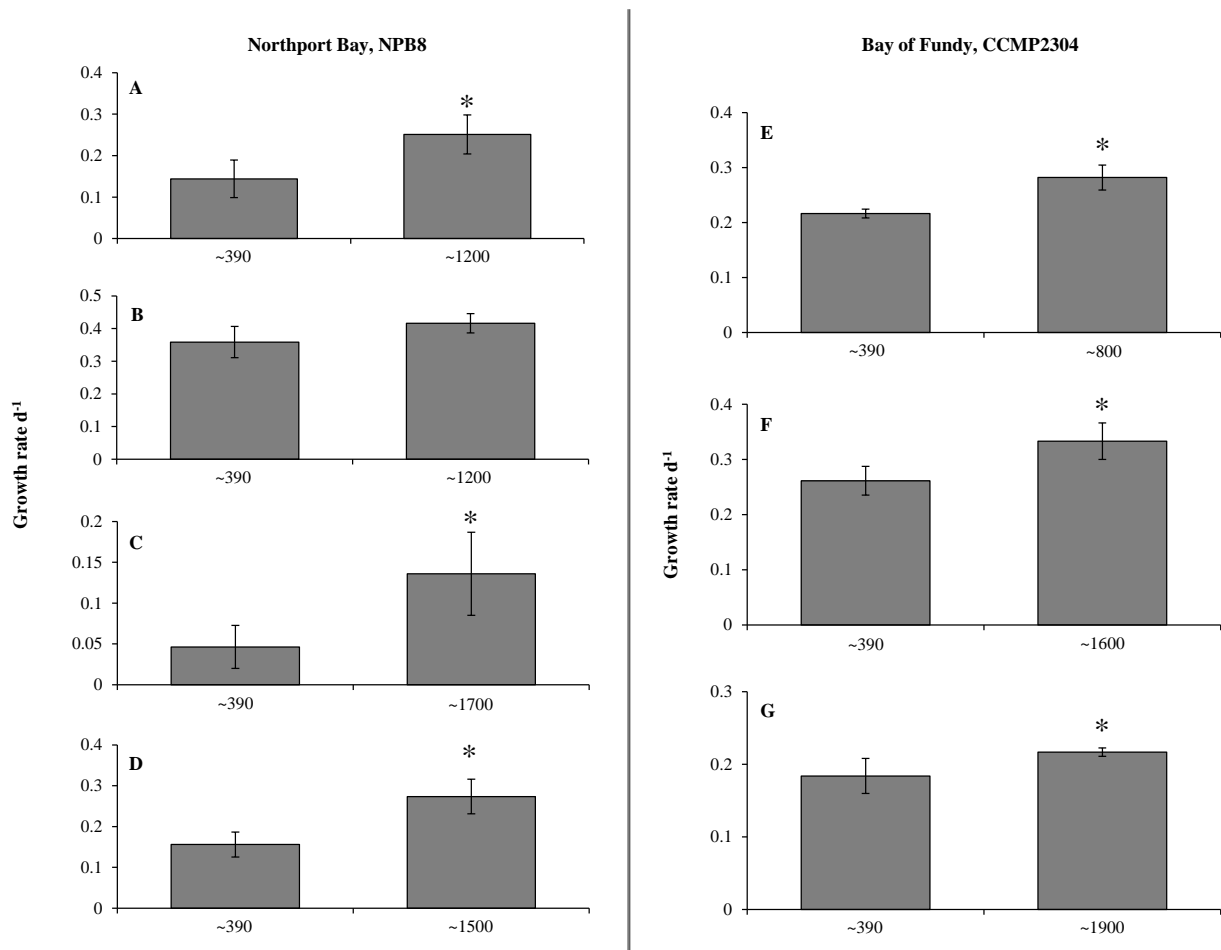
Figure 2. Growth rates (d^{-1}) of two *Alexandrium* strains (Northport Bay, NPB8 and Bay of Fundy, CCMP2304) under two levels of CO_2 (as in Table 1). Bars are means while error bars represent the SD of triplicate or quadruplicate measurements. Panels A-G represents experiments 1-7, respectively.
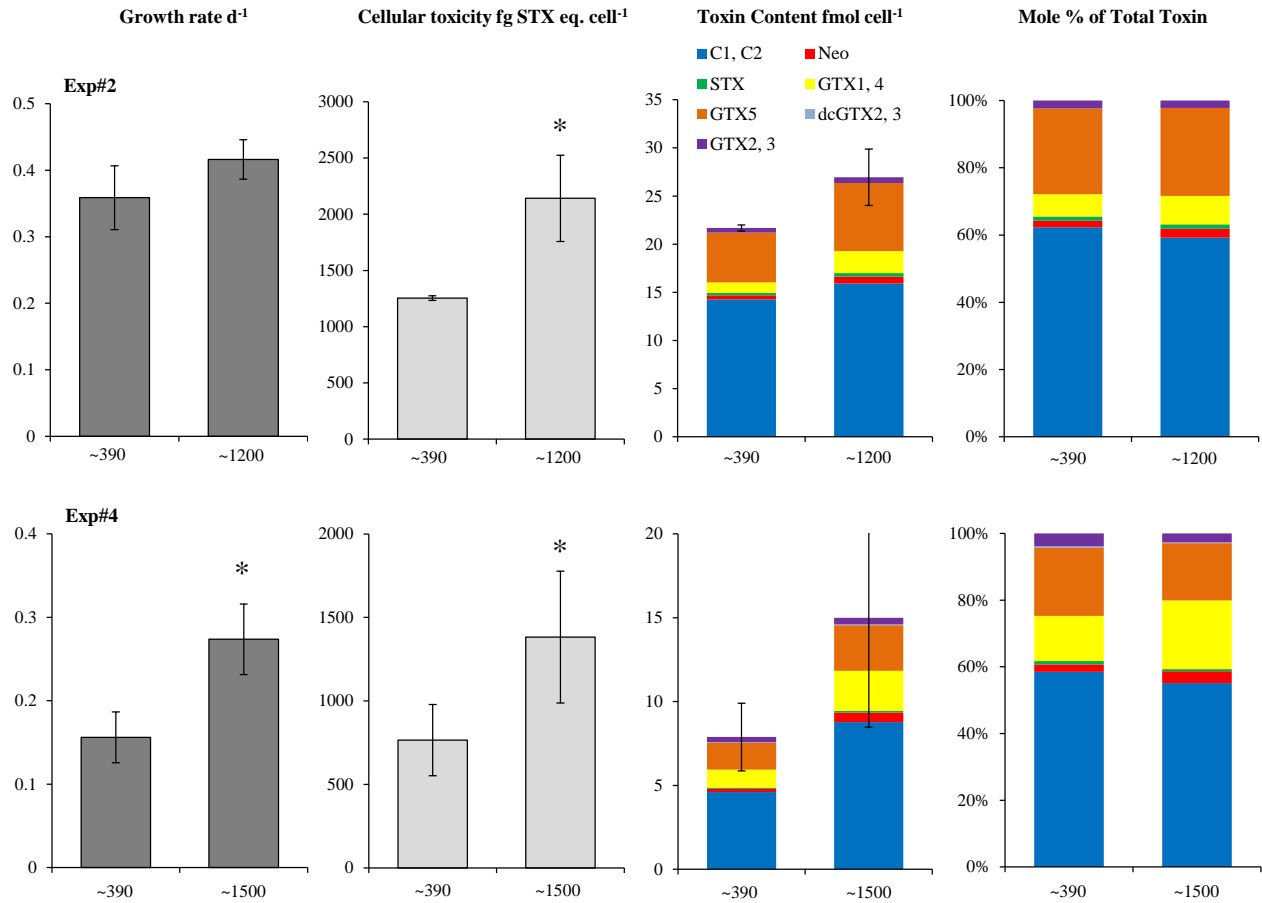
Figure 3. Growth rates (d^{-1}), cellular toxicity (fg STX eq. cell^{-1}), toxin content (fmol cell^{-1}) and percent molar toxin composition of the Northport Bay (NPB8) *Alexandrium* isolate under two levels of CO_2 (as in Table 1). Bars are means while error bars represent the SD of triplicate or quadruplicate measurements.
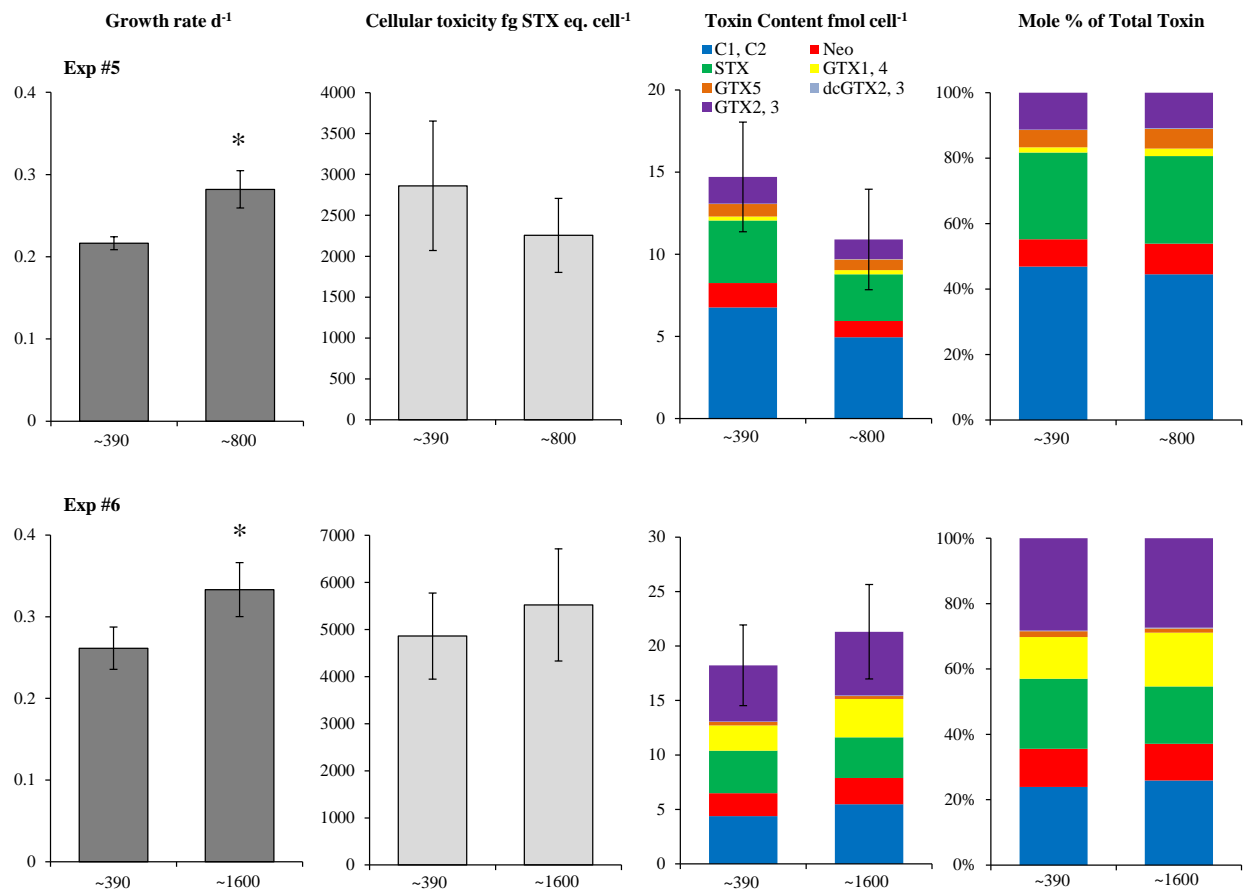
Figure 4. Growth rates (d^{-1}), cellular toxicity (fg STX eq. $cell^{-1}$), toxin content (fmol $cell^{-1}$) and percent molar toxin composition of the Bay of Fundy (CCMP2304) *Alexandrium* isolate under two levels of CO_2 (as in Table 1). Bars are means while error bars represent the SD of triplicate or quadruplicate measurements.
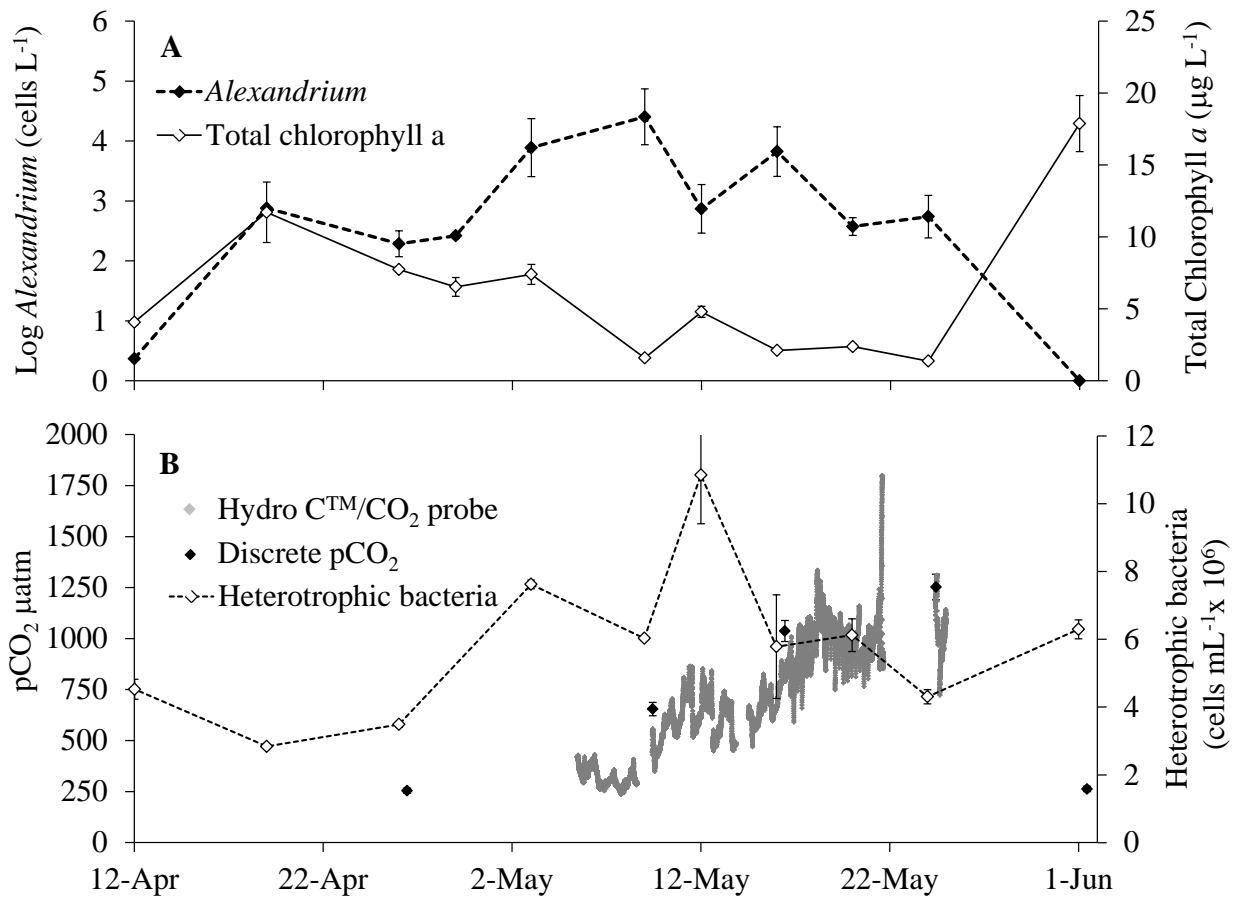
Figure 5. Northport Harbor, NY, USA, 2011: A) Log *Alexandrium* densities (cells L^{-1}) and total chlorophyll *a* (μg L^{-1}). B) pCO$_2$ (μatm) as measured by a HydroC™/CO$_2$ (Contros) probe and from discrete dissolved inorganic carbon (DIC) and pH measurements, and heterotrophic bacteria (cells mL^{-1} x 10^6).
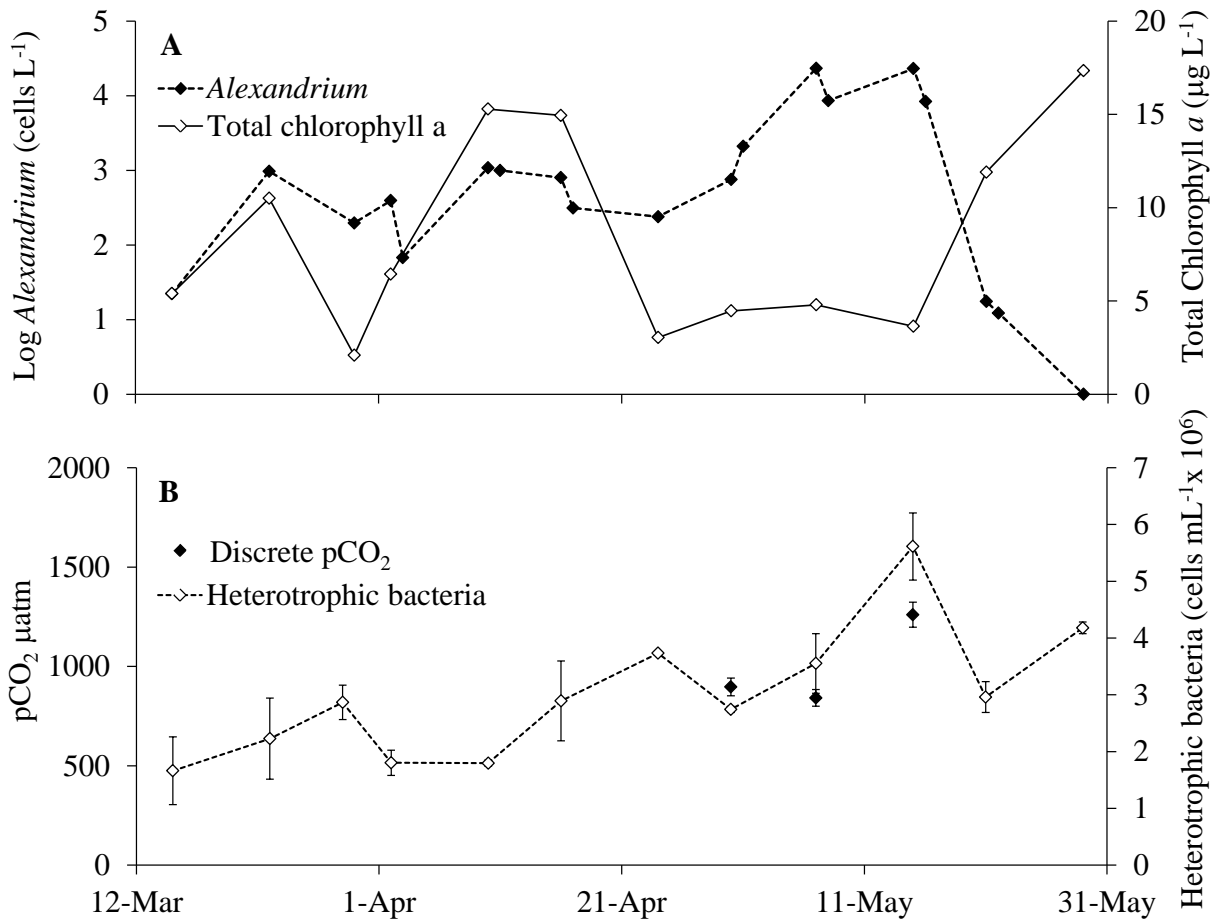
Figure 6. A) Log *Alexandrium* densities (cells L^{-1}) and total chlorophyll *a* (μg L^{-1}). B) pCO$_2$ (μatm) as determined from discrete dissolved inorganic carbon (DIC) and pH samples and heterotrophic bacteria (cells mL^{-1} x 10^6) for Northport Harbor, NY, USA during 2012.
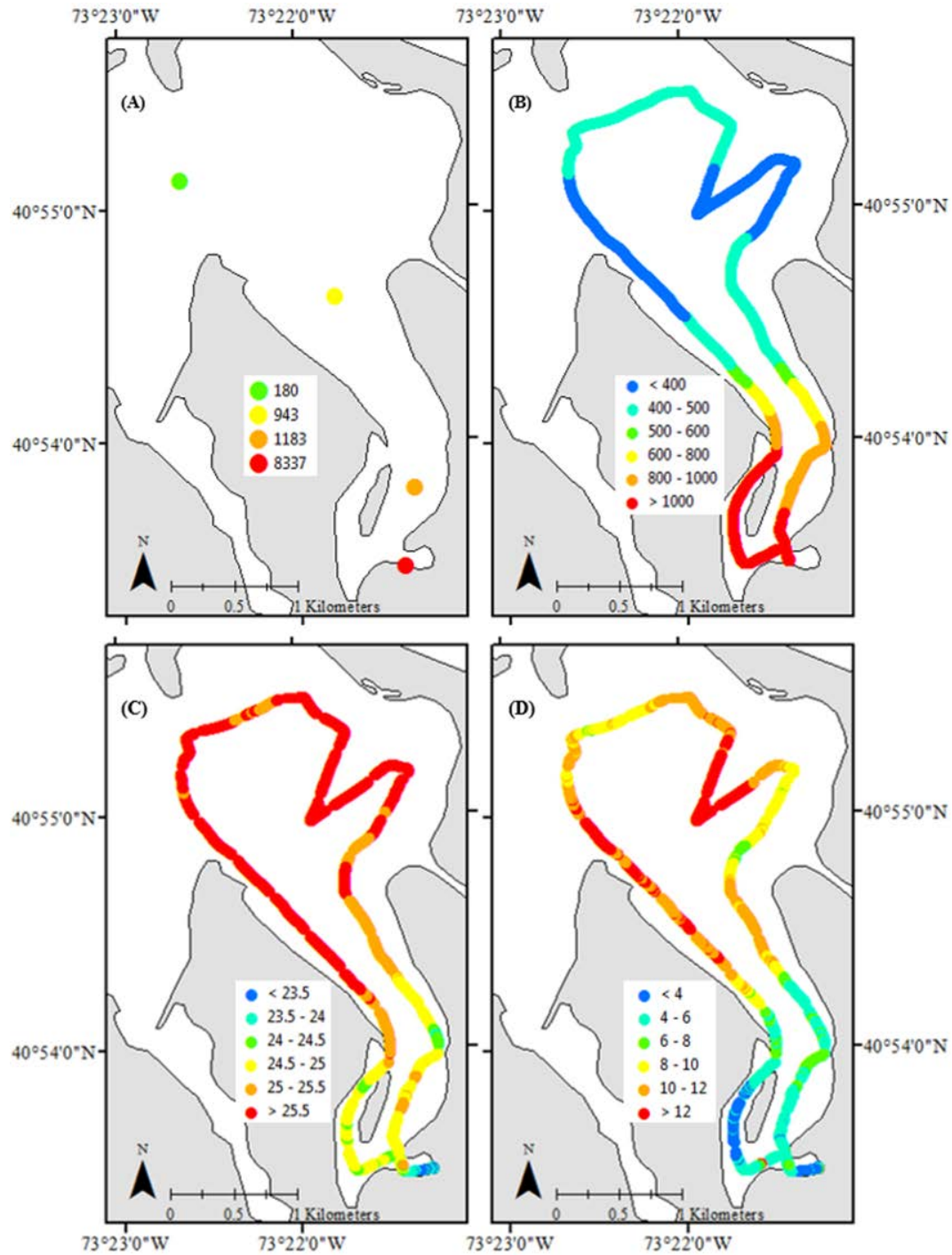
Figure 7. Maps of A) *Alexandrium* densities (cells L^{-1}), B) pCO$_2$ (μatm) as measured by a HydroC™/CO$_2$ (Contros) probe, and C) salinity and D) chlorophyll *a* (μg L^{-1}) as measured by a YSI 6920v2 probe, from a horizontal transect conducted in Northport Bay in May of 2012. Points in (A) represent individual samples/sites where cruise tracks in (B-D) represent multiple data points taken in close proximity via probes.

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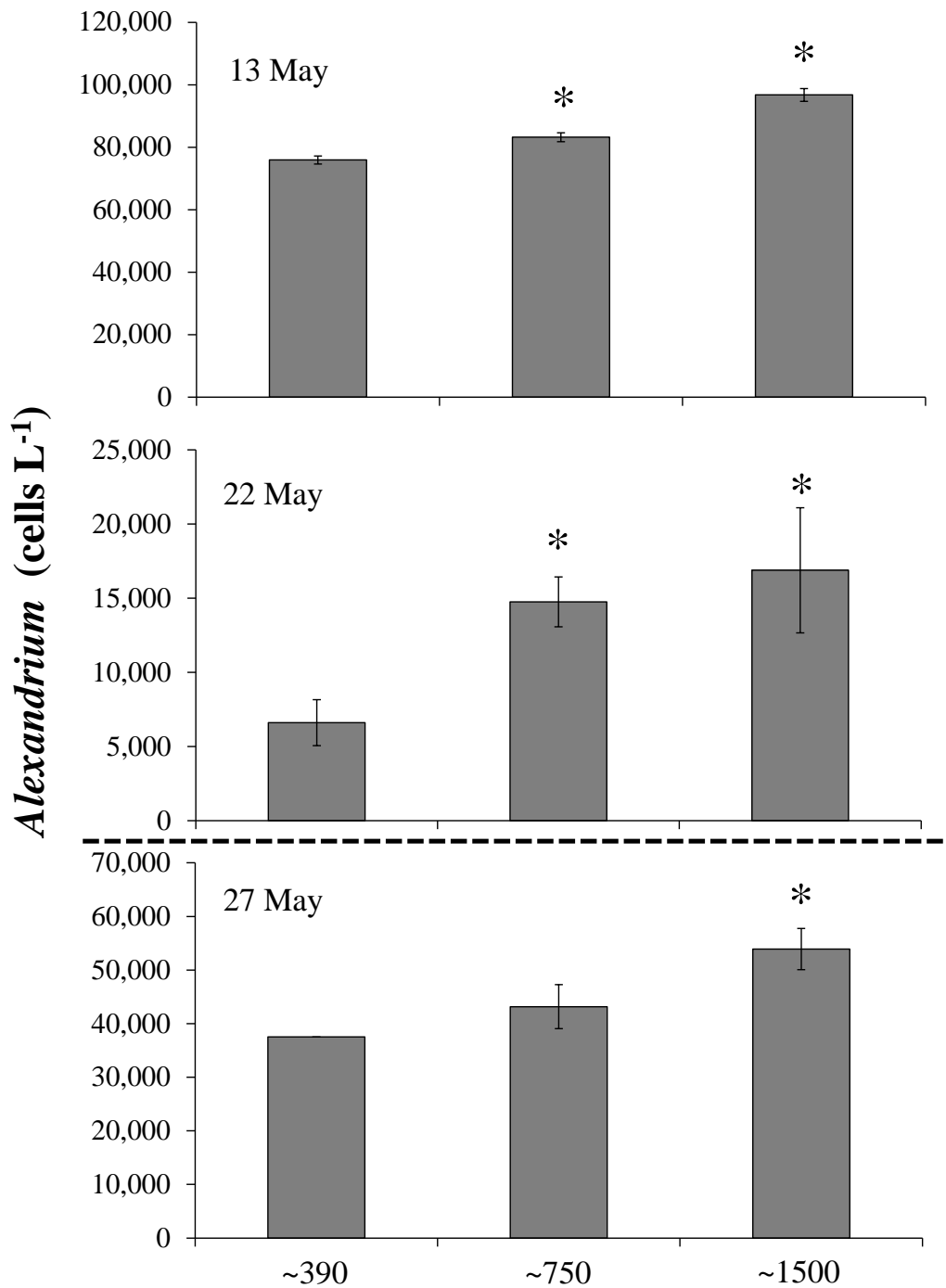
Figure 8. *Alexandrium* densities (cells L^{-1}) at the end of field incubations during which Northport Bay (13 and 22 May) and Mattituck Creek (27 May) water was subjected to varying levels of CO_2: ~390, ~750 and ~1500 μatm (Table 2). Bars are means while error bars represent the standard deviation of triplicate bottles. Dotted line represents the two different systems used for experiments.

Table 1. pH, dissolved inorganic carbon (DIC, $\mu mol\ L^{-1}$), calculated alkalinity (TA), calculated pCO_2 (μatm), and length of two- level CO_2 culture experiments (days). Values represent means and (SD).

Parameter	Northport Bay, NPB8		Bay of Fundy, CCMP2304	
	Ambient	High CO_2	Ambient	High CO_2
Experiment #1			*Experiment #5*	
pH	8.084 (0.002)	7.657 (0.002)	8.123 (0.005)	7.842 (0.004)
pCO_2 (μatm)	417 (8)	1191 (22)	436 (4)	895 (5)
Total DIC ($\mu mol\ L^{-1}$)	1643 (33)	1693 (22)	1893 (5)	1969 (9)
Alkalinity (TA)	1869 (35)	1773 (22)	2143 (8)	2105 (10)
Length of experiment (days)	15	15	24	24
Experiment #2			*Experiment #6*	
pH	8.062 (0.007)	7.744 (0.01)	8.054 (0.007)	7.582 (0.005)
pCO_2 (μatm)	440 (40)	1132 (41)	436 (5)	1617 (14)
Total DIC ($\mu mol\ L^{-1}$)	1639 (122)	1972 (34)	1597 (13)	1941 (5)
Alkalinity (TA)	1855 (127)	2082 (33)	1796 (16)	1994 (7)
Length of experiment (days)	27	27	12	12
Experiment #3			*Experiment #7*	
pH	8.026 (0.001)	7.516 (0.006)	8.009 (0.001)	7.495 (0.011)
pCO_2 (μatm)	441 (13)	1679 (3)	496 (5)	1922 (20)
Total DIC ($\mu mol\ L^{-1}$)	1509 (49)	1731 (25)	1629 (11)	1888 (26)
Alkalinity (TA)	1692 (53)	1768 (27)	1811 (11)	1917 (29)
Length of experiment (days)	15	15	12	12
Experiment #4				
pH	8.046 (0.001)	7.589 (0.006)		
pCO_2 (μatm)	483 (8)	1536		
Total DIC ($\mu mol\ L^{-1}$)	1734 (26)	1890		
Alkalinity (TA)	1937 (27)	1946		
Length of experiment (days)	12	12		

Table 2. pH, dissolved inorganic carbon (DIC, µmol L^{-1}), calculated alkalinity (TA), calculated pCO$_2$ (µatm) and length of incubation (days) during field experiments conducted in the spring of 2011. Values represent means and (SD).

Parameter	~390 µatm	~750 µatm	~1500 µatm
13-May			
pH	8.22 (0.01)	8.06 (0.20)	7.72 (0.04)
pCO$_2$ (µatm)	348 (10)	543 (226)	1199 (44)
Total DIC (µmol L^{-1})	1873 (46)	1846 (65)	1939 (126)
Alkalinity (TA)	2056 (49)	1972 (145)	1961 (137)
length of incubation (days)	4	4	4
22-May			
pH	8.52 (0.60)	7.88 (0.04)	7.64 (0.01)
pCO$_2$ (µatm)	235 (193)	916 (113)	1409 (19)
Total DIC (µmol L^{-1})	1496 (210)	2131 (81)	1888 (14)
Alkalinity (TA)	1851 (161)	2196 (71)	1888 (13)
length of incubation (days)	6	6	6
27-May			
pH	8.03 (0.01)	7.81 (0.02)	7.58 (0.01)
pCO$_2$ (µatm)	438 (27)	767 (81)	1439 (108)
Total DIC (µmol L^{-1})	1454 (74)	1547 (92)	1714 (108)
Alkalinity (TA)	1544 (75)	1588 (88)	1704 (106)
length of incubation (days)	4	4	4

Table 3. Cellular toxicity of saxitoxin derivatives (fg STX eq. cell^{-1}) from culture experiments conducted with the Northport Bay (NPB8) *Alexandrium* strain. Values represent the mean (SD) of triplicate or quadruplicate measurements. Asterisks indicate significant differences ($p<0.05$) between treatments and the control (~390µatm).

| | Saxitoxin derivatives fg STX eq cell $^{-1}$ | | | | | | | | |
	C1, C2	Neo	dcSTX	STX	GTX1, 4	GTX5	dcGTX2, 3	GTX2, 3	Total
Experiment #2									
390µatm	519 (90)	165 (136)	n.d.	112 (25)	343 (6)	141 (73)	n.d.	133 (13)	1253 (21)
1200µatm	626 (67)	286 (101)	n.d.	161 (83)	706 (90)*	197 (29)	n.d.	165 (34)	2141 (383)*
Experiment #4									
390µatm	188 (47)	76 (45)	n.d.	34 (10)	334 (84)	45 (11)	5 (1)	84 (20)	765 (213)
1500µatm	355 (205)	228 (139)	n.d.	47 (36)	750 (252)*	76 (44)	8 (6)	108 (56)	1383 (395)*

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Table 4. Cellular toxicity of saxitoxin derivatives (fg STX eq. cell^{-1}) from culture experiments conducted with the Bay of Fundy (CCMP2304) *Alexandrium* strain. Values represent the mean (SD) of triplicate or quadruplicate measurements. Asterisks indicate significant differences (p<0.05) between treatments and the control (~390μatm).

	C1, C2	Neo	dcSTX	STX	GTX1, 4	GTX5	dcGTX2, 3	GTX2, 3	Total
Experiment #5									
390μatm	275 (50)	590 (1)	n.d.	1646 (309)	74 (18)	21 (8)	4 (2)	447 (81)	2861 (791)
800μatm	202 (77)	397 (65)	n.d.	1226 (185)	80 (22)	18 (5)	5 (1)	328 (105)	2255 (453)
Experiment #6									
390μatm	178 (47)	842 (153)	n.d.	1682 (306)	729 (134)	9 (2)	6 (2)	1414 (291)	4860 (913)
1600μatm	221 (38)	964 (254)	n.d.	1608 (261)	1113 (297)	7 (1)	8 (2)	1599 (351)	5521 (1190)

Table 5. Toxicity of saxitoxin derivatives (fg STX eq. cell^{-1}) from field experiments conducted during the spring of 2011. Values represent the mean (SD). Asterisks indicate significant differences (p<0.05) between treatments and the control (~390µatm). n.d. = not detected.

	C1, C2	Neo	dcSTX	STX	GTX1, 4	GTX5	dcGTX2, 3	GTX2, 3	Total
13-May									
390µatm	1093 (280)	349 (372)	n.d.	845 (215)	107 (73)	587 (155)	n.d.	243 (71)	3072 (999)
750µatm	809 (162)	609 (446)	n.d.	504 (100)	372 (380)	606 (431)	n.d.	154 (5)	2362 (639)
1500µatm	1542 (532)	559 (106)	n.d.	1014 (126)	582 (119)	731 (84)	n.d.	329 (24)	4757 (852)
22-May									
390µatm	1944 (815)	243 (70)	n.d.	690 (372)	203 (101)	641 (123)	n.d.	562 (36)	4095 (1052)
750µatm	1456 (517)	104 (15)	n.d.	941 (533)	90 (24)	513 (209)	n.d.	712 (259)	3786 (1470)
1500µatm	1916 (544)	87 (28)	n.d.	749 (195)	178 (126)	328 (286)	n.d.	459 (401)	4110 (320)
27-May									
390µatm	1319 (323)	157 (64)	n.d.	256 (114)	606 (425)	287 (123)	n.d.	363 (24)	2815 (880)
750µatm	1635 (574)	30 (1)	n.d.	227 (55)	397 (285)	305 (62)	n.d.	433 (89)	3016 (921)
1500µatm	1251 (315)	23 (3)	n.d.	262 (42)	524 (355)	325 (40)	n.d.	448 (113)	2827 (784)

Saxitoxin derivatives fg STX eq cell^{-1}

Table 6. Diatom and non-*Alexandrium* sp. densities (cells mL^{-1}), and size fractionated chlorophyll *a* (µg L^{-1}) from field experiments conducted during the spring of 2011. Values are mean (SD). Asterisks indicate significant differences ($p<0.05$) between treatments and the control (~390µatm).

	Total Dinoflagellates (cells mL^{-1})	Total Diatoms (cells mL^{-1})	Total Chlorophyll *a* (µg L^{-1})	<20 µm Chlorophyll *a* (µg L^{-1})	>20 µm Chlorophyll *a* (µg L^{-1})
13-May					
390µatm	33 (7)	72390 (3649)	69 (31)	23 (4)	46 (27)
750µatm	20 (3)	43540 (7192)*	87 (25)	27 (4)	60 (25)
1500µatm	30 (9)	57477 (6791)*	113 (3)	31 (8)	82 (5)
22-May					
390µatm	29 (3)	161800 (2050)	67 (15)	46 (10)	21 (5)
750µatm	29 (6)	110030 (16989)*	58 (6)	47 (3)	11 (4)*
1500µatm	34 (3)	168833 (5618)	47 (4)	38 (6)	9 (2)*
27-May					
390µatm	171 (13)	2110 (786)	5 (1)	3 (1)	2 (0)
750µatm	154 (26)	2626 (669)	11 (10)	5 (5)	6 (5)
1500µatm	157 (18)	14067 (1916)*	14 (8)	6 (4)	7 (5)

Chapter 4

The emergence of *Dinophysis acuminata* blooms and DSP toxins in shellfish in New York waters

Hattenrath-Lehmann, T.K., Marcoval, M.A., Berry, D.L., Fire, S., Wang, Z., Morton, S.L., Gobler, C.J., 2013. The emergence of *Dinophysis acuminata* blooms and DSP toxins in shellfish in New York waters. Harmful Algae 26(0), 33-44.

Abstract

The dynamics of *Dinophysis acuminata* and its associated diarrhetic shellfish poisoning (DSP) toxins, okadaic acid (OA) and dinophysistoxin-1 (DTX1) as well as pectenotoxins (PTXs), were investigated within plankton and shellfish in Northport Bay, NY, USA, over a four year period (2008 - 2011). Over the course of the study, *Dinophysis* bloom densities ranged from ~ 10^4 to 10^6 cells L^{-1} and exceeded 10^6 L^{-1} in 2011 when levels of total OA, total DTX1, and PTX in the water column were 188, 86, and 2,900 pg mL^{-1}, respectively, with the majority of the DSP toxins present as esters. These cell densities exceed - by two orders of magnitude - those previously reported within thousands of samples collected from NY waters from 1971 to 1986. The bloom species was positively identified as *Dinophysis acuminata* via scanning electron microscopy and genetic sequencing (*cox1* gene). The *cox1* gene sequence from the *D. acuminata* populations in Northport Bay was 100% identical to *D. acuminata* from Narragansett Bay, RI, USA and formed a strongly supported phylogenetic cluster (posterior probability= 1) that included *D. acuminata* and *D. ovum* from systems along the North Atlantic Ocean. Shellfish collected from Northport Bay during the 2011 bloom had DSP toxin levels (1245 ng g^{-1} total OA congeners) far exceeding the USFDA action level (160 ng g^{-1} total OA of shellfish tissue) representing the first such occurrence on the East Coast of the U.S. *D. acuminata* blooms co-occurred with paralytic shellfish poisoning (PSP) causing blooms of *Alexandrium fundyense* during late spring each year of the study. *D. acuminata* cell abundances were significantly correlated with levels of total phytoplankton biomass and *Mesodinium spp.*, suggesting food web interactions may influence the dynamics of these blooms. Given that little is known regarding the combined effects of DSP and PSP toxins on human health and the concurrent accumulation and depuration of these toxins in shellfish, these blooms represent a novel managerial challenge.

Introduction

Harmful algal blooms are a growing human health and economic concern in many coastal regions. Paralytic shellfish poisoning (PSP) is a common, worldwide occurrence that is caused by dinoflagellates that produce the causative suite of toxins, saxitoxin (Anderson, 1994, 1997; Van Dolah, 2000; Lagos et al., 2003; Garcia et al., 2004; Glibert et al., 2005). In contrast, diarrhetic shellfish poisoning (DSP) is globally less commonly reported with recurring cases in Europe, South America (specifically Chile) and Southeast Asia (Hallegraeff, 1993; Van Dolah, 2000; Reguera et al., 2012). Dinoflagellates within the *Dinophysis* genus and to a lesser extent, *Prorocentrum lima* (Gayoso et al., 2002; Maranda et al., 2007), have been implicated in DSP events and closures around the world (Yasumoto et al., 1980; Hallegraeff, 1993; Van Dolah, 2000; Vale et al., 2008; Reguera et al., 2012). These dinoflagellates synthesize the causative toxins of DSP, namely okadaic acid (OA) and associated congeners, dinophysistoxins (DTXs), and/or pectenotoxins (PTXs) in the case of *Dinophysis* (Lee et al., 1989; Fux et al., 2011). While pectenotoxins do not cause DSP symptoms, they may be hepatotoxic to, and promote tumor formation in, mammals when injected intraperitoneally (Lee et al., 1989; Burgess and Shaw, 2001). Given the human health threats that these toxin producing blooms pose, as well as the observed global expansion of these events (Hallegraeff, 1993; Van Dolah, 2000) more investigations are needed in regions such as North America where previously DSP events have rarely been observed.

While PSP closures are a common occurrence in North America (Anderson, 1997; Todd, 1997; Anderson et al., 2005a, 2008; Jester et al., 2009; Hattenrath et al., 2010), there have been relatively few reports of DSP closures (Quilliam et al., 1991; Subba Rao et al., 1993; Todd, 1997; Tango et al., 2004; Deeds et al., 2010). On the east coast of North America, reports of

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DSP-producing blooms have primarily been attributed to the benthic dinoflagellate *Prorocentrum lima* (Marr et al. 1992; Morton et al., 1999; Lawrence et al., 2000; Maranda et al., 2007) with few reports of toxin-producing blooms of the *Dinophysis* genus (Cembella, 1989; Subba Rao et al., 1993), particularly in US waters (Maranda and Shimizu, 1987; Tango et al., 2004). The rarity of DSP closures due to *Dinophysis* blooms in North America is despite the recent isolation of toxin-producing *Dinophysis* clones from the northeast coast of North America (Hackett et al., 2009; Fux et al., 2011). While over a decade of monitoring and analyzing thousands of samples on Long Island (NY, USA) showed that twelve species of *Dinophysis* were present across multiple estuaries, shellfish containing DSP toxins were never observed in these regions (Freudenthal and Jijina, 1988).

Here we report on the dynamics of *Dinophysis acuminata* and its associated toxins, okadaic acid (OA), dinophysistoxin-1 (DTX1) and pectenotoxins (PTXs), both in the water column and in shellfish, during a four year period (2008 - 2011) in Northport Bay, New York. While investigating blooms of the PSP-producing dinoflagellate, *Alexandrium fundyense* (Hattenrath et al., 2010), the co-occurrence of *Dinophysis acuminata* was noted in Northport Bay (NY, USA). *Dinophysis acuminata* was positively identified via scanning electron microscopy (focusing on the contour of hypothecal plates, shape of sulcal list and areolation of plates) and genetic sequencing (*cox1* gene). Additionally, we report on the concurrent dynamics of *Alexandrium* and *Dinophysis*, PSP and DSP toxins, and the environmental conditions present during, and possibly facilitating the growth of, these blooms. Finally, we discuss the managerial, ecosystem, and human health implications of the co-occurrence of these toxic HABs in coastal ecosystems.

Materials and methods

Field sampling

Field samples were collected on a weekly to twice-weekly basis from March through September during 2008 through 2011. Samples were collected in Northport Harbor at 40.8916°N, 73.3572°W (site 2, Fig. 1 (circles); Hattenrath et al., 2010), which is a shallow (2 - 4m) well mixed system within the southeastern portion of the Northport-Huntington Bay complex, located on the north shore of Long Island, NY, USA. In 2011, cruises were conducted across six sites (4, 8, 9, 10, 16, and LIS; Fig.1) to assess the spatial extent of these blooms.

At each station, a YSI© probe was used to record surface temperature, salinity and dissolved oxygen. Total chlorophyll *a* was determined using GF/F (nominal pore size 0.7 µm) filters and measured using standard fluorometric techniques described in Parsons et al. (1984). Whole water samples were preserved in Lugol's iodine. Aliquots were settled in counting chambers and plankton were identified and enumerated using an inverted light microscope (Hasle, 1978). Cells larger than 10 µm were identified to at least genus level and grouped as ciliates, autotrophic nanoflagellates, dinoflagellates, and diatoms. *Dinophysis* cell densities were enumerated using a 1mL Sedgewick-Rafter slide under a compound microscope using whole water samples and concentrated water samples preserved in Lugol's iodine. Concentrated water samples were made by sieving 1 - 2L of water through either a 200 µm or 64 µm mesh (to eliminate large zooplankton) and then onto a 20 µm sieve that was backwashed into a 15mL centrifuge tube. Concentrates were made to increase the limit of detection as *Dinophysis* cell densities are often a relatively small portion of the total phytoplankton community and are therefore expressed as cells per L. Counts made on plankton concentrates were not significantly different from direct counts on whole water. Detection limits for whole water samples were

1,000 cells L^{-1} and 7 cells L^{-1} for concentrated samples. *Alexandrium fundyense* cell densities were enumerated using a highly sensitive molecular probe developed by Anderson et al. (2005b) and described at length in Hattenrath et al. (2010). Briefly, aliquots of phytoplankton concentrates (formalin and then methanol preserved) were hybridized with an oligonucleotide probe specific for the NA1 North American ribotype *Alexandrium fundyense/catenella/tamarense* with Cy3 dye conjugated to the 5' terminus (5'-/5Cy3/AGT GCA ACA CTC CCA CCA-3'). Cells were enumerated using a Nikon epifluorescence microscope with a Cy3™ filter set (Anderson et al., 2005b).

Dinophysis species identification

Morphological- Lugol's preserved samples from each bloom (2008 - 2011) were used for imaging and morphometric analysis (n=63 for all years). Between 10 and 20 images were taken from each bloom using a Nikon DigiSight Color Digital Camera System (DSVi1) mounted on an inverted light microscope (Nikon Eclipse TS100). Lengths and widths (not including the sulcal list; Reguera et al., 2012) of cells were determined using NIS-Elements Research Imaging Software and used to calculate length: width ratios. Additionally, *Dinophysis* cells were examined via scanning electron microscopy. Cells in Lugol's preserved samples were desalted using a 10% step gradient from seawater to freshwater on polycarbonate filter paper and dehydrated by using a step gradient of acetone (10%-100%), coated with 1.5 nm of gold using a Denton Desk IV sputter- edge coater (Moorestown, USA) and examined under the SEM JOEL 5600LV (Tokyo, Japan) as described by Morton et al. (2009).

Molecular analyses- Species identification was confirmed by sequencing the mitochondrial cytochrome c oxidase 1 (*cox1*) gene (Raho et al., 2008; Campbell et al., 2010; Papaefthimiou et al., 2010). A concentrated (5L) phytoplankton pellet (pelleted as in the next

section and preserved at -80°C) was collected from Northport Harbor during the peak of the 2011 bloom (27 June; 1.3×10^6 cells L^{-1}) when *Dinophysis* represented >90% of dinoflagellates in the water column. To extract nucleic acids, 1 mL of 2X CTAB buffer with fresh beta-mercaptonethanol was added to the cell pellet that was vortexed, heated to 50°C for 20 minutes, and frozen at -80°C until processing. Genomic DNA extraction was performed following Dempster et al. (1999). PCR amplification of the *cox1* gene was performed using the dinoflagellate specific primers Dinocox1F (5'-AAAAATTGTAATCATAAACGCTTAGG-3') and Dinocox1R (5'-TGTTGAGCCACCTATAGTAAACATTA-3') (Lin et al., 2002). PCR conditions were as in Raho et al. (2008), using an initial denaturation at 94°C for 10 minutes, 40 cycles at 94°C for 1 minute, 55°C for 1 minute and 72°C for 3 minutes, followed by a 10 minute extension step at 72°C. Reaction mixtures (25μl) were made with 12.5 μl of Go Taq Green (Promega), 1 μl of sample DNA (~500ng), 2.5 μl of a Dinocox1 forward and reverse primer mixture (4μM), and 9 μl of PCR water. The presence of a PCR product was confirmed using a 1% agarose gel and visualized using UV light.

The PCR product was cloned, as per the manufacturer's instructions, using a TOPO TA Cloning® kit (with pCR®2.1-TOPO® vector) with One Shot®TOP10F' Chemically Competent *E. coli* (Invitrogen). Ten resulting clones were sequenced at Stony Brook University's DNA Sequencing Facility using an ABI 3730 Genetic Analyzer with M13 Forward and M13 Reverse for the forward and reverse sequencing reactions, respectively.

Sequences were aligned, trimmed and assembled in Geneious Pro 5.5.2 (Drummond et al., 2011; http://www.geneious.com). Sequence similarity of the Northport, New York *Dinophysis sp.* to existing sequences deposited in NCBI was determined using BLAST. All deposited *cox1* sequences from the genus *Dinophysis* (n=35) were obtained from NCBI and used

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to construct a phylogenetic tree in Geneious Pro 5.5.2 using the dinoflagellate *Prorocentrum minimum* (accession #AF463415) as an outgroup. All sequences (n=37) were aligned and trimmed using ClustalW. A Bayesian inference tree was constructed using Mr. Bayes with the following parameters: general time reversible (GTR) substitution model, gamma rate variation, with 1.1 million generations at a subsampling frequency of 200 (Huelsenbeck and Ronquist, 2001).

Toxins in phytoplankton concentrates

Several liters of seawater were pre-sieved through a 200 μm mesh (to eliminate large zooplankton) and subsequently concentrated on a 20 µm sieve and backwashed into 15ml centrifuge tubes. Samples were centrifuged at 3000 rpm for 11 minutes and the supernatant aspirated without disturbing the cell pellet. Cell pellets were kept frozen at -20ºC until further analysis.

Analysis of DSP toxins- Algal pellets were resuspended in a known volume of either 100% or 80% aqueous methanol, homogenized by vortex mixing and probe-sonicated (Branson 1450 sonicator) on ice at 30% power, followed by centrifugation at 3400 x g for 10 min. The methanolic supernatants were filtered with a 0.2 μm syringe filter in preparation for analysis. Samples were analyzed for the presence of DSP toxins at NOAA's Marine Biotoxins Laboratory (Charleston, SC) using liquid chromatography (HP 1100 series HPLC; Agilent Technologies, Palo Alto, CA) coupled with tandem mass spectrometry (4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer; AB Sciex, Foster City, CA) using the method described by Gerssen et al. (2009) with modifications. LC separation was performed on X-Bridge^{TM} C18 (150×3 mm, 5 µm) column (Waters, Milford, MA) using a mobile phase of water (A) and acetonitrile/water (90:10, V/V) (B), both containing 6.7 mM ammonium hydroxide

under gradient elution at a flow rate of 0.4 mL min^{-1} (linear gradient from 1min of 10% B to 90% B at 12 min, hold for 3 min, then return to 10% B at 17 min and hold for 4 min). The detection of DSP toxins by MS was achieved by multiple reaction monitoring (MRM) in negative ion mode for OA, DTX1, and DTX2 (for OA and DTX2 with MRM transitions of *m/z* 803.5 →113.1and 255.1, for DTX1 with MRM transitions of *m/z* 817.5 → 113.1 and 255.1), and in positive ion mode for PTX11, PTX2, and their isomers (for PTX11 and its isomers with MRM transitions of *m/z* 892.5 →213.1 and 839.5, for PTX2 and its isomers with MRM transitions of *m/z* 876.5 → 213.1 and 823.5). Certified standards of OA, DTX1, DTX2, and PTX2 were available for toxin determination from NRC (Halifax, Canada) and RIKILT (Institute of Food Safety, The Netherlands). No standards were available for PTX11 and its isomers and PTX2 isomers; their concentrations were calculated approximately using PTX2 standards. PTX11 and its isomers showed identical product ion spectra but different LC retention time and their product ion spectra matched those published (Suzuki et al., 2003). PTX2 and its isomers also showed identical product ion spectra but different LC retention time. As such, all PTX concentrations were combined and reported as total PTXs (herein referred to as PTX). The detection limit was about 0.5 pg of OA, 0.65 pg of DTX1, 0.4 pg of DTX2, and 0.25 pg of PTX2 on LC column. The majority of toxin samples presented herein were not subjected to alkaline hydrolysis and therefore represent free toxins (i.e. esterified toxins are not included) and are therefore lower than the total OA (Deeds et al., 2010). However, to determine if esters were present in phytoplankton concentrates select samples (the peak of the *Dinophysis* blooms for 2008, 2010 and 2011) were hydrolyzed using the procedure described below.

Analysis of PSP toxins- Saxitoxin concentrations in plankton samples were determined via a competitive enzyme linked immunosorbent assay (ELISA). Cell pellets were acidified with 0.1 M HCl and subsequently analyzed for saxitoxin using an ELISA kit from Abraxis©, with toxin concentrations reported in STX equivalents. Cross-reactivities for the Abraxis© kit were as follows: 100% STX, 29% dcSTX, 23% GTX2,3, 23% GTX5B, 1.3% NEO, and <0.2% GTX1,4 (Hattenrath et al., 2010). We have found that concentrations of total saxitoxins in samples from our study sites analyzed with this assay are highly correlated with levels measured using via high performance liquid chromatography (HPLC) methods ($r^2 = 0.97$).

Analysis of DSP toxins in shellfish

During 2010 and 2011, netted bags containing the blue mussel, *Mytilus edulis***,** collected from regions without DSP toxins were deployed in the Northport-Huntington Bay complex (S1-S7; Fig. 1 (stars)). Mussel bags were collected sporadically from each site and mussels were shucked and frozen until analysis. Similarly, native soft shell clams (*Mya arenaria*) and ribbed mussels (*Geukensia demissa*) from Northport Harbor were harvested sporadically during the months of April through July (2011), shucked, and frozen until analysis. Samples of shellfish were homogenized and extracted in three volumes of 100% methanol, followed by centrifugation at 3000 x *g* for 5 min. The methanolic supernatants were filtered with a 0.2 μm syringe filter in preparation for analysis. Samples extract were analyzed as above by NOAA's Marine Biotoxins Laboratory. In addition to analyzing for free acids, samples were also subjected to alkaline hydrolysis for the determination of esterified toxins. A known volume of 2.5M sodium hydroxide solution was added to sample extract, placed in a water bath at 76ºC for 45 minutes, allowed to cool to room temperature, and then neutralized with a known volume of 2.5M hydrochloric acid solution (Mountfort et al., 2001).

Statistical analyses

A one way analysis of variance (ANOVA) was used to compare water quality parameters among years using SigmaStat within SigmaPlot 11.0; when data sets failed normality tests Kruskal–Wallis ANOVAs by ranks were performed. The extent to which all individual environmental parameters were correlated to each other was evaluated by means of a Spearman's rank order correlation matrix using SigmaStat within SigmaPlot 11.0.

Results

Species Identification

The *Dinophysis* sp. blooming in Northport Bay, NY, USA was identified as *Dinophysis acuminata* by morphological and molecular analyses. Cells were 47.29 ± 2.92 μm long by 29.28 ± 1.86 μm wide (length: width ratio = 1.62 ± 0.09) and somewhat oval-shaped, with a convex dorsal margin (Fig. 2). The left sulcal list had three noticeable ribs with the widest part of the cell occurring near the 3rd rib (Fig. 2a,b). The right sulcal list extended past the 2nd rib of the left sulcal list (Fig. 2a) and cells had a smooth thecal surface with small areolations (Fig. 2). Genomic DNA extracted from Northport Bay blooms yielded a 1,060bp consensus sequence of the *cox1* gene that was 100% identical to *D. acuminata* from Narragansett Bay, Rhode Island, USA (accession number EU130566) with 97% query coverage. A Bayesian phylogenetic tree constructed using *cox1* sequences from all *Dinophysis* spp. sequences on Genbank (as of September 2011) yielded strongly supported clusters (posterior probabilities= 0.75 - 1; Fig.3). Three clusters were monospecific for *D. rotundata*, *D. norvegica*, and *D. tripos*, respectively (Fig. 3). The *D. acuminata* from Northport Bay, NY, fell within a strongly supported cluster (posterior probability= 1) that included both *D. acuminata* and *D. ovum* originating from eastern

and western North Atlantic Ocean (Fig. 3). A final strongly supported cluster (posterior probability=1) contained both *D. acuminata* and *D. norvegica* (Fig. 3).

Dynamics of <u>Dinophysis</u>, <u>Alexandrium</u>, DSP, and PSP toxins

A *Dinophysis acuminata* bloom was observed every year from 2008 - 2011, usually starting in late April to May and lasting through the summer (Fig. 4). The largest *D. acuminata* bloom occurred in 2011, reaching ~1.3 million cells L^{-1}, followed by 2010, 2008, and 2009, with maximal densities of 116,000, 39,500, and 12,000 cells L^{-1}, respectively (Fig. 4). Transects across the Northport-Huntington Bay complex in 2011 showed that the highest *D. acuminata* densities were confined to Northport Harbor (site 2) with lower densities (ranging from 14 to 1,700 cells L^{-1}) occurring in other regions (Fig. 5). Blooms produced the DSP-causing toxins, okadaic acid and dinophysistoxin 1 (DTX1), as well as the pectenotoxins, PTX2, PTX11, and their isomers (Fig. 4). In general, PTX concentrations were usually the most abundant particulate toxin followed by esterified OA, esterified DTX1, free DTX1 and free OA (Fig 4 inset). Among the DSP toxins, esterified OA, esterified DTX1, free OA and free DTX1 represented 66%, 26%, 1% and 7%, respectively, of the total (Fig. 4 inset). DTX2 was not detectable within these blooms. Particulate toxin concentrations generally paralleled cell densities (R=0.796, p<0.001 for free OA; R=0.821, p<0.001 for free DTX1; and R=0.906, p<0.001 for PTX, for all of 2008, 2010 and 2011) with the exception of 2009 when PTX, DTX1, and OA were below detection limits (Fig. 4). Maximal particulate toxin levels during the study were as follows: total OA =188 pg mL^{-1}, total DTX1= 86 pg mL^{-1}, and PTX = 2,900 pg mL^{-1}, free OA = 4.2, free DTX1 = 20.4 pg mL^{-1}, esterified OA = 185 pg mL^{-1} and esterified DTX1 = 66 pg mL^{-1}. Mean cellular toxin quotas were 16 ± 12 fg $cell^{-1}$ for free OA, 62 ± 59 fg $cell^{-1}$ for

free DTX1 and 1,680 ± 1,314 fg cell^{-1} for PTX whereas total OA and total DTX1 quotas ranged from 148 – 759 fg cell^{-1} and 67 – 396 fg cell^{-1}, respectively, in select, hydrolyzed samples.

Dinophysis acuminata blooms co-occurred with another toxic dinoflagellate, *Alexandrium fundyense*, during late April to early June 2008 - 2011 (Fig. 6). The blooms were both concurrent (2008, 2009) and in succession (2010 and 2011; Fig. 6). Furthermore, the causative toxins of PSP and DSP produced by these HABs, saxitoxins and combined free okadaic acid congeners, respectively, were both observed in the plankton from May to early June of 2008 and 2011 (Fig. 7).

Co-occurring plankton communities and environmental conditions

During this four year study, *Dinophysis acuminata* densities were significantly correlated with multiple environmental parameters. *D. acuminata* abundances generally paralleled and were significantly correlated with water temperatures in Northport Harbor (R= 0.67, p<0.001) which ranged from 11.1 to 26.6ºC when *D. acuminata* cells were present in the water column (Fig. 6, Table 1). *D. acuminata* densities were also significantly correlated with chlorophyll *a* concentrations (R=0.42, p<0.001) and ciliate densities (Fig. 8; R=0.42, p<0.001), as well as with *Mesodinium spp.* densities during 2009 (Fig. 8; R=0.547, p<0.01). Salinity levels in Northport Bay ranged from 17.7 to 25.2 during *D. acuminata* blooms (mean = 23.1± 0.19) but did not co-vary with *D. acuminata* abundances (Table 1).

Shellfish toxicity

Both okadaic acid congeners (OA, DTX1) as well as pectenotoxins (PTX) were found in shellfish during the summer of 2010 and 2011 (Table 2, Fig. 9), while DTX2 was not detected. During 2010, toxic shellfish were collected on 28-June, one day prior to the peak of the 2010 bloom (Fig. 4), with site S4 having a higher toxin content (total OA congeners= 115 ng g^{-1}) than

site S3 (total OA congeners= 52 ng g^{-1}) which was closer to the documented bloom (Fig. 1, 9). During 2011, OA, DTX1 and PTX levels in shellfish ranged from 24 - 818 ng g^{-1}, 13 - 455 ng g^{-1}, and 3 - 115 ng g^{-1}, respectively (Table 2, Fig. 9), with the highest toxin concentrations (1245 ng g^{-1} total OA) found at site S3 (Woodbine Marina; Fig. 1) on 28-June, one day after the peak of the 2011 bloom and ~600m away from the site (2, Fig. 1) displaying the highest cell densities (Fig. 4). In 2011, five samples (four sites; S1, S2, S3 and S5) exceeded the USFDA action level (160 ng g^{-1} of shellfish tissue; black dotted line, Fig. 9). While four of these samples were collected from areas already closed to shellfishing due to coliform bacteria, one of these samples was collected from an area open to shellfish harvest (S5, Fig. 1).

In a manner similar to particulate toxins, total okadaic acid concentrations in shellfish (24 – 818 ng g^{-1}) were greater than total DTX1 concentrations (13 – 455 ng g^{-1}; Table 2, Fig. 4). Additionally, for all shellfish tissue analyzed, esterified toxins accounted for 74 – 98 % of total DSP toxins (Fig. 9). Native soft shell clams (*Mya arenaria*) and ribbed mussels (*Geukensia demissa*) collected from Scudder Beach contained DSP toxin concentrations exceeding the USFDA action level and had as much as 98% of their DSP toxins in esterified form (Fig. 9). The toxin profiles of blue mussels (*Mytilus edulis*) deployed for monitoring purposes were more variable with esterified toxins ranging from 74 – 90% of the total DSP toxins (Fig. 9).

Discussion

Comparison of NY *Dinophysis* blooms to other DSP events

This four year study documented the largest observed *Dinophysis* bloom (~1.3 x 10^6 cells L^{-1}, 2011) to occur in North America (exceeding the ~5 x 10^5 cells L^{-1} observed during a *D. norvegica* bloom in Bedford Basin, Canada; Subba Rao et al., 1993). Using both molecular and morphological analyses, the species blooming in Northport, NY, was identified as *D. acuminata*.

The species description, length, width, and length: width ratios of the Northport strain were well within the range of those reported globally for *D. acuminata* (Larsen and Moestrup, 1992; Zingone et al., 1998). Additionally, the Bayesian phylogenetic tree (Fig. 3) constructed using all available *Dinophysis cox*1 sequences was highly similar to the tree constructed by Papaefthimiou et al. (2010, in Fig. 3) with the Northport strain falling within Group I which also contained *D. ovum*, and is often referred to as the *D. acuminata* species complex (Papaefthimiou et al., 2010, Reguera et al., 2012).

Dinophysis acuminata bloom intensity varied interannually with the largest bloom ($>10^6$ cells L^{-1}; 2011) having toxin concentrations of 188, 86 and 2,900 pg mL^{-1} of total OA, total DTX1, and PTX, respectively. While *D. acuminata* densities during blooms in NY were extremely high, toxin concentrations were an order of magnitude lower than other *Dinophysis* blooms in North America (6.9 ng OA mL^{-1}; Campbell et al., 2010). Moreover, DSP toxins were present mostly as esters (92%) of OA and DTX1, whereas 83% of a Texas *Dinophysis* bloom sample contained free OA (Campbell et al., 2010; Deeds et al., 2010). Low bloom toxicity is due to the low toxin per cell quota found in *D. acuminata* from Northport Bay (396 ± 321 fg total OA $cell^{-1}$, 238 ± 164 fg total DTX1 $cell^{-1}$ (for select samples analyzed for total toxins), 1,680 ± 1,314 fg PTX $cell^{-1}$, 16 ± 12 fg free OA $cell^{-1}$, 62 ± 59 fg free DTX1$cell^{-1}$) which was within range of those reported in a culture study (OA= 18 fg $cell^{-1}$, DTX1= 203 fg $cell^{-1}$, PTX2= 20.4 pg $cell^{-1}$, and PTX11 isomer= 400 fg $cell^{-1}$) investigating a *D. acuminata* strain from the East Coast of the US (Hackett et al., 2009), but is lower than (in some cases by orders of magnitude) other field and lab studies of *Dinophysis* around the world (OA= 0 – 73 pg $cell^{-1}$, DTX1= 2.5 – 191.5 pg $cell^{-1}$, PTX2= 14.7 – 42.5 pg $cell^{-1}$; Cembella, 1989; Lee et al., 1989; Subba Rao et al., 1993; Raho et al., 2008; Kamiyama and Suzuki, 2009; Swanson et al., 2010). These differences

suggest that, on a per cell basis, *D. acuminata* populations from the northeast US are substantially less toxic than strains originating from other regions, with these intraspecific differences in toxicity being a common occurrence for this genus (Lee et al., 1989; Subba Rao et al., 1993; Reguera et al., 2012).

Although comparatively less toxic than other *Dinophysis* strains, blooms in Northport Bay did lead to high levels of DSP toxins in shellfish. This study documented the first occurrence of DSP toxins in shellfish exceeding the USFDA action levels on the East Coast of the US. Currently, USFDA action levels for DSP toxins are 16 µg total OA equivalents / 100 g shellfish tissue (= 160 ng g^{-1}), a value that includes both free and esterified okadaic acid and dinophysistoxins (US FDA, 2011). Unlike the European Union which includes total OA, DTX's and PTX's in their regulatory limits (EFSA, 2008), the USFDA currently makes no recommendations for the regulation of PTX (Deeds et al., 2010; US FDA, 2011) despite the potential human health implications (Burgess and Shaw, 2001). Additionally, given that some shellfish can rapidly transform PTX2 into PTX2 seco acid, a form that is not toxic to mice, the human health threat this toxin poses is unclear (Suzuki et al., 2001). In 2011, five shellfish samples collected from the Northport region contained DSP toxins (up to 1245 ng g^{-1} total OA congeners; Table 2, Fig. 9) exceeding the FDA action level, of which one of these samples (S5, Fig. 1) was collected from an area opened to shellfish harvest. The majority of DSP closures in North America have occurred in Canadian waters with maximal reported concentrations of 469 ng g^{-1} in scallops (Subba Rao et al., 1993), and 1,000 ng g^{-1} in mussels (Quilliam et al., 1991). Recently, closures have been implemented in Texas and Washington State with maximal reported concentrations of 470 ng g^{-1} in oysters (Deeds et al., 2010) and 960 ng g^{-1} in mussels (C. Hard, WA DOH, pers. comm.), respectively. In Portugal, however, concentrations of DSP

toxins in mussels approached 18,000 ng g^{-1} (Vale et al., 2008), an order of magnitude higher than Northport, NY. Prior to hydrolysis, only one shellfish sample from an area of Northport Harbor that was already closed to shellfish harvest (S3; 226 ng g^{-1}; Fig. 1, 9) exceeded the USFDA action level. After hydrolysis, however, total DSP toxin concentrations increased by 4 – 63 fold (depending on shellfish species), thereby increasing the number of samples over the USFDA action level (one to five) and expanding to an area (S5; Fig. 1, 9; Table 2) that was opened to harvest at the time of collection. This finding emphasizes the importance of analyzing for esterified toxins which in NY were 74 – 98 % of the total DSP toxins present. This was also the case in oyster samples from the Texas DSP event where only 2% of OA was in its free form while the rest was esterified and only detected after sample hydrolysis (Deeds et al., 2010).

This study documented species specific toxin profiles in shellfish collected from Northport Bay, NY. In all shellfish collected from Northport Bay, okadaic acid (OA) concentrations were higher than DTX1 concentrations, a finding similar to shellfish feeding on *Prorocentrum lima* which generally display OA as the dominant congener (Morton and Tindall, 1995; Morton et al., 1999; Maranda et al. 2007). Differences in the proportions of esterified vs. free forms of OA congeners between native and deployed shellfish species suggests that blue mussels may not be an ideal indicator species for DSP toxins as it did not accurately represent concentrations in native shellfish. Native shellfish contained almost exclusively DSP toxin esters (98 ± 1%) whereas deployed blue mussels possessed a significantly smaller fraction (81 ± 6%). Differential accumulation and depuration rates of DSP toxins in shellfish species have been observed previously (Vale, 2004; Vale, 2006; Reizopoulou et al., 2008). In addition to species-specific differences in toxins profiles, the toxin profiles of Northport Bay shellfish were regionally different from other shellfish exposed to *Dinophysis* blooms in North America. While

shellfish from Northport contained OA, DTX1, and their esters as well as PTXs, only OA and its esters were detected in oyster samples collected from the Texas *Dinophysis* bloom (Deeds et al., 2010) while only DTX1 and acyl-DTX1 were detected in shellfish from Washington State in 2011 (J. Deeds, USFDA, pers. comm.). This is not surprising given that toxin profiles for *Dinophysis* spp. are strain specific (Fux et al., 2011; references herein) and thus profiles in shellfish are likely to differ on a regional basis as well. Clearly, information on region- and species-specific toxin profiles in both cells and shellfish must be obtained to properly manage shellfish bed closures aimed to protect human health.

Factors promoting *Dinophysis* blooms in NY

Many *Dinophysis* spp. can be phagotrophic (Hallegraeff and Lucas, 1988; Hansen, 1991) and *D. acuminata* is a well-known obligate mixotroph (Park et al., 2006; Riisgaard and Hansen, 2009). *D. acuminata* densities in Northport were significantly correlated with chlorophyll *a* concentrations, indicating densities of algal prey and/or the photosynthetic ciliate, *Mesodinium sp.* (Crawford, 1989) paralleled bloom intensity. *D. acuminata* densities were also significantly correlated with ciliate abundances, a finding consistent with other field studies of *Dinophysis* (Sjöqvist and Lindholm, 2011) and the inability to maintain a *Dinophysis* culture without the addition of the ciliate, *Myrionecta rubra* (Park et al., 2006). Despite these correlations with potential planktonic prey, more research on the influence of plankton and other factors, such as nutrients, that may influence the growth and toxicity of *Dinophysis* is needed.

Several studies have demonstrated that *Dinophysis* blooms in estuarine ecosystems are related to hydrodynamic processes such as advection from offshore populations into embayments, and accumulation due to wind patterns and stratification (Subba Rao et al., 1993; Lindahl et al., 2007; Swanson et al., 2010; Sjöqvist and Lindholm, 2011). While physical

aggregation of cells may have occurred in Northport Bay, its relative importance is unclear. Northport Bay is a shallow well-mixed system that was never strongly stratified with respect to salinity or temperature (Anglès et al., 2012; Hattenrath-Lehmann and Gobler, pers. obs.) discouraging the occurrence of cell aggregation in thin layers associated with density gradients (Subba Rao et al., 1993; Lindahl et al., 2007; Sjöqvist and Lindholm, 2011). Further, *Dinophysis* concentrations were most dense within Northport Bay and were orders of magnitude lower outside (Fig. 5) indicating that physical advection of water masses from outside of Northport would dilute, not concentrate, *Dinophysi*s. If there were physical-biological interactions that were capable of specifically introducing *Dinophysis* cells into Northport Harbor, this enclosed, eutrophic area (Hattenrath et al., 2010) with low circulation may act as a 'bloom incubator' (Ryan et al., 2008), permitting retained cells to grow and accumulate and then stochastically seed outside regions. Given the highly eutrophic nature of this system (Hattenrath et al., 2010), further investigation regarding the role of nutrients in bloom occurrence here is warranted.

The expansion of *Dinophysis* blooms and the co-occurrence of HABs and algal toxins

The expansion and increased intensity of some HABs is a globally recognized phenomenon (Hallegraeff, 1993; Glibert et al., 2005). In Northport Bay, NY, monitoring conducted during the early 1980's found that peak *Alexandrium* densities were $\sim 10^2$ cells L^{-1} (Schrey et al., 1984). A long-term phytoplankton monitoring program conducted by the Nassau County (NY) Department of Health 25 years ago (thousands of samples analyzed, 1971 - 1986) found the highest *D. acuminata* densities (13,000 cells L^{-1}) occurred in the harbor adjacent to Northport Bay (Cold Spring Harbor; Freudenthal and Jijina, 1988). During these two investigations, PSP and DSP were not observed in shellfish (Schrey et al., 1984; Freudenthal and Jijina, 1988). Now, ~ 30 years later, these two HABs form annual blooms in Northport Bay,

with peak densities exceeding 1×10^6 cells L^{-1} and their respective toxins have been detected in shellfish at levels known to be toxic to humans (Hattenrath et al., 2010; present study). Beyond Northport, *Dinophysis* blooms in other NY locales such as Meetinghouse Creek, within the Peconic Estuary, have reached abundances exceeding 2×10^6 cells L^{-1} and have persisted for ~ 2 months (Hattenrath-Lehmann and Gobler, pers. obs.). The intensification of *Dinophysis* blooms in NY has occurred in parallel with new DSP outbreaks in other parts of the US. In 2008, oyster beds in Texas were closed to harvesting due to the presence of OA (Deeds et al., 2010) whereas during the summer of 2011, Sequim Bay in Washington State experienced its first DSP poisoning event (V. Trainer, NOAA, pers. comm.).

The emergence of DSP in an ecosystem that also experiences PSP is a serious and novel managerial challenge in the US. In 2008 and 2009, *Dinophysis* and *Alexandrium* blooms co-occurred and in 2010 and 2011, they occurred in immediate succession. In Northport Bay, shellfish beds that are already closed for approximately one month annually due to PSP may close for even longer periods in the future due to DSP events immediately following the PSP closures, potentially increasing the economic and recreational impact on the area. Few reports on the co-occurrence of marine HABs and their toxins exist. Recently, in California, domoic acid (ASP, *Pseudo-nitzshia*) and saxitoxin (PSP, *A. catenella*) were found co-occurring in both planktivorous fish and shellfish (Jester et al., 2009). In a manner similar to our study, the co-occurrence of both PSP and DSP- toxins in shellfish have been reported in South America (Garcia et al., 2004) and in Europe (Gago-Martinez et al., 1996). The proper management of these dual toxin events requires information that is currently unknown, including an understanding of the additive effects of multiple HAB toxins on shellfish toxin accumulation and depuration, as well as their cumulative short and long term effects on human health. Given that

HABs may continue to intensify in the future (Hallegraeff, 2010), the co-occurrence of HABs and their toxins may become more common and thus warrants further investigation, particularly given the unknown additive effects of multiple marine toxins on human health.

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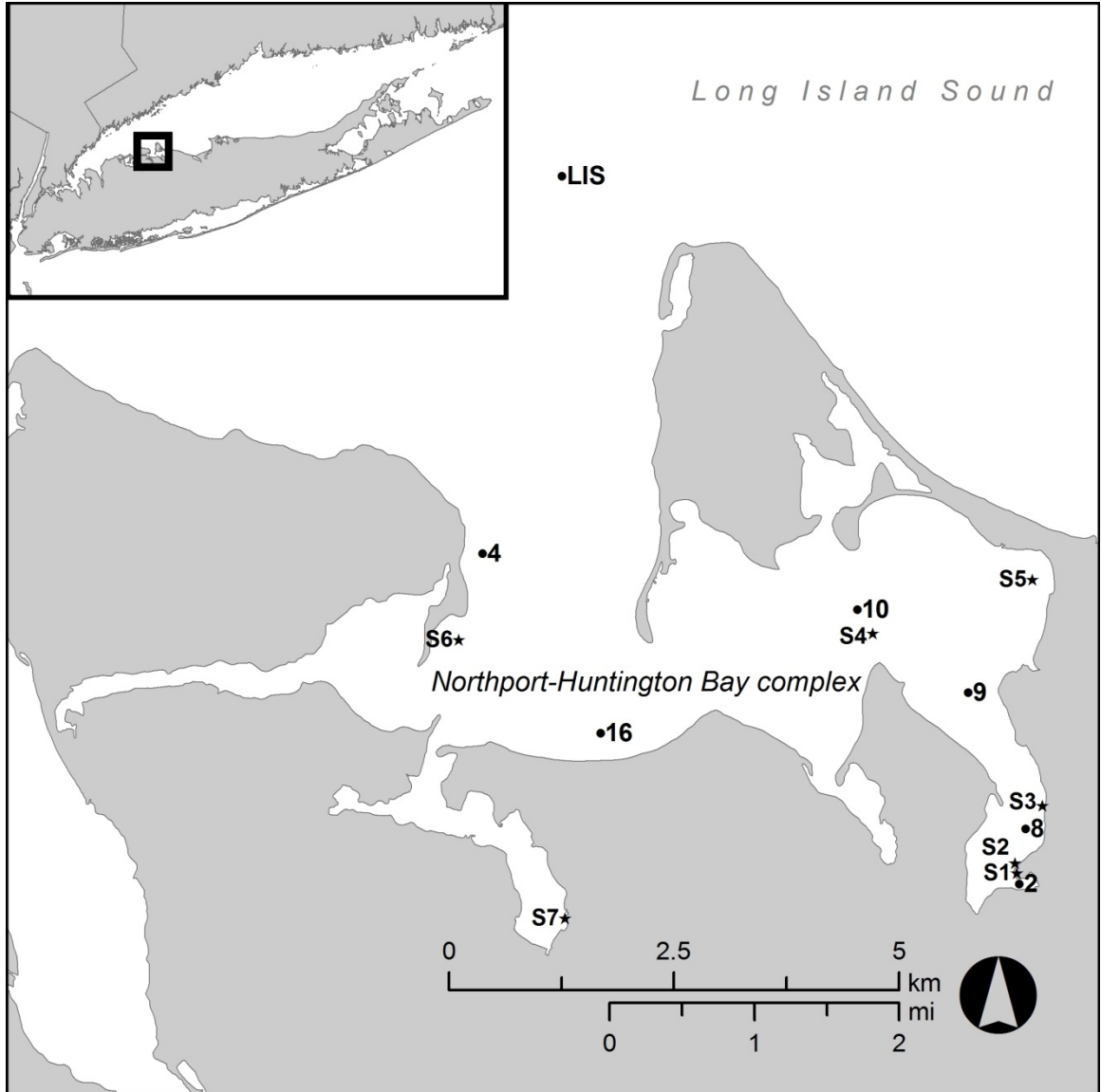
Figure 1. Field sampling (black circles) and shellfish collection (black stars) locations in Northport-Huntington Bay complex, New York, USA.
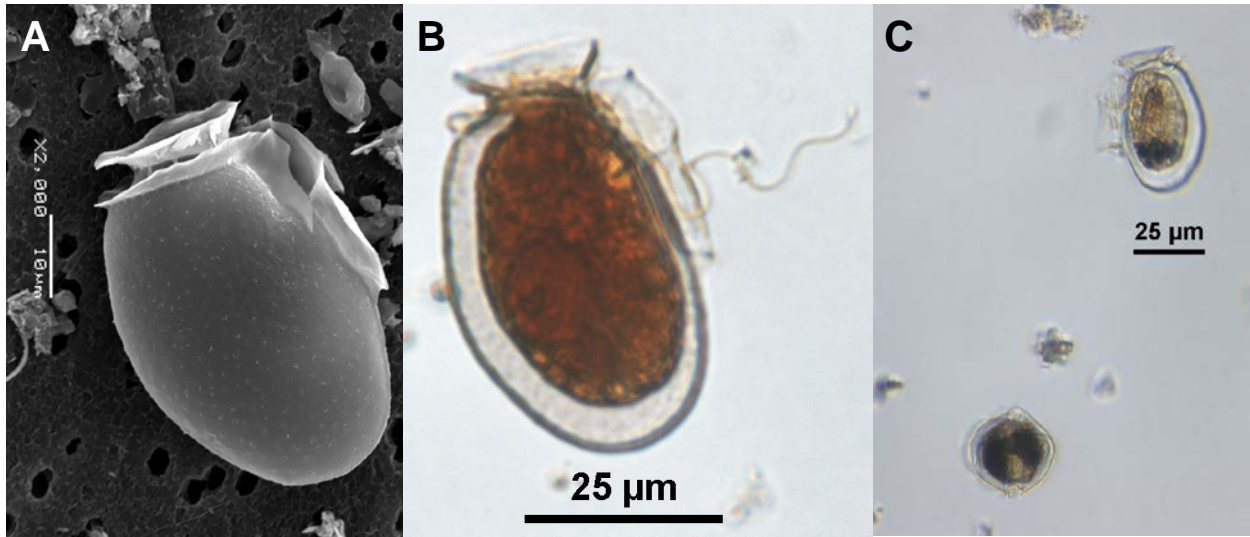
Figure 2. Images of *Dinophysis acuminata* cells from Northport Bay. (A) Scanning electron micrograph, (B) Light micrograph of a Lugol's iodine preserved cell, (C) light micrograph of the co-occurrence of *Dinophysis acuminata* (top right) and *Alexandrium fundyense* (bottom left).
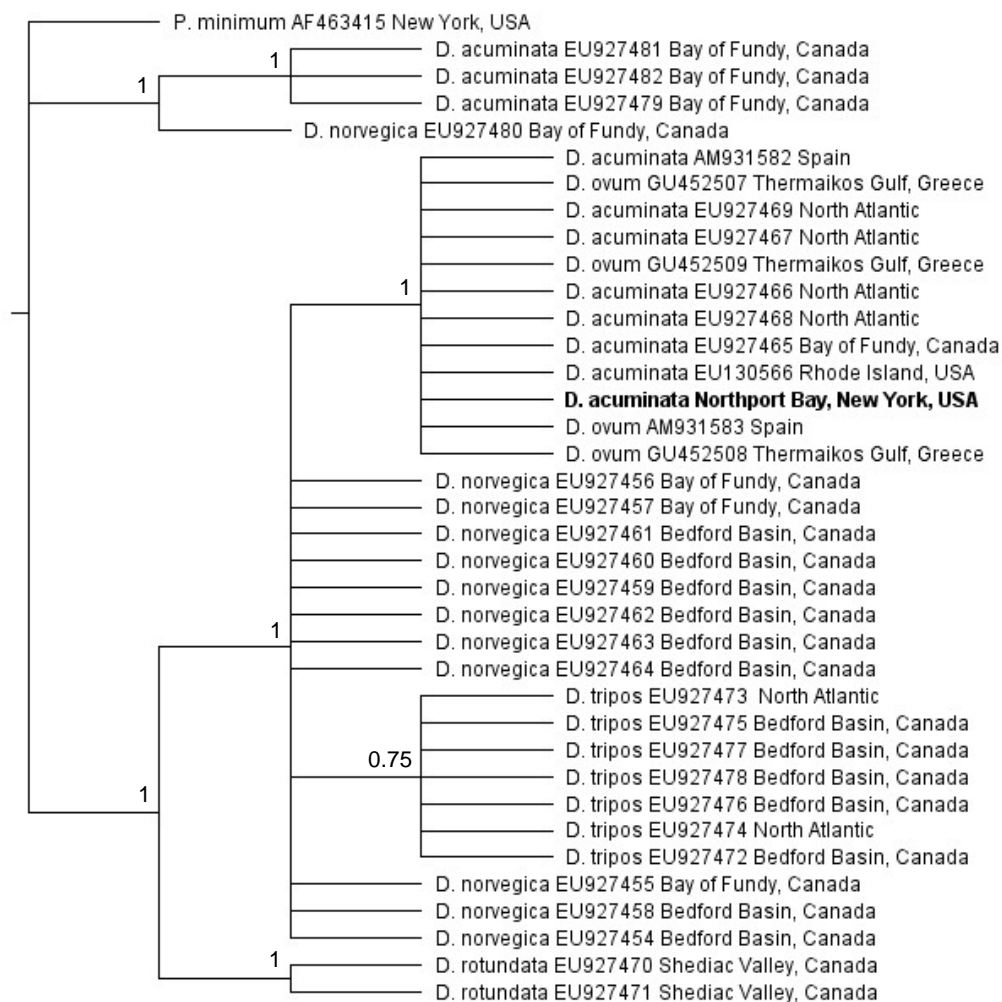
Figure 3. Phylogenetic tree based on *cox1* gene sequences from all *Dinophysis* spp. (n=35) deposited in NCBI as of September 2011 and the bloom species, *D. acuminata*, found in Northport Bay, NY, USA (in bold). The tree was constructed in Geneious Pro 5.5.2 (Drummond et al., 2011) using Mr. Bayes and rooted to the dinoflagellate, *Prorocentrum minimum* (accession #AF463415). Posterior probabilities are indicated next to the nodes.
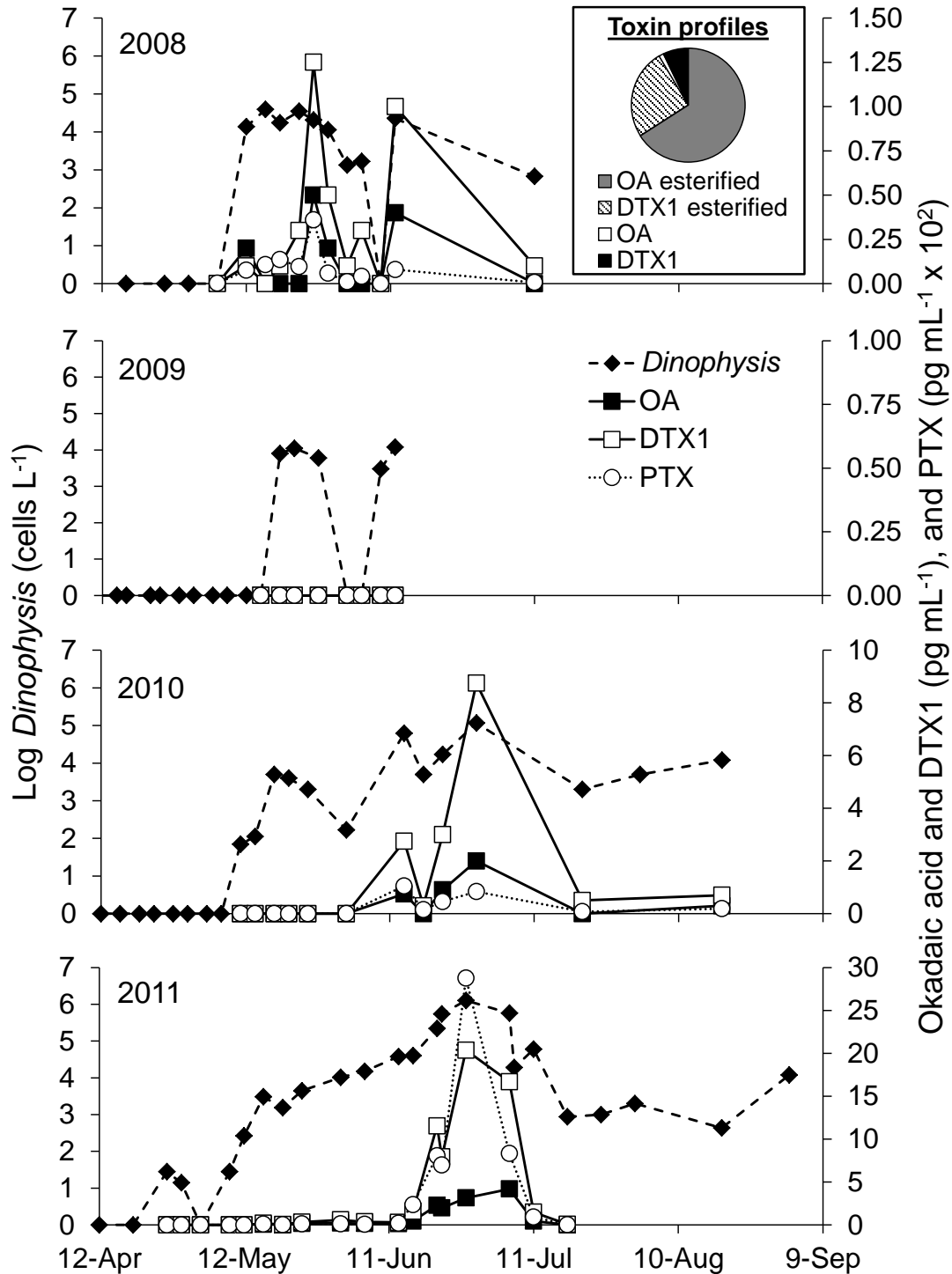
Figure 4. Log *Dinophysis acuminata* densities (cells L^{-1}), and the DSP toxins, okadaic acid (OA) and dinophysistoxin 1 (DTX1; pg mL^{-1}), and total pectenotoxins, PTX (pg mL^{-1} x 10^2) in phytoplankton concentrates from Northport Bay, NY, USA during 2008 - 2011. Inset: Toxin profile of hydrolyzed phytoplankton concentrates expressed as the mean of each toxins contribution to the total toxin profile.
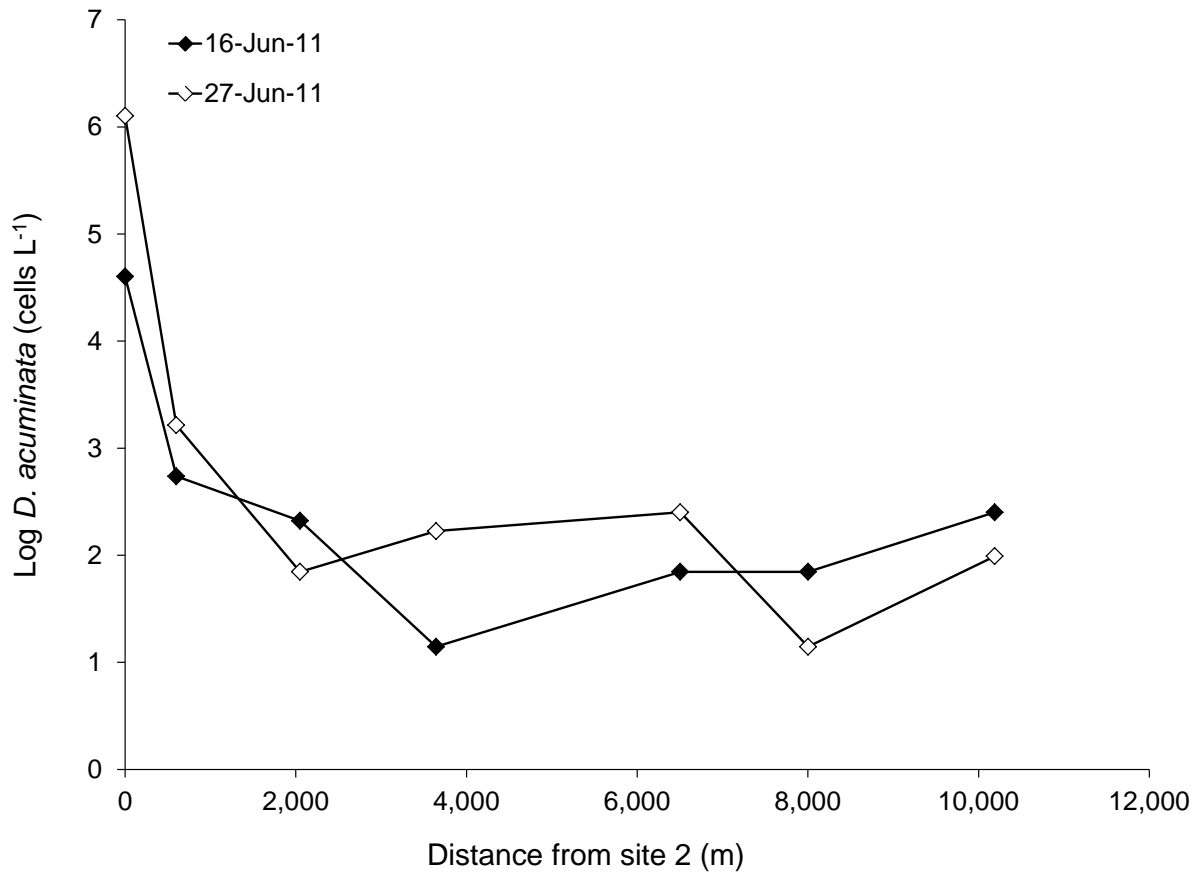
Figure 5. Log *Dinophysis acuminata* densities (cells L^{-1}) across Northport Bay, NY, USA, during 16 June and 27 June 2011 as a function of distance (m) from site 2.
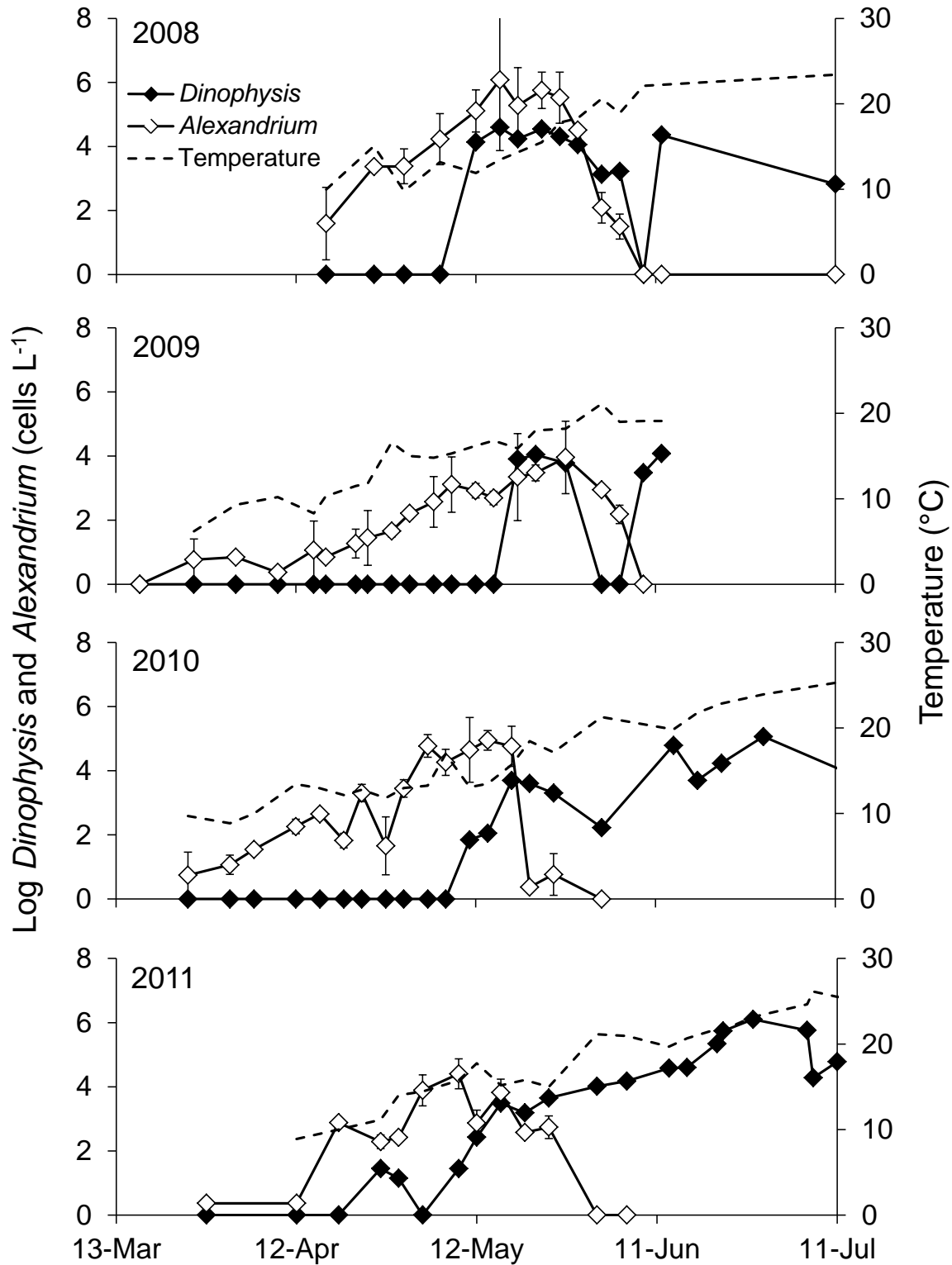
Figure 6. Log *Dinophysis acuminata* and *Alexandrium fundyense* densities (cells L^{-1}), and temperature (ºC) in Northport Bay, New York, USA, during 2008 - 2011 blooms.
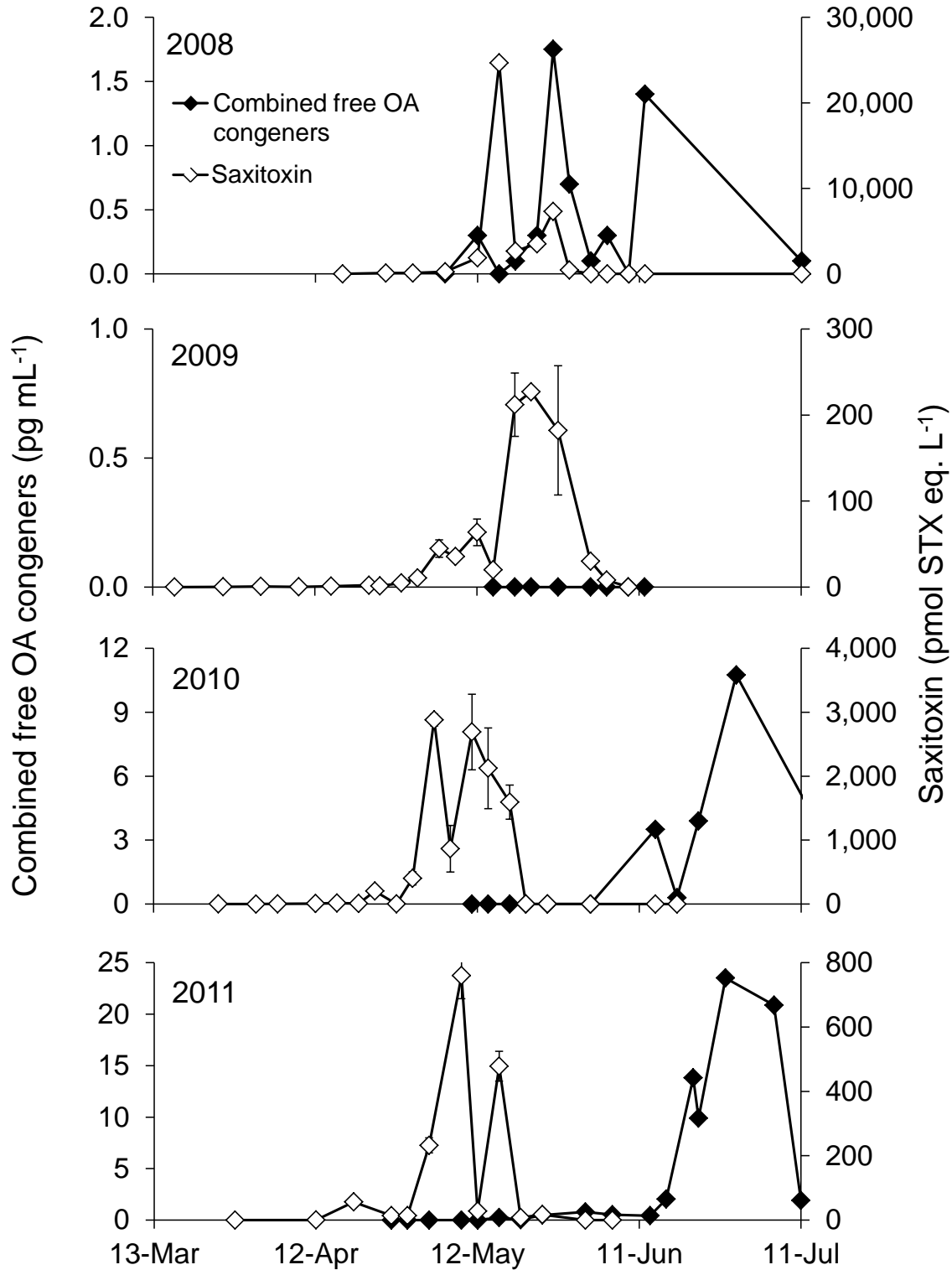
Figure 7. Combined free okadaic acid congeners (pg mL^{-1}) and saxitoxin concentrations (pmol STX eq. L^{-1}) in phytoplankton concentrates collected in Northport Bay, NY, USA during 2008 - 2011.
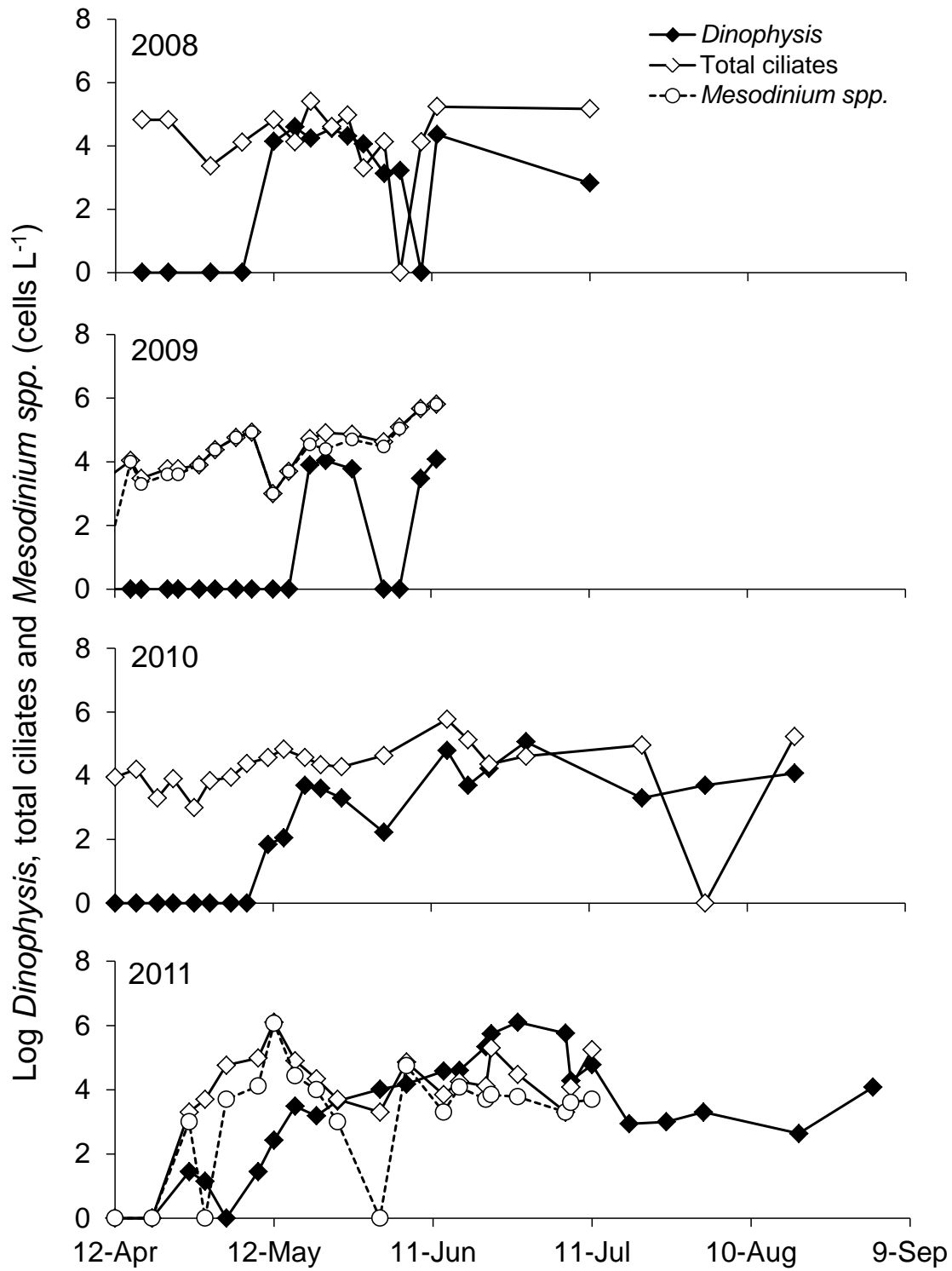
Figure 8. Dynamics of *Dinophysis acuminata*, total ciliates and *Mesodinium spp.* (cells L^{-1}) in Northport Bay, NY, USA during 2008 - 2011.
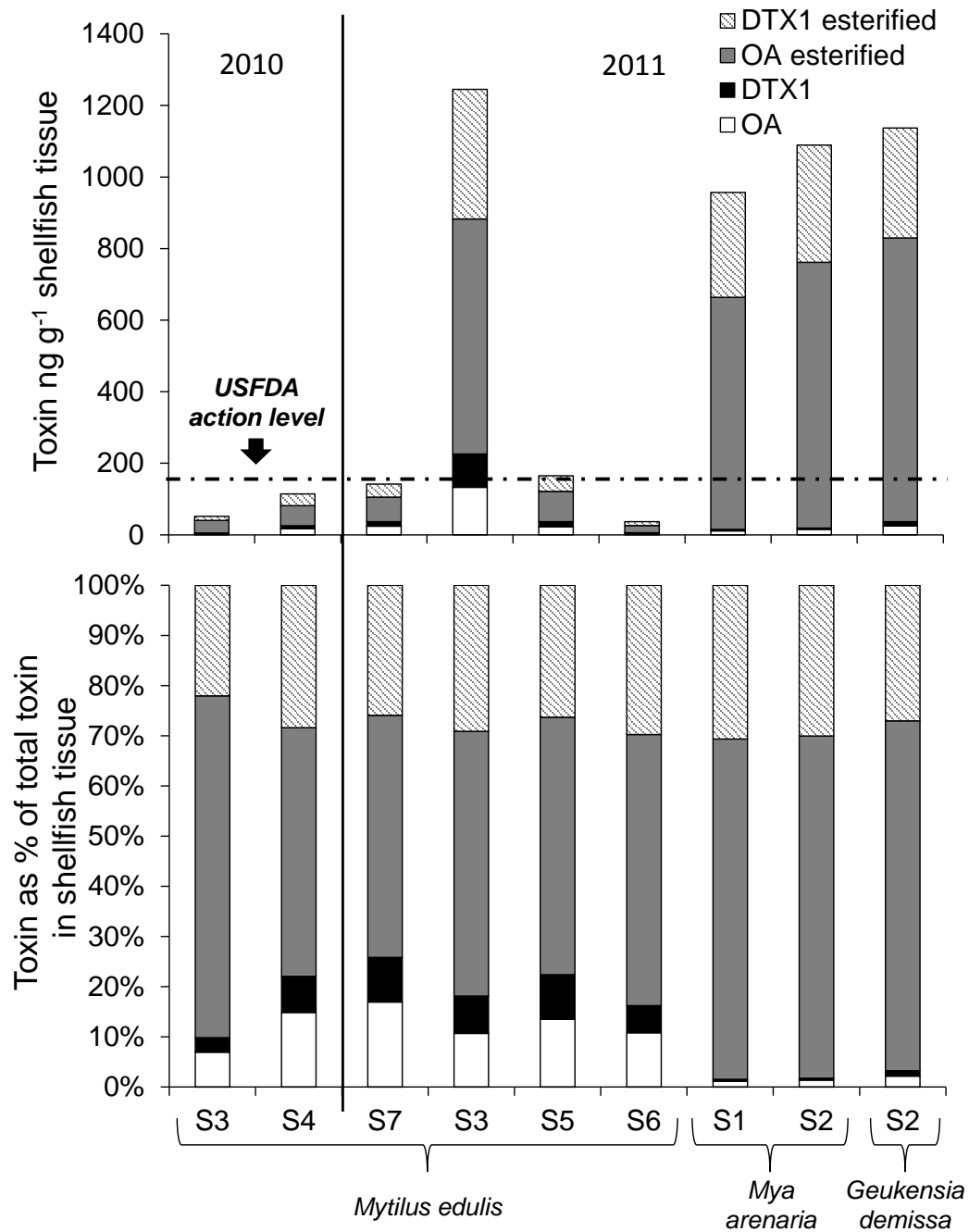
Figure 9. Top panel: Okadaic acid (OA), dinophysistoxin 1 (DTX1) and their esters (ng g^{-1}) measured in shellfish from the Northport-Huntington Bay complex located in New York, USA during 2010 and 2011. *Mytilus edulis* were hung in bags for monitoring purposes, whereas wild *Mya arenaria* and *Geukensia demissa* were harvested. The USFDA action level (160 ng g^{-1} of shellfish tissue) is indicated by the black dotted line. Bottom panel: Okadaic acid (OA), dinophysistoxin 1 (DTX1) and their esters as a percentage of total DSP toxins in shellfish tissue. Sites S1 - S7 as in Table 2.

Table 1.   Water quality parameters (temperature, total chlorophyll *a*, and salinity) in Northport Harbor, New York, during 2008 – 2011 blooms. Total chlorophyll *a* ($\mu g\ L^{-1}$) and salinity were averaged across the respective inclusive dates with standard errors indicated in parentheses while temperature (ºC) is given as a range.

	2008			2009			2010			2011		
	Alexandrium	*Dinophysis*	Both	*Alexandrium*	*Dinophysis*	Both	*Alexandrium*	*Dinophysis*	Both	*Alexandrium*	*Dinophysis*	Both
Inclusive dates	4/17- 6/5	5/12 - 7/11	4/17 - 7/11	3/26 - 6/5	5/19 - 6/12	3/26 - 6/12	3/25 - 5/25	5/11 -8/19	3/25 - 8/19	3/28 - 5/24	4/26 - 9/2	3/28 -9/2
Temperature (ºC)	9.7 - 20.6	11.9 - 23.4	9.7 - 23.4	6.2 - 21.1	15.9 - 21.1	6.2 - 21.1	8.8 - 18.4	13.1 - 26.4	8.8 - 26.4	8.9 - 17.7	11.1 - 26.6	8.9 - 26.6
Salinity (ppt)	22.7 (0.2)	21.8 (1.1)	22.0 (0.7)	24.0 (0.09)	24.0 (0.11)	24.0 (0.08)	21.5 (0.39)	23.4 (0.37)	22.4 (0.38)	24.0 (0.19)	23.8 (0.15)	23.8 (0.14)
Total chlorophyll *a* ($\mu g\ L{-1}$)	16.1 (2.89)	15.3 (2.76)	14.6 (2.45)	4.2 (0.55)	14.8 (6.38)	7.7 (2.45)	7.1 (1.5)	11.1 (2.58)	10.0 (1.66)	5.9 (1.4)	20.1 (7.5)	18.9 (6.54)

Table 2. Okadaic acid congener and pectenotoxin concentrations (ng g^{-1}) measured in shellfish collected from the Northport-Huntington Bay complex located in New York, USA. *Mytilus edulis* were monitoring species hung in bags, whereas *Mya arenaria* and *Geukensia demissa* were wild collected shellfish species. Samples were hydrolyzed therefore OA and DTX1 represent both free acids and esters. <dl indicates samples were below detection limit. Bold indicates sample above the FDA action level. OA=okadaic acid, DTX= dinophysistoxins, PTX= pectenotoxins.

Date	Shellfish Collection site	Location name	Longitude	Latitude	Shellfish species	OA	DTX1	DTX2	total OA congeners	PTX
28-Jun-2010	S3	Woodbine Marina	-73.35360	40.89880	*Mytilus edulis*	39	13	<dl	52	0.4
28-Jun-2010	S4	Northport Bay	-73.37560	40.91640	*Mytilus edulis*	74	41	<dl	115	7
20-Jun-2011	S7	Huntington Harbor	-73.41690	40.88840	*Mytilus edulis*	93	50	<dl	143	9
28-Jun-2011	**S3**	**Woodbine Marina**	**-73.35360**	**40.89880**	***Mytilus edulis***	**790**	**455**	**<dl**	**1245**	**115**
6-Jul-2011	**S5**	**Asharoken**	**-73.35440**	**40.92150**	***Mytilus edulis***	**107**	**58**	**<dl**	**165**	**5**
6-Jul-2011	S6	Huntington Bay	-73.43030	40.91650	*Mytilus edulis*	24	13	<dl	37	3
7-Jul-2011	**S1**	**South Scudder Beach**	**-73.35717**	**40.89211**	***Mya arenaria***	**660**	**297**	**<dl**	**957**	**66**
7-Jul-2011	**S2**	**North Scudder Beach**	**-73.35739**	**40.89311**	***Mya arenaria***	**758**	**331**	**<dl**	**1089**	**42**
7-Jul-2011	**S2**	**North Scudder Beach**	**-73.35739**	**40.89311**	***Geukensia demissa***	**818**	**319**	**<dl**	**1137**	**71**

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Chapter 5

The effects of inorganic and organic nutrients on the growth and toxicity of *Dinophysis acuminata* populations in New York estuaries

Abstract

Diarrhetic Shellfish Poisoning (DSP) is a globally significant human health syndrome most commonly caused by dinoflagellates of the genus *Dinophysis.* While blooms of harmful algae have frequently been linked to excessive nutrient loading, *Dinophysis* is a mixotrophic alga whose growth is typically associated with prey availability, and field studies of *Dinophysis* and nutrients have been rare. Here, I examined the temporal dynamics of *Dinophysis acuminata* blooms, DSP toxins, and nutrients (nitrate, ammonium, phosphate, silicate) over four years within two NY estuaries and I evaluated changes in the growth and toxicity of *D. acuminata* blooms during 14 nutrient amendment experiments performed over a three year period. *Dinophysis acuminata* blooms exceeding one million cells L^{-1} were observed in the New York estuaries, Meetinghouse Creek and Northport Bay. *D. acuminata* abundances were significantly correlated with ammonium, phosphate, and total dissolved phosphorus concentrations and were inversely correlated with nitrate. N- and P- containing inorganic and organic nutrients significantly enhanced *D. acuminata* densities in 12 of 14 experiments. Ammonium significantly increased cell densities in all but one experiment, while glutamine significantly enhanced cellular DSP content in 4 of 5 experiments examined. Nutrients may have directly or indirectly enhanced *D. acuminata* growth as densities of this mixotroph during experiments were significantly correlated with multiple members of the planktonic community (flagellates and *Mesodinium*). Collectively, this study demonstrates that nutrient loading and more specifically N-loading promotes the growth and toxicity of *D. acuminata* populations in coastal zones.

Introduction

The spatial and temporal expansion and increased intensity of HABs is a globally recognized phenomenon (Hallegraeff, 1993; Glibert et al., 2005). HABs associated with human health syndromes, for example, paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP) and diarrhetic shellfish poisoning (DSP), are a growing human health and economic concern in many coastal regions (Anderson et al., 2008; Anderson et al., 2012; Reguera et al., 2014). These dinoflagellate-related HABs are often associated with substantial economic losses due to the closure of toxic shellfish beds (Koukaras and Nikolaidis, 2004; Jin and Hoagland, 2008; Jin et al., 2008). Given the human health threats that these toxin producing blooms pose and the global increase in these events (Hallegraeff, 1993; Van Dolah, 2000) more research is needed to understand what promotes and sustains these blooms.

Anthropogenic loading of nutrients and organic matter are known to play a central role in the outbreak of multiple HABs (Hallegraeff, 1993; Anderson et al., 2002; Glibert et al., 2005; Anderson et al., 2008; Heisler et al., 2008). Several studies have demonstrated that inorganic nitrogen (ammonium, nitrate) promotes the growth of various HABs (Anderson et al., 2008; Heisler et al., 2008; Hattenrath et al., 2010; Xu et al., 2012). Similarly, multiple culture and field investigations have reported the stimulation of several HAB species by different dissolved organic substrates including urea, amino acids, peptides, humic acids, vitamins and uncharacterized organic substances (Mulholland et al., 2002; Dyhrman and Anderson, 2003; Glibert et al., 2005; Glibert et al., 2006; Glibert and Legrand, 2006; Anderson et al., 2008; Heisler et al., 2008; Tang et al., 2010). Such mixotrophic tendencies of these HAB species are not surprising given that the evolution of pathways to incorporate N from other sources beyond DIN would afford them a competitive advantage over other phytoplankton when inorganic sources are depleted (Stoecker et al., 2006; Anderson et al., 2008). Moreover, phagotrophy

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combined with phototrophy has been shown to significantly increase the growth rates of several

HABs in comparison to strict autotrophic growth (Jeong et al., 2005; Stoecker et al., 2006;

Burkholder et al., 2008). While the effects of both inorganic and organic nutrients on the growth

and toxicity of multiple HABs have been assessed, these effects are not fully understood for the

mixotrophic dinoflagellate, *Dinophysis acuminata*.

Diarrhetic Shellfish Poisoning (DSP) is a globally significant human health syndrome

most commonly caused by dinoflagellates of the genus *Dinophysis* (Hallegraeff, 1993; Van

Dolah, 2000; Reguera et al., 2012) with few cases associated with the dinoflagellate

Prorocentrum lima (Gayoso et al., 2002; Maranda et al., 2007). *Dinophysis* spp. synthesize

okadaic acid (OA) and dinophysistoxins (DTXs), the causative toxins of DSP, as well as the

pectenotoxins (PTXs; Lee et al., 1989; Fux et al., 2011) which are not associated with DSP but

may be hepatotoxic as well as promote the formation of tumors in mammals (Lee et al., 1989;

Burgess and Shaw, 2001). While DSP is common in regions of Europe, South America and Asia

(Hallegraeff, 1993; Van Dolah, 2000; Reguera et al., 2012), prior to 2008 North America had

experienced few *Dinophysis*-related closures with most of these events occurring in Canada

(Quilliam et al. 1991, Subba Rao et al., 1993; Todd, 1997; Tango et al., 2004). In recent years,

however, North America has witnessed an expansion of *Dinophysis* blooms yielding shellfish

toxicity over the USFDA action level (160 ng g^{-1} of shellfish tissue) on the east (NY; Hattenrath-

Lehmann et al., 2013), west (WA; Trainer et al., 2013) and Gulf coasts (TX; Campbell et al.,

2010; Deeds et al., 2010; Swanson et al., 2010) of the United States. Interestingly, *Dinophysis*

spp. were found in these regions (Freudenthal and Jijina, 1988; Dickey et al., 1992; Horner et al.,

1997; Trainer et al., 2013) even prior to the increased incidences of shellfish toxicity over the

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USFDA limit, suggesting these blooms have become larger and more intense in recent years. Understanding the factors promoting such phase shifts merit further exploration.

While the nutritional ecology of most harmful algae has been explored at length, research on that aspect of *Dinophysis* has been lacking. Only within the past decade have cultures of *Dinophysis* been established after positively identifying the food source of this obligate mixotroph, the ciliate *Mesodinium* (=*Myrionecta*), and establishing a three-step culture system involving cryptophytes (Park et al., 2006). Therefore, nearly all information known regarding *Dinophysis* and nutrients has been gleaned from field investigations, which have come to conflicting conclusions. Some correlative field studies have found no relationship between *Dinophysis* densities and nutrient concentrations (Delmas et al., 1992; Giacobbe et al., 1995; Koukaras and Nikolaidis, 2004). A study in South Africa using ^{15}N-labeled compounds found that *D. acuminata*–dominated communities had a high affinity for recycled N (ammonium) therefore giving them a competitive advantage in highly stratified, nitrate depleted waters (Seeyave et al., 2009). Additionally, the only study investigating the effects of nutrients on okadaic acid and its derivatives in *Dinophysis* found contrasting results for the two *Dinophysis* species investigated (Johansson et al., 1996). Finally, the most recent and comprehensive review of *Dinophysis* ecology focused solely on phagotrophy as a prime nutritional mode for this alga (Reguera et al., 2012). Given the limited and conflicting nature of results obtained to date, more research is needed to understand the effects of nutrients on the growth and toxicity of *Dinophysis*.

This multi-year (2008 – 2012) field investigation assessed the dynamics of *Dinophysis acuminata*, its associated toxins okadaic acid (OA), dinophysistoxins-1 (DTX1) and pectenotoxins (PTXs), as well as nutrients in two eutrophic New York estuaries. The effects of

nutrients (organic and inorganic, nitrogen- and phosphorus-containing) on the growth and toxicity of *Dinophysis acuminata* were examined by conducting nutrient amendment experiments using natural plankton communities from these two estuaries. In addition, correlation matrices were used to relate *Dinophysis* and its toxins to nutrients in the field and to relate *Dinophysis* to other members of the phytoplankton community during experiments. Finally, I discuss the potential role that nutrients play in the current expansion of *Dinophysis* blooms across North America.

Materials and Methods

Field sampling

Field samples were collected on a weekly to twice-weekly basis from March through September during 2008 through 2012. Samples were collected in Northport Harbor (40$^°$53.500′N, 73$^°$21.434′W; Fig. 1), a shallow (2 - 4m) well mixed system within the southeastern portion of the Northport-Huntington Bay complex, located on the north shore of Long Island, NY, USA (Hattenrath-Lehmann et al., 2013). Field samples were also collected weekly from March through August 2011 and 2012 from a second site in NY, Meetinghouse Creek, a tidal tributary located in the Peconic Estuary (40$^°$56.314′N, 72$^°$37.119′W; Fig.1).

Subsurface water (~0.25m) was filtered for nutrient analysis using precombusted (4 hr @ 450$^°$C) glass fiber filters (GF/F, 0.7 µm pore size) and frozen in acid washed scintillation vials. Filtrate was analyzed colorimetrically for nitrate, orthophosphate, ammonium and silicate (Jones, 1984; Parsons et al., 1984) using a spectrophotometeric microplate reader (SpectraMax® Plus 384, Molecular Devices). Total dissolved nitrogen and phosphorus (TDN, TDP) were analyzed by persulfate oxidation techniques (Valderrama, 1981) and dissolved organic nitrogen and phosphorus (DON and DOP) calculated by subtracting concentrations of nitrate and ammonium

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or orthophosphate from concentrations of TDN and TDP, respectively. These analyses provided complete recovery of nitrate, orthophosphate, ammonium, TDN, and TDP from Environmental Resources Associates (ERA, Golden, CO) certified reference material. Whole water samples were preserved in Lugol's solution. *Dinophysis* cell densities were enumerated using a 1mL Sedgewick-Rafter slide under a compound microscope using whole water samples and concentrated preserved water samples. Concentrates were made to increase the limit of detection as *Dinophysis* cell densities are often a relatively small portion of the total phytoplankton community and are therefore expressed as cells per L. Concentrated water samples were made by sieving 1 - 2L of water through either a 200 μm or 64 μm mesh (to eliminate large zooplankton) and then onto a 20 μm sieve that was backwashed into a 15mL centrifuge tube. Counts made on plankton concentrates were not significantly different from direct counts on whole water. Detection limits for whole water and concentrated samples were 1,000 cells L^{-1} and 7 cells L^{-1}, respectively.

Analysis of toxins in phytoplankton concentrates

Several liters of seawater were pre-sieved through a 200 μm mesh (to eliminate large zooplankton) and subsequently concentrated on a 20 µm sieve and backwashed into 15ml centrifuge tubes. Samples were centrifuged at 3000 rpm for 11 minutes and the supernatant aspirated without disturbing the cell pellet. Cell pellets were kept frozen at -20ºC until further analysis. Algal pellets were resuspended in a known volume of 100% aqueous methanol, homogenized by vortex mixing and probe-sonicated (Branson 1450 sonicator) on ice at 30% amplitude, followed by centrifugation at 3400 x g for 10 min. The methanolic supernatants were filtered with a 0.2 μm syringe filter in preparation for analysis. Samples were analyzed for the presence of DSP toxins and the co-eluted pectenotoxins at NOAA's Marine Biotoxins

Laboratory (Charleston, SC) using liquid chromatography (HP 1100 series HPLC; Agilent Technologies, Palo Alto, CA) coupled with tandem mass spectrometry (4000 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer; AB Sciex, Foster City, CA) using the method described by Gerssen et al. (2009) with modifications. LC separation was performed on a X-Bridge[TM] C18 (150 × 3 mm, 5 µm) column (Waters, Milford, MA) using a mobile phase of water (A) and acetonitrile/water (90:10, V/V) (B), both containing 6.7 mM ammonium hydroxide under gradient elution at a flow rate of 0.4 mL min^{-1} (linear gradient from 1min of 10% B to 90% B at 12 min, hold for 3 min, then return to 10% B at 17 min and hold for 4 min). The detection of DSP toxins by MS was achieved by multiple reaction monitoring (MRM) in negative ion mode for OA, DTX1, and DTX2 (for OA and DTX2 with MRM transitions of *m/z* 803.5 →113.1and 255.1, for DTX1 with MRM transitions of *m/z* 817.5 → 113.1 and 255.1), and in positive ion mode for PTX2 and its isomers (MRM transitions of *m/z* 876.5 → 213.1 and 823.5), and PTX11 and its isomers (MRM transitions of *m/z* 892.5 →213.1 and 839.5). Certified standards of OA, DTX1, DTX2, and PTX2 were available for toxin determination from NRC (Halifax, Canada) and RIKILT (Institute of Food Safety, The Netherlands). No standards were available for PTX11 and its isomers and PTX2 isomers; their concentrations were calculated approximately using PTX2 standards. PTX11 and its isomers showed different LC retention times and similar product ion spectra but differences at fragments between *m/z* 250 to 350 and some isomers could not match any of the published product ion spectra in this small mass range (Suzuki et al., 2003; Suzuki and Quilliam, 2011). PTX2 and its isomers also showed identical product ion spectra but different LC retention time. As such, all PTX concentrations were combined and reported as total PTXs (herein referred to as PTX). The detection limit was about 0.5 pg of OA, 0.65 pg of DTX1, 0.4 pg of DTX2, and 0.25 pg of PTX2 on LC column. The

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toxin samples presented herein were not subjected to alkaline hydrolysis and therefore represent free acids (i.e. esterified toxins are not included) and are therefore lower than the total OA (Deeds et al., 2010; Hattenrath-Lehmann et al., 2013).

Nutrient amendment experiments

To assess the impact of organic matter and nutrient loading on the growth and toxicity of *Dinophysis acuminata*, a series of nutrient amendment experiments were performed during 2008 (12-May, 19-May and 26-May), 2010 (14-June, 22-June and 28-June) and 2011 (6-June, 13-June, 21-June, 27-June and 6-July). Triplicate bottles (2.5 L) were filled with water from Northport Harbor. An unamended control was established along with several treatments including 20 µM nitrate, 20 µM ammonium, 10 µM urea (=20 µM N), 10 µM glutamine (=20 µM N), 2 µM phosphate, 100pM vitamin B_{12}, 20 µM ammonium + 100pM vitamin $B_{12,}$ and ~20 µM DON equivalent of high molecular weight organic matter from sewage treatment plant effluent (HMW STP) which also contained 5µM ammonium. High molecular weight (>1 kDa, Millipore) organic matter from sewage treatment plant effluent was isolated and concentrated from the Riverhead Sewer District plant which is located in Riverhead, NY, ~ 50 km east of Northport and ~2 km west of Meetinghouse Creek. High molecular weight organic matter was isolated via tangential flow filtration as described by Gobler and Sanudo-Wilhelmy (2003). The use of tangential flow filtration ensures that high molecular weight organic material is concentrated but inorganic nutrient concentrations remained unchanged (Gobler and Sanudo-Wilhelmy, 2003). Additional experiments were conducted using bloom water from another New York tributary, Meetinghouse Creek, during 2011 (6-April, 16-April and 9-May). Triplicate bottles (1 L) were filled with water from Meetinghouse Creek and an unamended control was established along with the treatments 100pM vitamin B_{12} and 100nM vitamin B_1. All treatment

concentrations were within the range of levels found in Long Island estuaries (Gobler et al., 2004; Koch et al., 2012), including Northport Bay (Hattenrath et al., 2010, this study), with the exception of glutamine. Concentrations of glutamine added to experimental bottles were not environmentally realistic but were added at equimolar levels of N for comparison with other nitrogen sources. All bottles were incubated for ~ 48h at light and temperature conditions matching those found at the study site after which toxin samples were collected as described above and *Dinophysis* cells and other members of the phytoplankton community were enumerated from samples preserved in Lugol's solution. Plankton were identified and enumerated using a 1mL Sedgewick-Rafter slide under a compound microscope. Cells larger than 10 µm were identified to at least genus level and grouped as dinoflagellates, flagellates, diatoms (pennate vs centric) and ciliates. Only *Dinophysis* densities were enumerated for Meetinghouse Creek experiments.

Statistical analyses

For all experiments, differences among treatments were elucidated by means of a one way analysis of variance (ANOVA) using SigmaStat within SigmaPlot 11.0; when data sets failed normality tests Kruskal-Wallis ANOVAs by ranks were performed. The Student-Newman-Keuls method was used for post hoc pairwise multiple comparison procedures. A one way ANOVA was similarly used for interannual comparisons of nutrients and nutrient ratios. Additionally, Spearman's rank order correlation matrices were used to assess the extent to which *Dinophysis* densities co-varied with other members of the plankton community during experiments. The extent to which all individual environmental parameters were correlated to each other was also evaluated by means of a Spearman's rank order correlation matrix. The

small bloom (Table 1) that occurred in 2009 during which okadaic acid or pectenotoxins were not detected (Hattenrath-Lehmann et al., 2013) was not considered in statistical analyses.

Results

Dynamics of Dinophysis, toxins and nutrients in New York estuaries

Dense *Dinophysis acuminata* blooms were observed in Meetinghouse Creek and Northport Bay, with peak densities typically occurring in late April to early May and June at temperatures of 13-16°C and 19-24°C (13-17°C in 2008), respectively. When *D. acuminata* was present in the water column, temperature and surface salinities ranged from 8.7 to 26.6°C and 17.7 to 26.0 (mean= 24.0 ± 1) for Northport Bay, and 10.6 to 26.7°C and 8.2 to 26.7 (mean= 21.7 ± 5.3) for Meetinghouse Creek. In Northport Bay, the largest *D. acuminata* bloom occurred in 2011 reaching ~1.3 million cells L^{-1}, followed by 2012, 2010, 2008, and 2009, with maximal densities of 123,000, 116,000, 39,500 and 12,000 cells L^{-1}, respectively (Table 1, Fig. 2). Paralleling *D. acuminata* densities, maximal particulate toxin concentrations in Northport Bay occurred in 2011 with free OA= 4.2 pg mL^{-1}, free DTX1= 20.4 pg mL^{-1}, and total PTX= 2,878 pg mL^{-1}, while toxins were not detected in 2009 (Table 1). In 2012, *D. acuminata* densities in Meetinghouse Creek surpassed those of Northport Bay reaching ~2.1 million cells L^{-1} (Table 1) during a bloom that persisted for ~2 months. Similar to Northport Bay, PTX was the most abundant particulate toxin measured in Meetinghouse Creek followed by free DTX1 and free OA, with concentrations of 6,765, 160.8 and 19.6 pg mL^{-1}, respectively (Table 1).

Overall, mean nutrient concentrations and ratios during the large 2011 Northport Bay bloom were not significantly different from any other bloom year and varied interannually (Table 2). Over the course of the 2011 *D. acuminata* bloom, mean concentrations of ammonium, nitrate, phosphate and silicate were 2.91 ± 0.7, 10.1 ± 1.51, 1.34 ± 0.27, and 31.5 ± 3.95μM,

respectively (Table 2). Dissolved organic N and P (DON, DOP) were 19.1 ± 1.33 and 0.85 ±

0.09 μM, while total dissolved N and P (TDN, TDP) were 32.1 ± 2.05 and 2.18 ± 0.22 μM,

respectively (Table 2).   Mean DIN:DIP, DON:DOP and TDN: TDP ratios were 16.2

± 3.48, 35.9 ± 13.7 and 16.4 ± 1.22, respectively (Table 2). Finally, DIN:Si, DON:Si and

TDN:Si were 0.5 ± 0.07, 0.79 ± 0.09 and 1.29 ± 0.12, respectively (Table 2).

During the four years when DSP toxins were detected (2008, 2010, 2011 and 2012),

nitrogen (ammonium, nitrate, TDN, DON) and phosphorus (phosphate, TDP, DOP)

concentrations were lower during the peak of the *Dinophysis acuminata* bloom compared to

before and after the peak of the bloom, although these decreases were rarely significant (Fig. 2,

Table 3). Ammonium, phosphate and TDP were significantly correlated with *D. acuminata*

abundances (R= 0.31, p<0.01; R= 0.30, p<0.01; and R= 0.36, p<0.001, respectively) while

nitrate was significantly, but inversely, correlated (R= -0.25, p<0.05; Table 4 and 5).

Ammonium was significantly correlated with DTX1 (R= 0.32, p<0.05) and total OA (R= 0.33,

p<0.05) concentrations on a per cell basis (fg cell^{-1}) while DOP was correlated with both OA

(R=0.31, p<0.05) and PTX (R=0.34, p<0.05) concentrations (pg mL^{-1}; Table 4 and 5). Some of

these correlations were stronger during individual sampling years (Table 4 and 5).

Nutrient Amendment Experiments

The addition of both inorganic and organic nutrients enhanced the growth of *Dinophysis*

acuminata in 13 of 14 experiments performed. In field experiments conducted over three years

in Northport Bay, ammonium consistently and significantly increased *Dinophysis* densities 4 -

625% compared to the control in nearly every experiment (10 of 11 experiments; p<0.01 for all;

Fig. 3). In 6 of 8 experiments performed, the addition of vitamin B$_{12}$ significantly increased

Dinophysis densities 7 - 125% compared to the control (p<0.05 for all; Fig. 3). High molecular

weight organic matter isolated from a sewage treatment plant (HMW STP) significantly increased *Dinophysis* densities up to 130% compared to the control in 3 of 5 experiments conducted (p< 0.05 for all; Fig. 3). Additions of nitrate, phosphate, urea, or glutamine yielded less consistent results, causing significant increases in *Dinophysis* densities up to 400, 225, 100 and 650%, respectively, in 1 of 3, 3 of 6, 1 of 3, and 4 of 8 experiments performed, respectively (Fig. 3). The addition of ammonium and vitamin B_{12} together was examined on 14, 22, and 28 June 2010 and yielded significant increases (up to 270%; p<0.001) in *Dinophysis* densities compared to the control on 14 and 28 June, with only the 14 June yield being significantly greater than either individual addition (Fig. 3). During the experiments conducted in Meetinghouse Creek in 2011, the addition of vitamins B_1 and B_{12} significantly enhanced the growth of *D. acuminata* in two of three experiments, increasing *Dinophysis* densities up to 250% (p<0.05) and 330% (p<0.05), respectively, compared to the control (6 and 16-April; Fig. 4).

Spearman's correlation matrices were generated using the final densities of *Dinophysis* and other members of the planktonic community during experiments performed in 2008, 2010 and 2011. For each year, *Dinophysis* densities were significantly, but inversely, correlated with the densities of autotrophic flagellates (2008: R= -0.46, p<0.05; 2010: R= -0.84, p<0.001; 2011: R= -0.82, p<0.001). In 2008 and 2010, *Dinophysis* densities were also significantly, but inversely, correlated with pennate diatoms (R= -0.54, 2008 and R= -0.34, 2010; p<0.05 for both years) and in 2011, *Dinophysis* densities were significantly, but inversely, correlated with the ciliate, *Mesodinium* (R= -0.28, p<0.01).

The addition of inorganic and organic nutrients enhanced the toxicity of *Dinophysis acuminata* in experiments conducted with Northport Bay bloom water during 2011. Glutamine significantly increased toxin content per cell (fg cell^{-1}) of okadaic acid (OA; p<0.05, 28-104%),

dinophysistoxin-1 (DTX1; $p<0.05$, 30-181%) and total pectenotoxins (total PTXs; $p<0.05$, 25-76%) compared to the control in 60%, 80%, and 40% of experiments conducted, respectively (Fig. 5). During the experiment conducted on 6 July, the HMW STP addition significantly ($p<0.01$) enhanced cellular content of OA, DTX1 and total PTX by 61, 76 and 72%, respectively (Fig. 5). In a single case (13-June), the addition of all nutrients caused a significant decrease (45-63%; $p<0.001$) in total PTX content compared to the control (Fig. 5). Contrastingly, the addition of all nutrients caused a significant increase (24-76%; $p<0.05$) in total PTX content during an experiment conducted on 6 July (Fig. 5).

Discussion

Prior to this study, the role of nutrient loading in the occurrence of *Dinophysis* blooms has been largely ignored and links between nutrient loading and DSP events have never been made. This multi-year field investigation documented two high density ($>10^6$ cells L^{-1}), toxic *Dinophysis acuminata* blooms in two different New York estuaries, Meetinghouse Creek and Northport Bay. Cell densities and cellular toxin quotas, in some cases, were correlated with ammonium concentrations during blooms and ammonium consistently enhanced the growth of *D. acuminata* during experiments. Additionally, organic nitrogen compounds such as glutamine and high molecular weight organic matter from STPs enhanced the cellular toxicity of *D. acuminata* during experiments. Finally, densities of *D. acuminata* during experiments were inversely correlated with densities of multiple plankton groups suggesting that trophic interactions and/or competition influenced the net growth of *D. acuminata*. Collectively, this work provides new insight regarding the role of nutrient loading in the occurrence of HABs caused by this mixotrophic alga.

The effects of nutrients on *Dinophysis* blooms

Dinophysis acuminata is a mixotrophic dinoflagellate and to date, the role of nutrients in the occurrence of DSP events has received little attention (Reguera et al., 2012). There were multiple lines of evidence generated by this study, however, that demonstrate that nutrients promote *D. acuminata* blooms. While nutrient levels within Northport Harbor were high before and after the onset of blooms, during blooms concentrations of nitrate, ammonium, and phosphate were always lower, sometimes by more than five-fold, indicating there was a significant nutrient demand during these events. Both DIN:DIP and DIN:Si ratios during the peak of the bloom were lower than those found before and after the peak of the bloom, specifically evidencing a strong N demand by the *Dinophysis* blooms. The DIN:DIP only fell below the Redfield ratio (16:1) during the peak of the large ($>10^6$) 2011 bloom, perhaps indicating a stronger N demand during that large bloom year. During other periods, inorganic N:P ratios were close to Redfield (20.6 ± 2.5), while organic N:P were substantially above it (35.3 ± 3.85) suggesting that although autotrophs had an adequate balance of nutrients, heterotrophs had excess N relative to P. Finally, silicate levels were high and DIN:Si and DON:Si were always below 1 indicating that diatoms were unlikely to have been limited by the supply of silicon during this study (Brzezinski, 1985; Smayda, 1990; Gobler et al., 2006).

During this investigation the progression of *D. acuminata* blooms coincided with seasonally-influenced biogeochemical cycling of nutrients. As blooms develop during late spring and early summer, flow rates of nitrate-enriched groundwater (Young et al., 2013) become minimal (Steenhuis et al., 1985; Gobler and Sanudo-Wilhelmy, 2001) while benthic fluxes of phosphate and ammonium become maximal (Boynton and Kemp, 1985; Boynton et al., 1995). Consistent with this idea, *D. acuminata* were positively correlated with ammonium and

phosphate concentrations and negatively correlated with nitrate suggesting processes that enhance the production of recycled ammonium and phosphate, such as benthic fluxes, may promote *D. acuminata* blooms. While correlations between some nutrients and *Dinophysis* densities were significant, they accounted for less than half of the variability in this population suggesting additional modes of nutrition may contribute toward these events, namely mixotrophy and specifically phagotrophy (Koukaras and Nikolaidis, 2004; Davidson et al., 2012). Anderson et al. (2008) linked eutrophication to increases in mixotrophic (phagotrophic) HABs such as *K. veneficum* and *Pfiesteria* spp. Burkholder et al. (2008) hypothesized that most HABs found in eutrophic environments are mixotrophs that are stimulated directly by nutrients, and/or indirectly by elevated nutrients that increase their algal prey. This may be the case for *Dinophysis* blooms in New York which occur in eutrophied environments with significant point and non-point sources of nutrients.

While relationships between *Dinophysis acuminata* abundances and nutrients in the field were modest, the experimental addition of nutrients to bloom water clearly and consistently (93% of experiments) yielded enhanced *Dinophysis acuminata* densities. Similar to other HABs, *D. acuminata* densities were significantly enhanced by both inorganic (nitrate, ammonium, phosphate) and organic (glutamine, vitamins B_1 and B_{12}, high molecular weight sewage effluent) nutrients (Mulholland et al., 2002; Glibert and Legrand, 2006; Anderson et al., 2008; Heisler et al., 2008; Tang et al., 2010). The mode by which nutrients promote *Dinophysis* populations may be either direct via nutrients used by *Dinophysis* or indirect via the increases in the abundance of *Dinophysis*' prey. There was a significant correlation between *Dinophysis* and its known primary prey, *Mesodinium*, during one field year (2009, $R= 0.547$, $p < 0.01$; Hattenrath-Lehmann et al., 2013). Furthermore, during nutrient amendment experiments conducted with bloom water

over a three year period, there were significant inverse correlations with multiple members of the planktonic community including those that can be considered both direct (*Mesodinium*) and indirect (autotrophic flagellates) prey of *Dinophysis*. Given the broad range of nutrients capable of promoting the growth of *Dinophysis*, I hypothesize that many of these nutrients are stimulating the growth of various members of the microbial food web which benefit *Dinophysis* as direct (e.g. *Mesodinium*) or indirect (e.g. autotrophic flagellates) prey.

B-vitamins consistently yielded elevated densities of *Dinophysis* (e.g. 8 of 8 experiments in 2011). A recent survey of 47 strains of harmful algae found that 96% had an absolute requirement for vitamin B_{12} and 80% had an absolute requirement for vitamin B_1 (Tang et al., 2010), and further studies have also noted the ability of picomolar levels of vitamin B_{12} to promote the growth of HABs during blooms in NY waters (Koch et al., 2013; Koch et al., 2014). The vitamin requirements of *Dinophysis* spp. and *Mesodinium* spp. are unknown, however. Tang et al. (2010) further documented that the cryptophyte, *Rhodomonas salina,* also had absolute requirements for vitamin B_1 and $B_{12.}$ Hence, like other nutrients, the ability of vitamins to promote the growth of *Dinophysis* could be direct or indirect if high vitamin levels promote the growth of other cryptophytes, such as *Teleaulax* spp., which are consumed by *Mesodinium* spp., which are, in turn, consumed by *Dinophysis*.

The most consistent result (10 out of 11 experiments) in the present study was the significant increase in *Dinophysis acuminata* densities with the enrichment of ammonium. This finding was consistent with the significant correlation between *Dinophysis* abundance and ammonium concentration during this study and with a study conducted in South Africa which found *D. acuminata*–dominated populations had a high affinity for ammonium (Seeyave et al., 2009). Trainer et al. (2013) attributed the greater frequency of *Dinophysis* blooms in

Washington to higher than average flow from the Fraser River during the summers of 2011 and 2012. While the authors linked the enhanced river flow to stratification that promoted those events (Trainer et al., 2013), tributaries are significant nutrient sources to estuaries (Bricker et al., 2008) and frontal boundaries, where *Dinophysis* blooms occurred (in WA), are often associated with high nutrient levels (Townsend et al., 2005). In addition to ammonium, other regenerated forms of N such as glutamine and high molecular weight sewage effluent enhanced *Dinophysis* densities in 4 of 5 experiments in 2011. Several other studies have demonstrated that dinoflagellates typically prefer regenerated N (ammonium) over new N (nitrate; Dortch, 1990; Collos et al., 2007; Hattenrath et al., 2010; Gobler et al., 2012). Collectively, this study demonstrates that regenerated nitrogen and ammonium, in particular, can promote *D. acuminata* blooms.

The effect of nutrients on *Dinophysis* toxicity

There have been very few lab or field studies on the environmental factors that influence *Dinophysis* toxicity. Both Tong et al. (2011) and Nielsen et al. (2012, 2013) demonstrated that light intensity (15–300µmol photons $m^{-2}s^{-1}$) had no effect on toxin quotas for *D. acuminata* and *D. acuta*; Tong et al. (2011), however, found that *D. acuminata* toxin production and toxin content varied during the growth cycle and toxin production required light. While the effect of light on *Dinophysis* toxicity was not the subject of the present study, during blooms in Northport Bay DSP toxin (OA and DTX1) concentrations and cellular quotas were significantly correlated with inorganic and organic nutrients as well as nutrient ratios during individual years. Over the entire study, the cellular toxin content of *Dinophysis* was correlated with ammonium concentration. In experiments conducted with *D. acuminata* bloom water, however, the addition of glutamine increased cellular toxin quotas of OA, DTX1 and total PTX in every experiment

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performed, while the addition of other nutrients had less consistent effects. A study investigating the effects of nutrients on okadaic acid congeners in *Dinophysis* found contrasting results for the two *Dinophysis* species investigated (Johansson et al., 1996). While *D. acuminata* had higher OA concentrations under N-deficient conditions, levels were highest in *D. acuta* during nutrient sufficient conditions (+N+P; Johansson et al., 1996). An investigation using cultures of the benthic DSP-producing dinoflagellate, *Prorocentrum lima*, found that either N- or P- limitation increased OA concentrations (Vanucci et al., 2010). Consistent with the present study, Nagai et al. (2011) found that concentrations of PTX2, OA and DTX1 significantly increased in *D. acuminata* with the addition of organic substances (obtained from sonicating a *M. rubra* culture). The toxin production in the organic substances treatment, however, was lower than that of *Dinophysis* actively feeding on live *M. rubra* (Nagai et al., 2011), which is likely a function of the positive relationship between toxin production and growth rate which is indirectly linked to the ingestion of prey (Tong et al., 2011; Nielsen et al., 2013). Unlike other toxins associated with human health syndromes, such as saxitoxin and domoic acid, okadaic acid, dinophysistoxins and the co-ocurring pectenotoxins do not have nitrogen associated with their structures and are large molecules (molecular weight >800) that are structurally rich in C. As such, it is possible the organic C from substances like glutamine and high molecular weight sewage treatment plant water may promote toxin production. Biosynthesis studies have demonstrated that okadaic acid is synthesized from units of acetate, glycine and glycolate (Kalaitzis et al., 2010). Given the regularity with which glutamine enhanced okadaic acid and other DSP toxin levels during enrichment experiments, a further hypothesis is that glutamine is involved in toxin synthesis as a precursor or cofactor in the synthesis reactions. Clearly, more research is needed to understand the effects of nutrients on the toxicity of *Dinophysis*.

Regardless, this study clearly demonstrates that glutamine enhances the toxin content of *Dinophysis*.

During this multi-year study, DSP toxins and *Dinophysis* densities were promoted by and significantly correlated with multiple nutrients. Ammonium consistently enhanced the growth of *Dinophysis* while glutamine consistently enhanced its toxicity. Correlations between *Dinophysis* and multiple plankton groups during experiments suggested that the mechanism by which nutrients promote *Dinophysis* may be indirect via effects on prey items. Controlled culture experiments are needed to further resolve the effects of nutrients on both the phototrophic and mixotrophic growth of *Dinophysis acuminata*. Regardless, this study demonstrates that enhanced nutrient and organic matter loading in coastal zones are likely to promote both the growth and toxicity of *Dinophysis* blooms.

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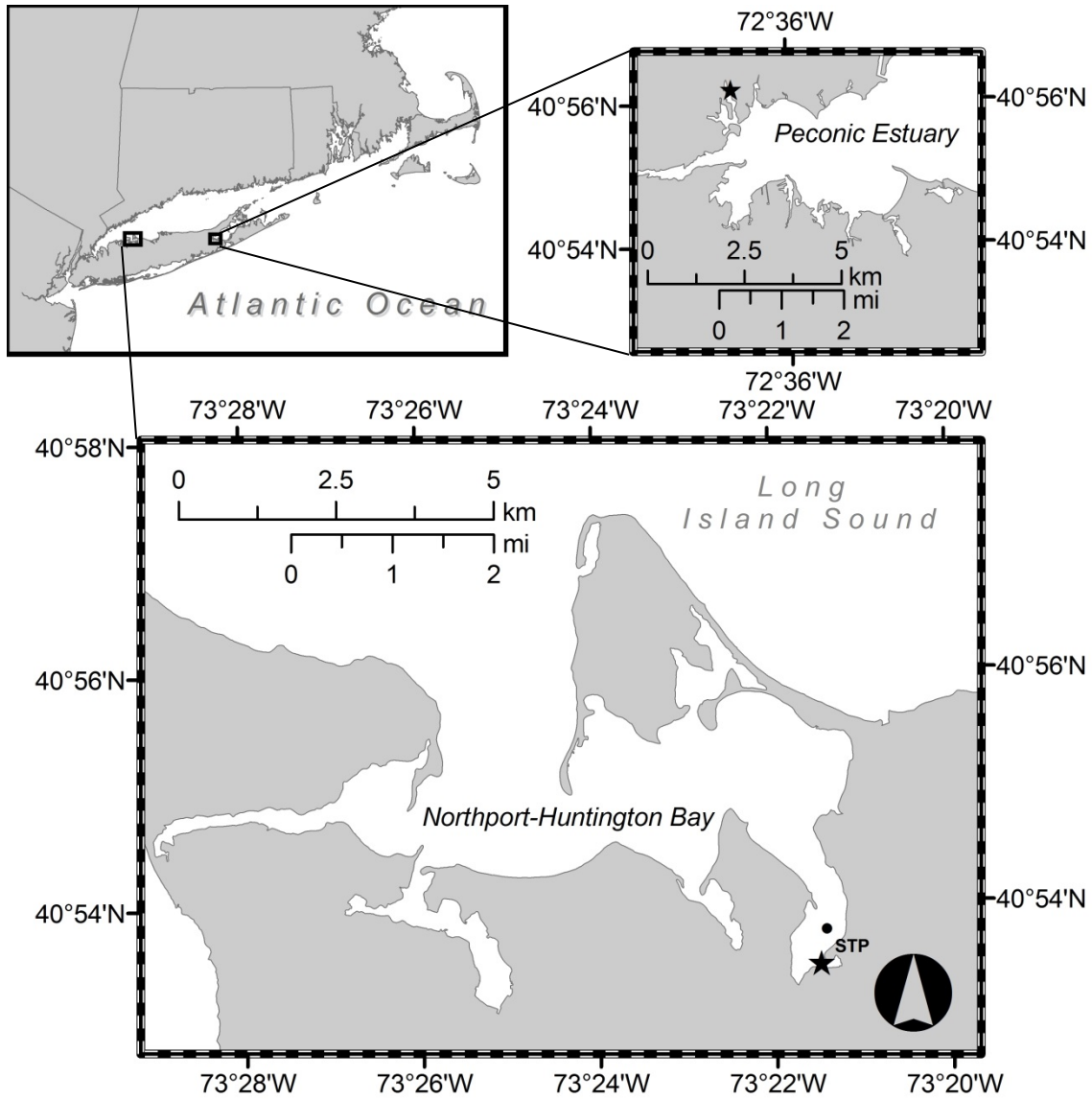
Figure 1. Field sampling (star) and sewage treatment plant outflow (circle) locations in the Northport-Huntington Bay complex and Meetinghouse Creek, New York, USA.
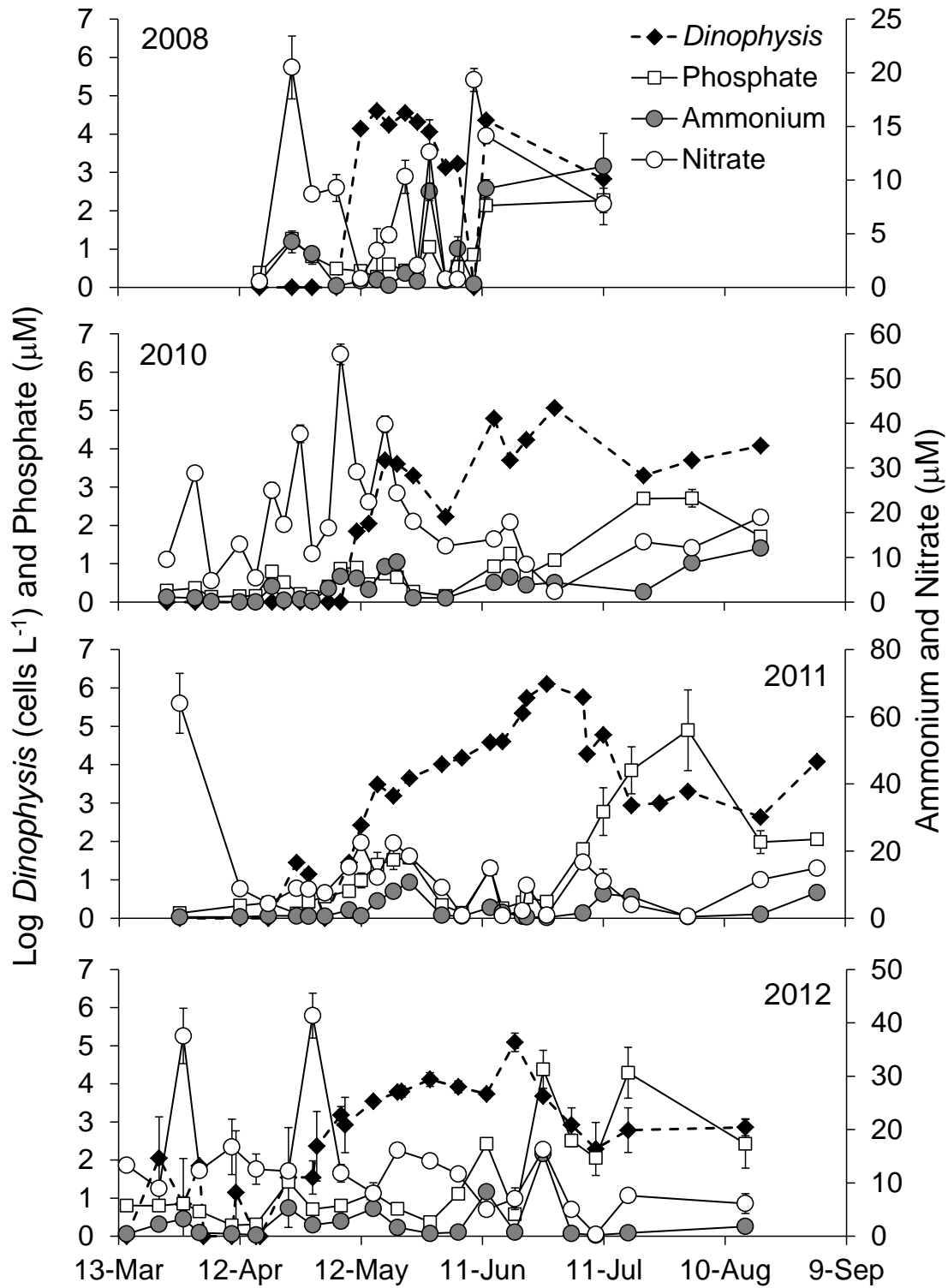
Figure 2. Log *Dinophysis acuminata* densities (cells L^{-1}), and the inorganic nutrients, ammonium, nitrate and phosphate (μM) in Northport Bay, NY, USA during 2008-2012.
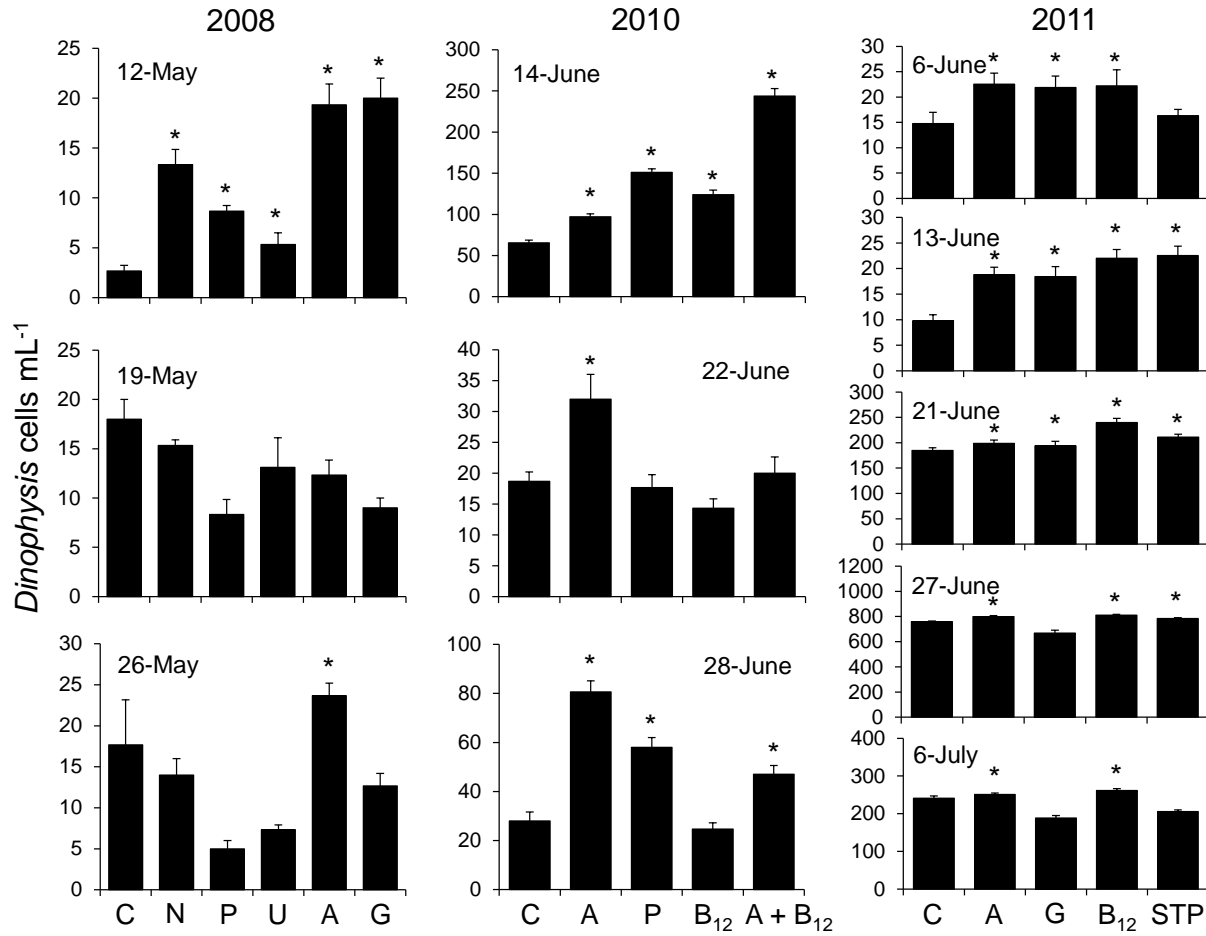
Figure 3. *Dinophysis acuminata* densities (cells mL^{-1}) at the end of nutrient amendment experiments conducted during 2008, 2010 and 2011 using water collected from Northport Bay, New York. Bars are means while error bars represent the SD of triplicate bottles. Asterisks indicate treatments that significantly increased compared to the unamended control. C= Control, N= Nitrate, P= Phosphate, U= Urea, A= Ammonium, G= Glutamine, B$_{12}$= vitamin B$_{12}$, A+ B$_{12}$= Ammonium + vitamin B$_{12}$, STP= high molecular weight sewage treatment plant organic matter.

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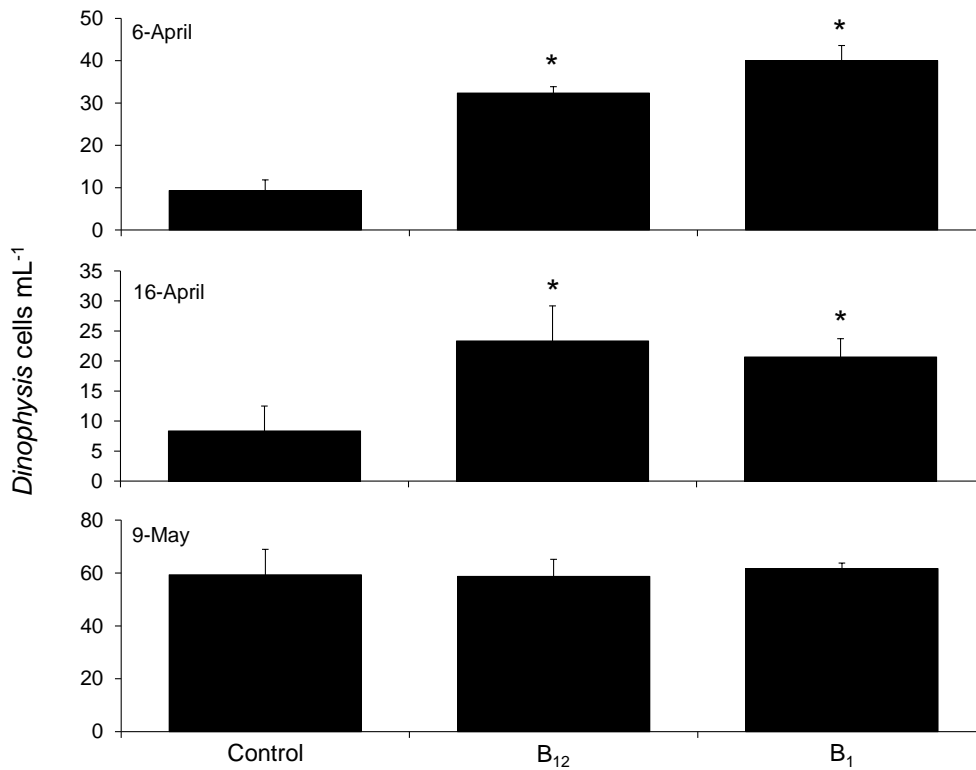
Figure 4. *Dinophysis acuminata* densities (cells mL^{-1}) at the end of nutrient amendment experiments conducted during 2011 using water collected from Meetinghouse Creek, New York. Bars are means while error bars represent the SD of triplicate bottles. Asterisks indicate treatments that are significantly different from the unamended control. C= Control, B$_{12}$= vitamin B$_{12}$ and B$_1$= vitamin B$_1$.
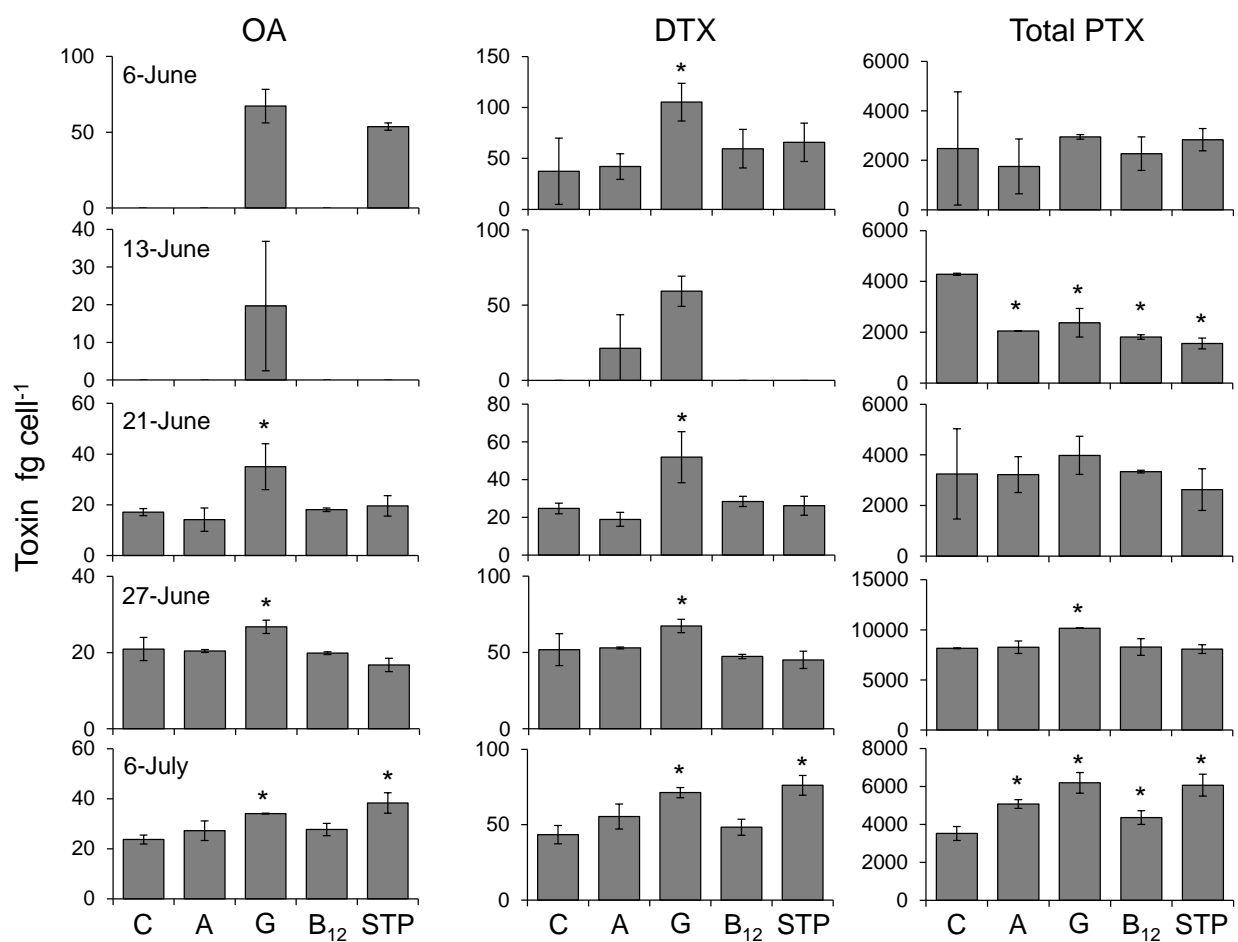
Figure 5. Okadaic acid (OA), dinophysistoxins 1 (DTX1) and total pectenotoxins (total PTX) as fg cell^{-1} for nutrient amendment experiments conducted using Northport Bay bloom water during 2011. Bars are means while error bars represent the SD of triplicate bottles. Asterisks indicate treatments that are significantly different from the unamended control. Abbreviations as in figure 2.

Table 1. Peak *Dinophysis acuminata* densities and toxin concentrations in two New York estuaries, Northport Bay and Meetinghouse Creek. n.d.= not detected.

Location	Year	*Dinophysis acuminata* (cells L^{-1})	Free OA (pg mL^{-1})	Free DTX1 (pg mL^{-1})	Total PTX (pg mL^{-1})
Northport Bay	2008	39,500	0.5	1.25	36.1
	2009	12,000	nd	nd	Nd
	2010	116,000	2.0	8.75	105.2
	2011	1,266,000	4.2	20.4	2877.6
	2012	123,000	4.6	18.0	1112.7
Meetinghouse Creek	2011	38,000			
	2012	2,123,000	19.6	160.8	6764.8

Table 2. Inorganic nutrients (ammonium, nitrate, phosphate, silicate), total dissolved and organic nutrients (μM) as well as nutrient ratios over the course of *Dinophysis acuminata* blooms in Northport Bay from 2008-2012. Samples were averaged across the respective inclusive dates with standard errors indicated in parentheses.

	2008	2010	2011	2012	All years
Inclusive dates	5/12 - 7/11	5/11 -8/19	4/26 - 9/2	3/23 - 8/15	
Ammonium (μM)	3.38 (1.29)	5.23 (0.93)	2.91 (0.70)	2.78 (0.86)	3.42 (0.46)
Nitrate (μM)	6.97 (1.92)	17.96 (2.65)	10.1 (1.51)	13.2 (2.35)	12.10 (1.13)
Phosphate (μM)	0.84 (0.22)	1.10 (0.23)	1.34 (0.27)	1.47 (0.29)	1.24 (0.14)
Silicate (μM)	22.9 (2.46)	35.0 (3.32)	31.5 (3.95)	41.3 (4.97)	33.7 (2.22)
TDN (μM)	26.7 (3.06)	38.6 (3.28)	32.1 (2.05)	42.8 (2.48)	35.7 (1.48)
DON (μM)	16.7 (1.68)	15.4 (1.98)	19.1 (1.33)	26.8 (2.36)	20.2 (1.10)
TDP (μM)	1.15 (0.16)	2.28 (0.33)	2.18 (0.22)	2.20 (0.38)	2.03 (0.16)
DOP (μM)	0.37 (0.11)	1.18 (0.17)	0.85 (0.09)	0.74 (0.11)	0.80 (0.07)
DIN:DIP	12.4 (2.34)	35.1 (7.46)	16.2 (3.48)	20.8 (4.49)	20.6 (2.50)
DIN:Si	0.41 (0.09)	0.71 (0.09)	0.50 (0.07)	0.51 (0.08)	0.53 (0.04)
DON:DOP	54.7 (9.78)	22.8 (8.96)	35.9 (13.7)	48.2 (6.89)	35.3 (3.85)
DON:Si	0.85 (0.15)	0.50 (0.09)	0.79 (0.09)	0.82 (0.11)	0.75 (0.05)
TDN: TDP	26.0 (2.85)	22.4 (4.31)	16.4 (1.22)	28.7 (4.19)	22.9 (1.73)
TDN:Si	1.25 (0.13)	1.20 (0.13)	1.29 (0.12)	1.33 (0.16)	1.28 (0.07)

Table 3. Mean nutrient concentrations and ratios at the peak of the bloom compared to before and after the peak of the bloom (when cells where present) for Northport Bay 2008 – 2012. Values are means with standard errors in parentheses. 2009 was excluded given the low *Dinophysis* densities. Values that are in bold are those that are significantly different from each other as determined by a t-test.

	2008		2010		2011		2012	
	bloom peak	before and after	bloom peak	before and after	bloom peak	before and after	bloom peak	before and after
Inclusive dates	5/16 - 5/26	5/12, 5/29 - 7/11	6/14 - 6/29	5/11 - 6/2, 7/21 -8/19	6/6 - 7/11	4/26 -6/1, 7/18 -9/2	5/29 - 6/19	3/23 - 5/22, 6/26 - 8/15
Ammonium (μM)	0.7 (0.2)	4.9 (1.8)	4.5 (0.4)	5.5 (1.4)	2.0 (0.8)	3.5 (1.0)	2.6 (1.9)	2.8 (1.0)
Nitrate (μM)	5.2 (1.8)	8.0 (2.9)	10.7 (3.4)	21.2 (3.0)	7.2 (2.4)	12.0 (1.8)	9.5 (2.1)	14.2 (2.9)
Phosphate (μM)	0.4 (0.07)	1.1 (0.3)	1.0 (0.1)	1.1 (0.3)	1.0 (0.3)	1.6 (0.4)	1.1 (0.5)	1.6 (0.3)
Silicate (μM)	23.4 (4.8)	22.7 (3.1)	29.9 (2.6)	37.2 (4.6)	31.0 (6.5)	31.9 (5.2)	38.7 (6.1)	42.0 (6.1)
TDN (μM)	22.2 (2.8)	29.4 (4.4)	34.5 (4.0)	40.5 (4.4)	28.0 (3.1)	34.7 (2.6)	**52.3 (4.7)**	**40.3 (2.6)**
DON (μM)	16.3 (1.7)	16.9 (2.6)	19.3 (1.4)	13.7 (2.6)	18.9 (1.7)	19.2 (1.9)	**40.3 (5.9)**	**23.2 (1.6)**
TDP (μM)	**0.7 (0.1)**	**1.4 (0.2)**	2.6 (0.2)	2.1 (0.5)	2.1 (0.3)	2.3 (0.3)	1.7 (0.5)	2.3 (0.5)
DOP (μM)	0.3 (0.1)	0.6 (0.2)	1.6 (0.2)	1.0 (0.2)	**1.1 (0.1)**	**0.7 (0.1)**	0.6 (0.1)	0.8 (0.1)
DIN:DIP	13.9 (4.7)	11.5 (2.8)	15.4 (3.2)	43.8 (9.4)	9.9 (1.7)	20.1 (5.3)	17.4 (7.6)	21.7 (5.4)
DIN:Si	0.27 (0.07)	0.49 (0.12)	0.53 (0.14)	0.78 (0.10)	**0.29 (0.07)**	**0.63 (0.10)**	0.37 (0.12)	0.55 (0.09)
DON:DOP	68.9 (15.8)	43.3 (11.0)	12.5 (0.89)	27.4 (12.9)	19.5 (3.3)	24.3 (3.3)	**76.3 (18.9)**	**40.7 (6.1)**
DON:Si	0.78 (0.13)	0.90 (0.23)	0.67 (0.12)	0.42 (0.11)	0.82 (0.16)	0.77 (0.11)	1.1 (0.25)	0.74 (0.11)
TDN: TDP	32.5 (5.2)	22.3 (2.7)	13.5 (1.5)	26.4 (5.8)	14.5 (1.8)	17.6 (1.6)	42.1 (14.3)	25.2 (3.6)
TDN:Si	1.0 (0.17)	1.4 (0.18)	1.2 (0.23)	1.2 (0.18)	1.1 (0.15)	1.4 (0.18)	1.5 (0.35)	1.3 (0.18)

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Table 4. Spearman rank correlations for inorganic nutrients, *Dinophysis acuminata* and toxins for Northport Bay, NY 2008 through 2012. Values represent correlation coefficient R and corresponding p-values in parentheses. Significant (p<0.05) correlations are highlighted in bold.

Parameter 1	Parameter 2	Year 2008	2010	2011	2012	All years
Ammonium	Dinophysis (cells L^{-1})	0.171 (0.532)	**0.663 (0.000390)**	0.143 (0.502)	0.267 (0.249)	**0.305 (0.00512)**
	OA (pg mL^{-1})	0.213 (0.498)	0.116 (0.699)	-0.256 (0.297)	0.169 (0.552)	0.0576 (0.672)
	DTX1 (pg mL^{-1})	0.474 (0.111)	0.0821 (0.783)	-0.205 (0.407)	0.0414 (0.880)	0.0984 (0.469)
	Total OA congeners (pg mL^{-1})	0.450 (0.136)	0.0821 (0.783)	-0.205 (0.407)	0.0803 (0.773)	0.107 (0.430)
	OA (fg cell^{-1})	0.273 (0.377)	0.2 (0.513)	0.212 (0.392)	0.234 (0.407)	0.209 (0.121)
	DTX1 (fg cell^{-1})	**0.592 (0.0387)**	-0.0299 (0.921)	**0.468 (0.0494)**	0.114 (0.682)	**0.317 (0.0175)**
	Total OA congeners (fg cell^{-1})	**0.570 (0.0478)**	-0.00746 (0.974)	0.427 (0.0759)	0.148 (0.605)	**0.332 (0.0126)**
	PTX (pg mL^{-1})	0.00699 (0.974)	0.224 (0.512)	-0.433 (0.117)	0.119 (0.670)	-0.00792 (0.956)
	PTX (fg cell^{-1})	-0.139 (0.681)	-0.515 (0.116)	0.0857 (0.762)	-0.0792 (0.773)	0.0115 (0.938)
Nitrate	Dinophysis (cells L^{-1})	-0.109 (0.686)	-0.114 (0.592)	-0.142 (0.504)	-0.352 (0.125)	**-0.254 (0.0205)**
	OA (pg mL^{-1})	0.0876 (0.766)	**-0.599 (0.0359)**	-0.246 (0.321)	0.123 (0.670)	-0.185 (0.171)
	DTX1 (pg mL^{-1})	-0.0287 (0.921)	**-0.709 (0.00903)**	-0.303 (0.214)	0.219 (0.444)	-0.220 (0.104)
	Total OA congeners (pg mL^{-1})	-0.0643 (0.834)	**-0.709 (0.00903)**	-0.303 (0.214)	0.243 (0.390)	-0.221 (0.101)
	OA (fg cell^{-1})	0.187 (0.542)	-0.508 (0.0843)	-0.112 (0.650)	0.180 (0.521)	-0.104 (0.442)
	DTX1 (fg cell^{-1})	-0.437 (0.150)	**-0.687 (0.0126)**	-0.0845 (0.736)	0.219 (0.444)	-0.216 (0.110)
	Total OA congeners (fg cell^{-1})	-0.465 (0.123)	**-0.672 (0.0154)**	-0.109 (0.656)	0.224 (0.435)	-0.203 (0.133)
	PTX (pg mL^{-1})	-0.217 (0.484)	-0.576 (0.0736)	-0.349 (0.212)	-0.0682 (0.808)	-0.259 (0.0696)
	PTX (fg cell^{-1})	-0.588 (0.0665)	-0.382 (0.258)	-0.226 (0.425)	-0.517 (0.0560)	-0.130 (0.377)
Phosphate	Dinophysis (cells L^{-1})	-0.240 (0.381)	**0.656 (0.000484)**	0.225 (0.286)	0.202 (0.388)	**0.289 (0.00821)**
	OA (pg mL^{-1})	0.150 (0.635)	0.312 (0.306)	-0.0712 (0.773)	0.00721 (0.976)	-0.0264 (0.846)
	DTX1 (pg mL^{-1})	0.241 (0.442)	**0.608 (0.0333)**	-0.0219 (0.928)	-0.297 (0.293)	0.0138 (0.919)
	Total OA congeners (pg mL^{-1})	0.179 (0.557)	**0.608 (0.0333)**	-0.0219 (0.928)	-0.258 (0.364)	0.0210 (0.877)
	OA (fg cell^{-1})	0.0936 (0.766)	0.320 (0.295)	-0.0585 (0.811)	0.0938 (0.738)	0.0180 (0.895)
	DTX1 (fg cell^{-1})	0.183 (0.557)	**0.720 (0.00707)**	0.335 (0.169)	-0.234 (0.407)	0.179 (0.185)
	Total OA congeners (fg cell^{-1})	0.204 (0.513)	**0.728 (0.00620)**	0.268 (0.278)	-0.185 (0.511)	0.184 (0.173)
	PTX (pg mL^{-1})	-0.266 (0.389)	0.442 (0.182)	-0.165 (0.562)	-0.0836 (0.762)	-0.113 (0.431)
	PTX (fg cell^{-1})	0.188 (0.583)	0.0303 (0.919)	0.0286 (0.916)	0.180 (0.521)	0.0738 (0.616)

Table 5. Spearman rank correlations for total dissolved and organic nutrients, *Dinophysis acuminata* and toxins for Northport Bay, NY 2008 through 2012. Values represent correlation coefficient R and corresponding p-values in parentheses. Significant (p<0.05) correlations are highlighted in bold. TDN was excluded given that it was not correlated with any of the listed parameters.

Parameter		Year				
1	2	2008	2010	2011	2012	All years
DON	Dinophysis (cells L^{-1})	-0.218 (0.426)	0.217 (0.304)	0.0593 (0.780)	**0.536 (0.0147)**	0.128 (0.249)
	OA (pg mL^{-1})	-0.0792 (0.800)	0.374 (0.215)	0.377 (0.120)	0.0469 (0.868)	-0.0127 (0.926)
	DTX1 (pg mL^{-1})	0.298 (0.329)	**0.579 (0.0446)**	0.366 (0.131)	0.290 (0.308)	0.155 (0.252)
	Total OA congeners (pg mL^{-1})	0.236 (0.442)	**0.579 (0.0446)**	0.366 (0.131)	0.231 (0.416)	0.131 (0.333)
	OA (fg cell^{-1})	0.0390 (0.886)	0.233 (0.456)	-0.303 (0.217)	-0.0469 (0.868)	-0.179 (0.187)
	DTX1 (fg cell^{-1})	0.542 (0.0623)	**0.623 (0.0285)**	-0.135 (0.586)	0.212 (0.453)	0.113 (0.405)
	Total OA congeners (fg cell^{-1})	0.556 (0.0547)	**0.593 (0.0387)**	-0.195 (0.431)	0.197 (0.492)	0.0848 (0.533)
	PTX (pg mL^{-1})	-0.0210 (0.939)	**0.661 (0.0332)**	0.446 (0.105)	0.414 (0.134)	0.0840 (0.560)
	PTX (fg cell^{-1})	0.552 (0.0892)	0.236 (0.490)	0.275 (0.332)	0.444 (0.105)	0.167 (0.255)
TDP	Dinophysis (cells L^{-1})	-0.0619 (0.822)	**0.657 (0.000471)**	0.328 (0.116)	0.176 (0.452)	**0.360 (0.000876)**
	OA (pg mL^{-1})	0.0355 (0.904)	0.466 (0.117)	0.0500 (0.837)	0.177 (0.532)	0.191 (0.158)
	DTX1 (pg mL^{-1})	0.171 (0.588)	**0.717 (0.00801)**	0.112 (0.650)	-0.217 (0.444)	0.178 (0.189)
	Total OA congeners (pg mL^{-1})	0.107 (0.733)	**0.717 (0.00801)**	0.112 (0.650)	-0.187 (0.511)	0.187 (0.167)
	OA (fg cell^{-1})	-0.0703 (0.817)	0.441 (0.143)	0.0797 (0.748)	0.227 (0.425)	0.159 (0.240)
	DTX1 (fg cell^{-1})	0.420 (0.165)	**0.739 (0.00466)**	0.383 (0.114)	-0.173 (0.542)	0.245 (0.0681)
	Total OA congeners (fg cell^{-1})	0.384 (0.206)	**0.754 (0.00336)**	0.347 (0.153)	-0.119 (0.670)	0.251 (0.0622)
	PTX (pg mL^{-1})	-0.431 (0.150)	**0.673 (0.0290)**	-0.174 (0.542)	-0.0484 (0.856)	0.141 (0.326)
	PTX (fg cell^{-1})	0.243 (0.468)	-0.0182 (0.946)	-0.0549 (0.844)	0.220 (0.435)	0.203 (0.166)
DOP	Dinophysis (cells L^{-1})	-0.0128 (0.954)	**0.510 (0.0112)**	0.265 (0.209)	0.0158 (0.942)	0.199 (0.0713)
	OA (pg mL^{-1})	-0.366 (0.233)	0.508 (0.0843)	0.252 (0.305)	0.447 (0.105)	**0.306 (0.0221)**
	DTX1 (pg mL^{-1})	-0.254 (0.415)	**0.668 (0.0154)**	0.197 (0.426)	0.0341 (0.904)	0.226 (0.0931)
	Total OA congeners (pg mL^{-1})	-0.293 (0.340)	**0.668 (0.0154)**	0.197 (0.426)	0.0584 (0.832)	0.236 (0.0795)
	OA (fg cell^{-1})	-0.344 (0.263)	0.441 (0.143)	0.137 (0.580)	0.462 (0.0907)	0.232 (0.0851)
	DTX1 (fg cell^{-1})	-0.0423 (0.886)	**0.631 (0.0263)**	-0.285 (0.248)	0.0535 (0.844)	0.108 (0.429)
	Total OA congeners (fg cell^{-1})	-0.0988 (0.749)	**0.638 (0.0242)**	-0.199 (0.421)	0.102 (0.715)	0.131 (0.334)
	PTX (pg mL^{-1})	-0.228 (0.456)	**0.818 (0.00150)**	0.262 (0.356)	0.0286 (0.916)	**0.339 (0.0161)**
	PTX (fg cell^{-1})	0.0486 (0.865)	0.176 (0.607)	-0.178 (0.532)	0.244 (0.390)	0.281 (0.0529)

Chapter 6

The effects of inorganic and organic nutrients on the growth of a New York isolate of

Dinophysis acuminata

Abstract

Diarrhetic Shellfish Poisoning (DSP) is a globally significant human health syndrome most commonly caused by dinoflagellates of the genus *Dinophysis.* While recent evidence (Chapter 5) suggests that blooms of this mixotrophic dinoflagellate are promoted by nutrient loading, specifically nitrogen (N) loading, it is still unclear whether these effects are direct (nutrient stimulation of *Dinophysis*) or indirect (nutrient stimulation of prey) as this alga is mixotrophic. Moreover, culture studies addressing the effects of nutrients on *Dinophysis* have been extremely rare. Therefore, I established an isolate of *Dinophysis acuminata* from New York waters and conducted controlled culture experiments to assess the effects of nutrients on the growth of this dinoflagellate with and without its prey, *Mesodinium rubrum* (=*Myrionecta rubra*). Cultures grown with and without prey grew significantly faster with high molecular weight sewage treatment plant effluent, glutamine or ammonium as their sole N source than respective controls, while nitrate grown cultures yielded significantly more rapid growth only when fed large amounts of *M. rubrum.* In contrast to prior studies with field populations of *D. acuminata* (Chapter 5), vitamin B_{12} had no effect on *D. acuminata* growth. Growth rates of *D. acuminata* increased with the amount of *M. rubrum* added and were significantly faster than cultures grown without prey. While the effects of nutrients on *D. acuminata* were smaller at the highest *M. rubrum* densities, nutrients consistently enhanced the growth of all *D. acuminata* cultures grown with and without prey. This is the first study to demonstrate that inorganic and organic nutrients are capable of directly promoting the growth of *D. acuminata* and support the hypothesis that enhanced nutrient loading in coastal ecosystems can promote DSP producing blooms of this species.

Introduction

Harmful algae, dinoflagellates in particular, are known to possess a diverse array of nutritional strategies (Jacobson and Anderson, 1996; Stoecker et al., 2006; Burkholder et al., 2008), including phototrophy, heterotrophy in the form of osmotrophy (uptake of dissolved organic compounds) or phagotrophy (ingestion of particles or prey), and mixotrophy when a combination of phototrophy and heterotrophy are employed. The nutritional strategy of dinoflagellates may change depending on ambient inorganic nutrient concentrations and light levels and it has been hypothesized that limitations of light or nutrients may cause a switch from phototrophy to mixotrophy to supplement cellular nutrition (Burkholder et al., 2008, and references therein), with these triggers often being species-specific (Stoecker et al., 2006). Originally, it was further hypothesized that mixotrophs should possess lower growth rates than organisms having strictly a heterotrophic or phototrophic strategy due to the energy requirements to maintain the cellular machinery to utilize both nutritional modes (Raven, 1997; Burkholder et al., 2008). However, mixotrophy has been shown to significantly increase the growth rates of several harmful algae in comparison to strict phototrophy (Jeong et al., 2005; Stoecker et al., 2006; Burkholder et al., 2008, and references therein). Moreover, it has recently been proposed that mixotrophy may not be exclusive to nutrient poor habitats but may be more prevalent in eutrophied estuaries than originally hypothesized due to low light levels and/or unbalanced nitrogen-to-phosphorous (N:P) ratios (Burkholder et al., 2008). Overall, mixotrophy may play a large part in the growth and maintenance of harmful algal blooms (HABs; Anderson et al., 2002; Smayda, 2002; Burkholder et al., 2008) as well as the succession of the phytoplankton community (Anderson et al., 2002; Jeong et al., 2005). Given the important role that mixotrophy plays in HAB ecology more research is needed, particularly within regions that are heavily

influenced by anthropogenic nutrient loading and prone to frequent HABs such the East Coast of North America.

Diarrhetic Shellfish Poisoning (DSP) is a globally significant human health syndrome most commonly caused by dinoflagellates of the genus *Dinophysis* (Hallegraeff, 1993; Van Dolah, 2000; Reguera et al., 2012). *Dinophysis* spp. synthesize okadaic acid (OA) and dinophysistoxins (DTXs), the causative toxins of DSP, as well as the co-eluted pectenotoxins (PTXs; Lee et al., 1989; Fux et al., 2011) which are not associated with DSP but may be hepatotoxic as well as promote the formation of tumors in mammals (Lee et al., 1989; Burgess and Shaw, 2001). While DSP is a common occurrence in regions of Europe, South America and Asia (Hallegraeff, 1993; Van Dolah, 2000; Reguera et al., 2012), prior to 2008 the US had experienced only a single precautionary closure based on the presence of high *D. acuminata* densities (>3 x 10^4 cells L^{-1}), although subsequent testing revealed that shellfish were well below the regulatory closure limits (Tango et al., 2004). In recent years, however, the US has experienced an expansion of *Dinophysis* blooms causing shellfish to accumulate DSP toxins to levels exceeding the USFDA toxicity threshold (160 ng g^{-1} of shellfish tissue) on the East (NY; Hattenrath-Lehmann et al., 2013), West (WA; Trainer et al., 2013) and Gulf coasts (TX; Campbell et al., 2010; Deeds et al., 2010; Swanson et al., 2010). Interestingly, *Dinophysis* spp. were found in these regions prior to the shellfish toxicity events (Freudenthal and Jijina, 1988; Dickey et al., 1992; Horner et al., 1997; Trainer et al., 2013), suggesting these DSP-producing blooms have become more intense in recent years. While recent evidence (Chapter 5) suggests that blooms of *Dinophysis acuminata* in New York are promoted by nutrient loading, specifically N loading, it is still unclear whether these effects are direct or indirect (via effects of prey).

In contrast to many harmful algae, the nutritional ecology of *Dinophysis* is poorly understood. The first ever *Dinophysis* culture was only recently established after a three-step culture system was developed (Park et al., 2006). Specifically, *Dinophysis* sequesters and utilizes plastids (kleptoplastids) from *Mesodinium rubrum* (= *Myrionecta rubra*; Minnhagen and Janson, 2006; Wisecaver and Hackett, 2010; Minnhagen et al., 2011) which they, in turn, obtain from their cryptophyte prey (Hansen et al., 2012). Given this very recent advance, the very large majority of the information known regarding *Dinophysis* and nutrients has been gleaned from field investigations, which have come to contradictory conclusions (Delmas et al., 1992; Giacobbe et al., 1995; Johansson et al., 1996; Koukaras and Nikolaidis, 2004; Seeyave et al., 2009, Chapter 5). Thus far, *Dinophysis* culture research has focused on differences in toxicity and toxin profiles between species and geographic isolates (Hackett et al., 2009; Kamiyama and Suzuki, 2009; Fux et al., 2011), toxin production and/or excretion (Nagai et al., 2011; Tong et al., 2011; Smith et al., 2012; Nielsen et al., 2013), the effects of prey concentration (*Mesodinium rubrum*) on growth (Kim et al., 2008; Kamiyama and Suzuki, 2009) as well as toxin production (Nielsen et al., 2012), and the effects of light intensity on the growth and/or toxicity of *Dinophysis* (Kim et al., 2008; Tong et al., 2011; Nielsen et al., 2012, 2013). In addition, recent investigations on North American isolates are not ecologically relevant given the temperatures at which they are cultured (4-10°C) are not within the range of those found during DSP-producing blooms (13-24°C in NY; Chapter 5) because the prey used are not from a temperate location (Hackett et al., 2009; Tong et al., 2010; Fux et al., 2011; Tong et al., 2011; Smith et al., 2012). Furthermore, studies assessing the effects of nutrients on the growth and toxicity of *Dinophysis* have not been conducted. Given that recent evidence from field experiments (Chapter 5) suggests that nutrients either directly or indirectly (via prey) enhance the growth and toxicity of

Dinophysis acuminata, culture experiments specifically isolating the effects of nutrients on both autotrophic (direct) and mixotrophic (indirect) cultures are clearly needed.

In 2013, a *Dinophysis acuminata* culture was established from Meetinghouse Creek, a eutrophic, tidal tributary located in the Peconic Estuary, NY, USA, that experiences annual blooms of *Dinophysis,* with densities exceeding 2 million cells L^{-1}. The autotrophic (no prey) and mixotrophic (with *Mesodinium rubrum*) growth of *Dinophysis* was assessed over a gradient of prey-to-*Dinophysis* ratios. The effects of multiple inorganic and organic nutrient sources on *Dinophysis* growth rates were examined using both autotrophic (starved) and mixotrophically (fed) conditioned *Dinophysis* cultures. Similarly, cultures grown with and without nutrients were also grown with and without prey to isolate the effects of nutrients on *D. acuminata* when *M. rubrum* is present (mixotrophic) and when it is not (autotrophic). These are the first experiments to isolate the direct and indirect effects of nutrients on *Dinophysis* spp.

Materials and Methods

Cultures and culturing conditions

Cultures of the cryptophyte, *Teleaulax amphioxeia* (K-0434, Scandinavian Culture Collection of Algae and Protozoa), and the ciliate, *Mesodinium rubrum* (MBL-DK2009), were isolated in 2009 from Helsingør Harbor, Denmark, and were generously provided by PJ Hansen. *M. rubrum* was provided with *T. amphioxeia* weekly at a ratio of ~10:1 (prey: predator) and following complete consumption of the cryptophyte were fed to *Dinophysis acuminata* isolates weekly at a ratio of ~10:1 (prey: predator). During May 2013, clonal isolates of *D. acuminata* were established from Meetinghouse Creek, a tidal tributary located in the Peconic Estuary (40°56.314′N, 72°37.119′W), using 12-well culture plates (Corning, Corning, NY, USA). Two viable *D. acuminata* isolates were combined to mimic field populations and grown out in 2L

Pyrex Erlenmeyer flasks for experiments. All cultures were maintained in sterile $f/2$ (-Si) medium (883μM nitrate and 36μM phosphate; Guillard and Ryther, 1962) made from autoclaved and 0.2 μm-filtered aged coastal Atlantic Ocean water (40.7969°N, 72.4606°W) adjusted to 25psu and kept at 18°C in an incubator with a 12:12 h light:dark cycle, illuminated by a bank of fluorescent lights that provided a light intensity of ~100 µmol quanta m^{-2} s^{-1} to cultures. Consistent with all prior culture studies of this genus, cultures were non-axenic as antibiotics were lethal to *Dinophysis*.

Growth experiments with varying Mesodinium *ratios*

Growth rates of *Dinophysis acuminata* were assessed over a gradient of predator-to-prey (*Mesodinium rubrum*) ratios during a month-long experiment. Stock cultures of well-fed *D. acuminata* and *M. rubrum* (*T. amphioxeia* completely consumed) were diluted to appropriate treatment densities in triplicate using $f/2$ (-Si) medium in 500mL Erlenmeyer flasks and incubated as above. Initial concentrations of 100 *D. acuminata* cells mL^{-1} were established for all treatments and for a no prey control. *M. rubrum* was added at prey: predator ratios of 2:1 (200 cells mL^{-1}), 5:1 (500 cells mL^{-1}) and 10:1 (1,000 cells mL^{-1}) with additional no predator controls established for each ratio. Every three to four days, a 5mL aliquot from each flask was fixed in Lugol's iodine (final concentration= 2%) and cells were enumerated with a 1mL Sedgewick-Rafter chamber using a compound microscope.

Growth rates were calculated during the exponential growth phase (persisting 4-8 days depending on food source ratio) using the formula: $\mu= \ln(B_t/B_0)/t$, where B_0 and B_t are the initial and final cell densities and t is the incubation duration in days. Differences in growth rates among treatments were evaluated with a One Way ANOVA with post hoc Tukey multiple comparisons tests using Sigma Stat software embedded within Sigma Plot 11.0. Additionally,

growth rates of *D. acuminata* over the varying *M. rubrum* ratios were fitted using linear regression as well as a Michaelis-Menten model using curve fitting functions within Kaleidagraph (Synergy Software; version 4.5) and μmax (maximum growth rate) and K_s (half saturation constant, as prey concentration) were derived.

Ingestion rates were calculated as per Tong et al. (2010) using the following two equations: (1) $dx/dt = \mu_x \times x - U \times y$ and (2) $dy/dt = \mu_y \times y$, which assumes that the concentrations of *y* (*D. acuminata*) and *x* (*M. rubrum*) increase exponentially, with respective growth rate constants, μ_y and μ_x. The mortality of *M. rubrum* from *Dinophysis* ingestion (*U*) is a function of $U \times y$ and was calculated during the first four days of experiments to ensure that food was not limiting (Jakobsen and Hansen, 1997).

Growth of cultures with multiple nutrient sources and prey densities

Experiments were conducted to isolate the effects of nutrients and prey on *Dinophysis acuminata* growth. Prior to the initiation of the experiments a one-month, two-phase, preconditioning period was implemented. For both phases, *M. rubrum* was added at ~1:1 ratio to reflect prey densities in the field where *Dinophysis* spp. is typically food-limited (Kim et al., 2008; Riisgaard and Hansen, 2009). The first phase was a grow-out period (two weeks) in which *D. acuminata* was grown in nutrient replete medium (*f*/2 -Si) and fed *M. rubrum* at a ratio of ~1:1 three times (day 0, day 7 and day 11). After the two week period the culture was sieved through a 10μm mesh (to rid the culture of residual *M. rubrum* and nutrients) and subsequently washed into autoclaved, 0.2μm-filtered, aged seawater. These *D. acuminata* cells were then used to initiate phase two of the preconditioning period when they were starved or fed for another two weeks. The starved *D. acuminata* culture was maintained in autoclaved, 0.2μm-filtered, aged seawater. The fed culture was maintained in nutrient replete medium (*f*/2 -Si, made

from autoclaved, 0.2μm-filtered, aged seawater) and fed *M. rubrum* at a ~1:1 ratio four times during the two week period (every three to four days). Given the two week period without prey, starved cultures were assumed to be photosynthetic while fed cultures were considered mixotrophic. Upon the end of phase two, the starved and fed *D. acuminata* cultures were sieved through a 10μm mesh and were added into autoclaved, 0.2μm-filtered, aged seawater to rid the fed culture of residual *M. rubrum* and nutrients and ensure cultures used for experiments had identical background nutrient levels (Table 1).

Preconditioned cultures (starved and fed) were used for experiments exploring the effects of different nutrients on the growth of *D. acuminata*. For each of the two conditions (starved and fed), a control (Table 1) was established in addition to the following treatments: ammonium (50μM), nitrate (50μM), glutamine (25μM = 50μM N), vitamin B_{12} (100pM) and high molecular weight sewage treatment plant effluent (HMW STP; 50μM N), treatments matching and thus complementing field experiments conducted in Chapter 5. High molecular weight (>1 kDa, Millipore) organic matter from sewage treatment plant effluent was isolated and concentrated from the Riverhead Sewer District plant which is located in Riverhead, NY, ~2 km west of Meetinghouse Creek (Chapter 5). High molecular weight organic matter was isolated via tangential flow filtration as described by Gobler and Sañudo-Wilhelmy (2003). The use of tangential flow filtration ensures that high molecular weight organic material is concentrated but inorganic nutrient concentrations remained unchanged (Gobler and Sañudo-Wilhelmy, 2003). The first experiment (time series) was performed using six-well culture plates (10 mL per well) with a single dose of nutrients and aliquots removed every 4 days for cell enumeration over a 12 day period. A second experiment was shorter term (6 days) and complementary to the first experiment (used 50ml polystyrene flasks, Thermo Scientific™) but was performed with

nutrients added in two equal doses (day 0 and day 3) to match the above mentioned total concentrations (50 µM N) but to avoid potential toxic effects of high ammonium (Taylor et al., 2006) seen in initial experiments. A third experiment was performed where the addition of *M. rubrum* (at ratios of 2:1 and 10:1) was used to assess the effects of nutrients on *D. acuminata* when *M. rubrum* is present and absent. This experiment was conducted for 6 days in six-well culture plates, nutrients were added in two equal doses as above and used *Dinophysis* cells preconditioned as 'fed'. At the end of experiments, aliquots were preserved in Lugol's iodine and cell densities were enumerated as above. Differences in cell densities among treatments were elucidated using two-way ANOVAs with post hoc Tukey multiple comparisons tests where either pre-conditioning (starved or fed), or food level (with or without *M. rubrum*) and nutrient source were the main effects using Sigma Stat software embedded within Sigma Plot 11.0.

Bacterial experiments

The combined effects of nutrients and *Dinophysis acuminata* on the heterotrophic bacterial community within cultures were examined. To remove *D. acuminata* without changing bacterial densities, a portion of the well mixed stock flask pre-conditioned as fed (as above) was filtered through a 5 µm polycarbonate filter. Six-well plates with and without *D. acuminata* were then established in parallel to assess differences in the growth of the heterotrophic bacterial community. A no nutrient control was established and nutrients (HMW STP and glutamine) that elicited an increase in *D. acuminata* densities in the previous experiments were added and run in parallel with the short term experiment described in the above nutrient experiments section. At the start and end of experiments, whole water samples were preserved in 10% buffered formalin (0.5% v/v final), stored at -80°C, and analyzed flow cytometrically to quantify the abundance of heterotrophic bacteria. Samples were stained with SYBR Green I and heterotrophic bacteria

were quantified using a FACScan (BD[®]) flow cytometer (Jochem, 2001). To determine the potential contribution of grazing on heterotrophic bacteria to the growth of *Dinophysis acuminata*, abundances of both populations were converted to C equivalents using previously published carbon contents of 895 pg cell[-1] (Riisgaard and Hansen, 2009) and 20 fg cell[-1] (Fukuda et al., 1998; Ducklow, 2000), for *Dinophysis acuminata* and heterotrophic bacteria, respectively.

Nutrient analyses

To determine ambient nutrient concentrations in stock cultures (Table 1), filtrate was made using precombusted (4 hr @ 450°C) glass fiber filters (GF/F, 0.7 µm pore size) and frozen in acid washed scintillation vials. Filtrate was analyzed colorimetrically for nitrate, ammonium and urea (Jones, 1984; Parsons et al., 1984) using a spectrophotometeric microplate reader.

Results

Growth of Dinophysis acuminata *under varying* Mesodinium rubrum *ratios*

The growth rates of *Dinophysis acuminata* cultures varied over the spectrum of *Mesodinium rubrum* : *D. acuminata* ratios (Fig.1, 2). *D. acuminata* growth rates were low (0.07 ± 0.01 d^{-1}) in the absence of prey reaching peak densities of 258 ± 58 cells mL^{-1}. With increasing prey ratios (2:1, 5:1, and 10:1) the growth rates of *D. acuminata* increased significantly (0.15 ± 0.04, 0.20 ± 0.01, 0.28 ± 0.01 d^{-1}; p<0.01, Tukey) with maximal *D. acuminata* densities of 303 ± 85, 873 ± 106, and 1549 ± 65 cells mL^{-1}, respectively (Fig. 1, 2). For all prey ratios, *M. rubrum* was consumed by *D. acuminata* within the first 8 days of the experiment (Fig. 1). *M. rubrum* growth in control cultures without *D. acuminata* was negligible, ranging from 0 to 0.04 d^{-1}. Ingestion rates of *M. rubrum* by *D. acuminata* over the range of feeding ratios (2:1, 5:1, and 10:1) were 0.29 ± 0.04, 0.46 ± 0.07 and 0.31 ± 0.06 cells per *D. acuminata* per day, respectively, with ingestion rates at a 5:1 (prey:predator) ratio significantly

(p<0.05, Tukey) higher than others. *D. acuminata* growth rate as a function of prey:predator ratio (Fig. 2B) was fit better to a linear function (R^2=0.91) than a Michaelis-Menten equation (R^2= 0.79), which had a maximum growth rate of 0.36 ± 0.07 d^{-1} and a prey:predator ratio of 3.23 ± 1.61 (323 ± 161 cells mL^{-1} *M. rubrum*) sustaining half the maximum growth rate (K$_s$), likely because saturation was not reached (as evidenced by the calculated maximum growth rate which was not reached during the experiments).

The effects of nutrients on starved and fed Dinophysis acuminata

    Time series- Pre-conditioning (starved vs fed) and nutrients significantly affected *D. acuminata* densities and there was a significant interaction between these factors as the response to preconditioning differed among the different nutrients investigated (p<0.001 for all, two-way ANOVA on final densities; Fig. 3). In starved cultures, the addition of HMW STP significantly (p<0.01, Tukey) increased cell densities by 25% compared to the control while all other nutrient additions slightly decreased *D. acuminata* densities (by 2-13%; Fig. 3). In the fed cultures, however, HMW STP, glutamine, and ammonium additions increased *D. acuminata* densities by 64, 24 and 13%, respectively, compared to the control (p<0.01; Tukey) while changes due to the addition of nitrate and B$_{12}$ were negligible (Fig. 3).

    Short term experiment- Pre-conditioning (starved vs fed) and nutrients significantly affected *D. acuminata* densities and there was a significant interaction between these factors as the response to preconditioning differed among the different nutrients investigated (p<0.001 for all, two-way ANOVA; Fig. 4). *D. acuminata* densities in control fed cultures increased by 200 cells mL^{-1} while growth in control starved cultures was negligible (Fig. 4). Compared to control cultures, the addition of HMW STP and glutamine significantly increased *D. acuminata* densities by 120 and 150% for starved, and 97 and 62% for fed cultures, respectively (p<0.001, Tukey;

Fig. 4). The addition of ammonium significantly decreased (77%) *D. acuminata* densities in starved cultures, but significantly increased (60%) densities in fed cultures (p<0.001, Tukey; Fig. 4). Similar to the previous experiment, the addition of B_{12} and nitrate had little effect on *D. acuminata* densities (Fig. 4).

The effects of varying ratios of Mesodinium rubrum *and nutrients on* Dinophysis acuminata

Prey availability (with vs without *M. rubrum*) and nutrients significantly affected *D. acuminata* growth at both 2:1 and 10:1 feeding ratios and there was a significant interaction between these factors as the response to nutrients differed among the different prey levels investigated (p<0.001 for all, two-way ANOVA; Fig. 5). Compared to initial *D. acuminata* concentrations, control cultures without *M. rubrum* grew 1.3 to 1.5-fold, while control cultures fed *M. rubrum* at 2:1 and 10:1 ratios grew 1.8 and 6.3-fold, respectively (Fig. 5). Across all nutrient treatments, the addition of *M. rubrum* significantly (p<0.001, Tukey) increased *D. acuminata* densities 1.4 to 1.9-fold at a 2:1 feeding ratio, and 2.8 to 4.9-fold at a 10:1 ratio, compared to their respective no *M. rubrum* treatments (Fig. 5). Densities of *D. acuminata* cultures fed a 2:1 prey ratio significantly increased with the addition of HMW STP (53%) and ammonium (55%), while their no *M. rubrum* counterparts only significantly increased with the addition of HMW STP (53%; p<0.001 for all, Tukey; Fig. 5), compared to their respective controls. When *M. rubrum* was fed to cultures at ratios of 10:1, *D. acuminata* densities increased with the addition of HMW STP (16%), nitrate (20%), ammonium (23%) and glutamine (40%) compared to the control (p<0.001, Tukey), while no *M. rubrum* cultures examined in parallel significantly increased with the addition of HMW STP (76%), ammonium (50%) and glutamine (44%; p<0.01, Tukey; Fig. 5). Consistent with other experiments the addition of B_{12} had no effect on *D. acuminata*. While effects of nutrients on *D. acuminata* were less pronounced at

higher *M. rubrum* densities (10:1) compared to those grown on lower densities or without *M. rubrum* (Fig. 5), some nutrients significantly enhanced the growth of *D. acuminata* cultures at all prey levels; the highest *D. acuminata* densities were achieved in cultures with high *M. rubrum* densities and nutrients added.

The effects of nutrients and D. acuminata *on bacterial densities*

Both nutrients and the presence of *Dinophysis acuminata* (with vs. without) significantly ($p<0.01$, two-way ANOVA) affected heterotrophic bacterial densities in fed cultures of *D. acuminata*, but there was no interaction between these factors (Fig. 6A). In cultures with *D. acuminata*, the addition of HMW STP and glutamine significantly ($p<0.05$, Tukey) increased heterotrophic bacterial densities 71 and 205%, respectively, whereas in cultures without *D. acuminata* bacterial densities increased 120 and 280%, respectively (Fig. 6A). Heterotrophic bacteria densities in cultures without *Dinophysis* were 8 to 41% higher than cultures with *Dinophysis* (Fig. 6A), suggesting that *Dinophysis* had an inhibitory effect (i.e. allelopathy, nutrient competition) on bacteria or consumed them. Contrary to patterns in heterotrophic bacterial abundances, HMW STP and glutamine significantly ($p<0.001$, Tukey) increased *D. acuminata* densities 97 and 62%, respectively, compared to the control (Fig. 6B). *D. acuminata* growth represented increases in biomass of 1.7 ± 0.75, 10.9 ± 1.3, and 7.6 ± 0.97 x 10^5 pg C mL^{-1} for the control, HMW STP and glutamine, respectively (Fig. 6C). The differences in heterotrophic bacterial carbon concentrations in treatments with and without *D. acuminata* were 2 to 3 orders of magnitude lower than these C specific increases in *D. acuminata* (Fig. 6C). As such, decreases in heterotrophic bacterial carbon due to the presence of *D. acuminata* represented less than 2% of the carbon increase in *Dinophysis* and thus did not contribute appreciably to increases in *D. acuminata* biomass during experiments.

Discussion

While recent evidence (Chapter 5) suggests that blooms of the mixotrophic dinoflagellate, *Dinophysis*, are stimulated by nutrient loading, specifically N loading, it is still unclear whether these effects are direct (via nutrient uptake by *Dinophysis*) or indirect (via nutrient stimulation of prey) and, thus far, research aimed towards resolving these effects has not been conducted. During this study, an isolate of *Dinophysis acuminata* from a New York estuary was established and a series of controlled culture experiments were performed to assess the effects of nutrients on the growth of this dinoflagellate in the absence and presence of prey. High molecular weight sewage treatment plant effluent and glutamine significantly and consistently enhanced the growth of *D. acuminata* in both starved and fed cultures compared to respective controls, while ammonium did so only when cultures were pre-conditioned as fed, and nitrate significantly increased densities of fed cultures only when *D. acuminata* was offered *Mesodinium rubrum* ratios of 10:1. In contrast, the addition of vitamin B_{12} had no effect on *D. acuminata* densities in any experiment. In general, across all nutrient treatments, growth rates of fed cultures were significantly higher than those of their starved counterparts. While effects of nutrients on *D. acuminata* were less dramatic at higher *M. rubrum* densities (10:1) compared to those grown with lower densities or without *M. rubrum*, some nutrients consistently and significantly enhanced the growth of both starved and fed cultures in all experiments. Moreover, heterotrophic bacterial biomass could not account for the growth of *Dinophysis*, indicating that nutrients and not bacterivory supported *Dinophysis* growth in cultures where *M. rubrum* was not added. Collectively, this is the first culture study to unequivocally demonstrate that *Dinophysis* growth rates can be directly stimulated by N loading.

Growth and ingestion rates of *Dinophysis acuminata* compared to other cultures

Growth rates of *Dinophysis* spp. cultures have been found to range from 0.06 to 0.95 d^{-1} and have been shown to be a function of temperature, prey concentration, and light (Park et al., 2006; Kim et al., 2008; Kamiyama and Suzuki, 2009; Riisgaard and Hansen, 2009; Tong et al., 2010; Nagai et al., 2011; Nielsen et al., 2012, 2013). My growth rate experiments conducted at 18°C with a *D. acuminata* culture established from Meetinghouse Creek, NY yielded autotrophic growth rates (no prey) of ~0.1 d^{-1} and mixotrophic growth rates of 0.15 to 0.28 d^{-1} (when offered *Mesodinium rubrum* and nutrients) with a maximal growth rate of 0.36 ± 0.07 d^{-1} when data were fitted to a modified Michaelis-Menten equation. It is possible, however, that my growth rates were underestimated due to the 3 to 4 day gap between sampling points. These patterns are consistent with another study that demonstrated that *D. acuminata* growth rates increase with increasing prey concentration, yielding phototrophic growth rates of 0.19 d^{-1} and a maximal mixotrophic growth rate of 0.91 d^{-1} (Kim et al., 2008). Additionally, my mixotrophic growth rates are within range of those observed for another North American strain of *D. acuminata* cultured using a different cryptophyte strain, *Geminigera cryophila*, and *M. rubrum*, both isolated from the Ross Sea (0.11 and 0.23 d^{-1} at 4 and 10°C, respectively; Tong et al., 2010). Similarly, *D. acuminata* cultured using the same *M. rubrum* and *T. amphioxeia* cultures as the present study yielded growth rates of 0.13 to 0.51 d^{-1} (Nielsen et al., 2012). While cross-comparison of growth rates among studies is difficult given the different culturing conditions (prey concentration, temperature, light, etc.), overall the growth rate of the New York strain of *D. acuminata* was within the range observed in other culture studies.

Ingestion rates of *Mesodinium* by *Dinophysis* have previously been shown to be a function of prey concentration and light intensity (Kim et al., 2008; Riisgaard and Hansen, 2009;

Tong et al., 2010). Tong et al. (2010) demonstrated that ingestion rates varied over the *D. acuminata* growth cycle and were 0.03 to 2.67 and 0 to 2.8 *M. rubrum* cells *Dinophysis* $^{-1}$ d^{-1} for 4 and 10°C, respectively, with temperature having a minor effect on these rates. Riisgaard and Hansen (2009) measured *D. acuminata* ingestion rates from 4 to 11 *M. rubrum* cells *Dinophysis* $^{-1}$ d^{-1}, which varied with prey concentration.   Kim et al. (2008) found that ingestion rates increased with prey concentration (maximal ingestion rates of 3.2 cells *Dinophysis*$^{-1}$ d^{-1}) and light intensity. During the present study, ingestion rates of the NY *D. acuminata* strain were lower than other culture studies, ranging from 0.29 to 0.46 cells *Dinophysis*$^{-1}$ d^{-1}, and varied with prey concentration (5:1 prey:predator ratio yielded the highest ingestion rates). Given that *M. rubrum* was completely consumed by *D. acuminata* within the first 8 days of experiments, these are not steady state ingestion rates or changes in ingestion rates over the course of the *Dinophysis* growth cycle (Tong et al., 2010).   Moreover, culturing conditions were different among all studies, further complicating comparisons. However, the near normal growth rates of the NY isolate of *D. acuminata* coupled with lower ingestion rates may indicate that the NY isolate may rely less on phagotrophy for nutrition than other strains studied to date.

Dinophysis **and nutrients**

Investigations on the effects of nutrients on the growth of the mixotrophic dinoflagellate, *Dinophysis*, are rare, contradictory and often focus on field correlations and experiments that are unable to separate out potential indirect effects of nutrients on prey items (Delmas et al., 1992; Giacobbe et al., 1995; Koukaras and Nikolaidis, 2004; Seeyave et al., 2009, Chapter 5).   While some correlative field studies have found no relationship between *Dinophysis* densities and nutrient concentrations (Delmas et al., 1992; Giacobbe et al., 1995; Koukaras and Nikolaidis, 2004), Seeyave et al. (2009) demonstrated by ^{15}N-labeling *Dinophysis* dominated-communities

(91% of total biomass as C) that these communities have a high affinity for ammonium. Recent ecosystem-based studies in NY estuaries (Chapter 5) have suggested that *Dinophysis* growth rates can be promoted by both inorganic (nitrate, ammonium) and organic N (glutamine, HMW STP). The only two culture studies investigating the effects of nutrients on *Dinophysis* growth concluded that both organic matter originating from sonicated *M. rubrum* cultures (Nagai et al., 2011) and dissolved inorganic nitrogen (DIN) had no effect on the growth of *D. acuminata* cultures (Tong et al., 2013). The present culture study, in agreement with Chapter 5, demonstrated that both inorganic and organic nutrients significantly enhanced the growth of *D. acuminata*. More specifically, I was able to demonstrate that these nutrient effects were direct as enhancements were seen in cultures of *D. acuminata* where no *M. rubrum* was added.

Similar to Chapter 5, the addition of high molecular weight sewage treatment plant effluent and glutamine significantly and consistently enhanced the growth of *D. acuminata*. While it is unclear whether nitrogen or carbon from these organic nutrients are stimulating *D. acuminata*, given that these enhancements were observed in starved cultures it suggests that *Dinophysis* may be capable of osmotrophy. While Nagai et al. (2011) didn't observe any growth rate enhancements with the addition of organic substances they demonstrated that organics enhanced *Dinophysis* toxicity. A study by Graneli et al. (1997) focusing on the nutritional capabilities of *Dinophysis*, however, could not resolve whether dark C uptake by *Dinophysis* was due to phagotrophy or osmotrophy.

While ammonium significantly and consistently increased *Dinophysis* densities in experiments using field populations (Chapter 5), this was the case in only fed cultures of *Dinophysis acuminata*. When *Dinophysis* was starved, the addition of ammonium inhibited growth likely due to toxicity (Taylor et al., 2006). I suggest that when *Dinophysis* is starved, the

threshold of ammonium toxicity is lower compared to when it has been well fed, perhaps due to fewer functioning plastids and an overall compromised cell physiology. Additionally, observations (Fig. 5 bottom panel) suggest that *Dinophysis* densities are enhanced by ammonium when it is actively feeding on *M. rubrum* as well as when it has recently fed and therefore likely continues to grow on reserve nutrition (Reguera et al., 2012). Regardless, collective observations from this study and Chapter 5 demonstrate that ammonium stimulates *Dinophysis acuminata* growth when cells have recently fed and are, thus, likely physiologically healthy.

In contrast to other nutrients examined, nitrate significantly increased *D. acuminata* densities only when *D. acuminata* was also offered *Mesodinium rubrum* ratios of 10:1. This is consistent with field experiments in Chapter 5 where only one third of experiments showed a significant increase in *D. acuminata* densities with the addition of nitrate. Since nitrate only enhanced *D. acuminata* growth in the presence of high prey densities, it is the only nutrient that may have indirectly stimulated the growth of *D. acuminata* through the initial uptake of nitrate by its prey, *M. rubrum*. Field observations and correlations, as well as nutrient uptake studies, demonstrated that *M. rubrum* is capable of using nitrate, ammonium and DON (Wilkerson and Grunseich, 1990; Crawford et al., 1997; Herfort et al., 2012; Hansen et al., 2013). Myung et al. (2013) also demonstrated that a culture of *M. rubrum* was able to grow for ~6 weeks in the absence of its cryptophyte prey, further supporting its autotrophic capabilities and the role *M. rubrum* may play in the indirect stimulation of *D. acuminata* blooms. During the present study, however, *M. rubrum* did not grow in control cultures that were nutrient replete and without *Dinophysis* or cryptophyte prey. This finding strongly suggests that increases in *Dinophysis* growth rates in the presence of prey and nutrients were direct effects (i.e. due to true mixotrophy)

rather than being due to an indirect effect (i.e. nutrient use by *M. rubrum* prey and subsequent consumption by *Dinophysis*), and that nutrients enhanced *Dinophysis* biomass.

Mixotrophic vs autotrophic growth rates

Mixotrophy has been shown to significantly increase the growth rates of several HABs in comparison to strict phototrophy (Jeong et al., 2005; Stoecker et al., 2006; Burkholder et al., 2008, and references therein; Kim et al., 2008). Similar to other studies, this study found that across all nutrient treatments, mixotrophic growth rates (with prey and nutrients) were significantly higher than those grown with nutrients only (Table 2). While the addition of nutrients increased the growth rates of cultures with and without prey compared to their respective controls, maximal growth rates were obtained when *Dinophysis* was fed *M. rubrum* at a 10:1 ratio in combination with the addition of glutamine (0.36 ± 0.01 d^{-1}; Table 2). While bacterial biomass could not support the changes in *Dinophysis* biomass during this study, past studies assessing phototrophic vs mixotrophic growth in algal cultures, such as *D. acuminata* (Kim et al., 2008), *Heterocapsa triquetra*, *Prorocentrum micans* and *Lingulodinium polyedrum* (Jeong et al., 2005) have not used antibiotics and therefore were likely non-axenic. In those cases, the importance of bacterivory in cultures deemed phototrophic cannot be dismissed.

***Dinophysis* and bacterial associations**

All *Dinophysis* culture investigations conducted thus far have maintained cultures under non-axenic conditions (Park et al., 2006; Kim et al., 2008; Hackett et al., 2009; Kamiyama and Suzuki, 2009; Riisgaard and Hansen, 2009; Tong et al., 2010; Fux et al., 2011; Nagai et al., 2011; Tong et al., 2011; Nielsen et al., 2012; Smith et al., 2012; Nielsen et al., 2013), and yet, to date no study has assessed the relationship between bacteria and *Dinophysis*. In this study, heterotrophic bacterial abundances were lower in the presence of *D. acuminata* compared to

parallel treatments without *D. acuminata*, suggesting this alga inhibited bacterial growth via nutrient competition, allelopathy, or bacterivory, with the later creating the prospect that nutrient effects on *D. acuminata* could be indirect. There were multiple lines of evidence that demonstrate this was not the case, however. *D. acuminata* growth was highest with the addition of HMW STP while the decrease in bacterial abundances was higher in the glutamine treatment. Additionally, reductions in heterotrophic bacterial biomass in the presence of *D. acuminata* accounted for <2% of the observed growth by *Dinophysis* in treatments. Finally, while bacteria are well known for their ability to liberate ammonium from organic N compounds (Kirchman, 2008) and the growth of *D. acuminata* was promoted by organic N compounds, the growth response to organic N was often larger than the response from equimolar amounts of ammonium. And, *D. acuminata* growth was also promoted by inorganic N. Therefore, bacterial remineralization of organic N could not account for the growth response of *D. acuminata* during experiments, meaning that *D. acuminata* growth responses to organic N were higher than would have been predicted if remineralized to ammonium by bacteria. Collectively, this suggests that nutrient assimilation supported the growth by *Dinophysis* in cultures where *M. rubrum* was not added. While bacterivory could explain the changes seen in heterotrophic bacteria abundances when *D. acuminata* was present, other processes such as an allelopathic effect of *Dinophysis* on bacteria, attachment of heterotrophic bacteria to *Dinophysis*, and competition for nutrients may also reduce the levels of bacteria when grown in the presence of *D. acuminata*.

While some *Dinophysis spp.* are known to harbor endosymbiotic bacteria (Lucas and Vesk, 1990), their association with free-living heterotrophic bacteria is largely unknown (Berland et al., 1995; Reguera et al., 2012). In this study, the inability to culture *Dinophysis* with the use of antibiotics is suggestive of a close association between *Dinophysis* and bacteria.

Moreover, a recent study demonstrated that vitamins (B_1 and B_{12}) significantly enhanced *D. acuminata* densities in bloom water from two different estuaries (Chapter 5), providing a potential link to the *Dinophysis*-bacteria relationship given that bacteria are the primary B-vitamin producers (Raux et al., 2000). A series of recent studies have demonstrated that the growth of phytoplankton can be strongly dependent on vitamin B_{12} production by bacteria (Croft et al., 2005; Kazamia et al., 2012). Further, other NY field studies have shown that HABs can be promoted by the addition of B-vitamins (Koch et al., 2013) and recent culture studies have demonstrated that 96% and 80% of harmful algae strains surveyed (*n*=47) were vitamin B_{12} and B_1 auxotrophs, respectively (Tang et al., 2010). The present study, however, found that *Dinophysis acuminata* cultures were not affected by the addition of vitamin B_{12} with or without *Mesodinium rubrum* present. This may be due, in part, to bacterial production of B_{12} in these non-axenic cultures. In an ecosystem setting, however, where *Dinophysis*, *Mesodinium* and cryptophytes co-occur, an indirect effect of vitamins on *Dinophysis* cannot be ruled out given that Tang et al. (2010) found that other cryptophytes such as *Rhodomonas salina* are vitamin B auxotrophs and thus could benefit from enhanced vitamins and indirectly support *Dinophysis*. This study in combination with previous work (Chapter 5) demonstrates a strong association between *Dinophysis* and bacteria and suggests it could be linked to a requirement for B-vitamins.

Conclusion

This study is the first to demonstrate that inorganic and organic nutrients enhance the growth of *Dinophysis acuminata* cultures with and without ciliate prey present. Additionally, this study, in agreement with Reguera et al. (2012), demonstrated that *Dinophysis* spp. are excellent survivors, as *D. acuminata* cultures were able to sustain high densities over a 3 week period without the addition of its food source, *M. rubrum*. This can have important implications

for human health as it has been demonstrated that *Dinophysis acuminata* retains its toxins as long as cells are viable even when starved (Smith et al., 2012). This work represents the first North American *Dinophysis* strain cultured using temperate prey items throughout the three-step culturing process (i.e. *Mesodinium rubrum* and *Teleaulax amphioxiea*) and grown at temperatures within the range seen during blooms in North America, therefore making these culturing experiments environmentally realistic compared to currently published work on other North American strains. More work should be conducted using locally established prey isolates to better constrain the effects of prey concentration and nutrients on *D. acuminata* growth. In addition, given the new information presented here on heterotrophic bacterial-*Dinophysis* associations, future studies are needed to better resolve these interactions. Overall, this study suggests that *Dinophysis* blooms are directly stimulated by nutrient loading.

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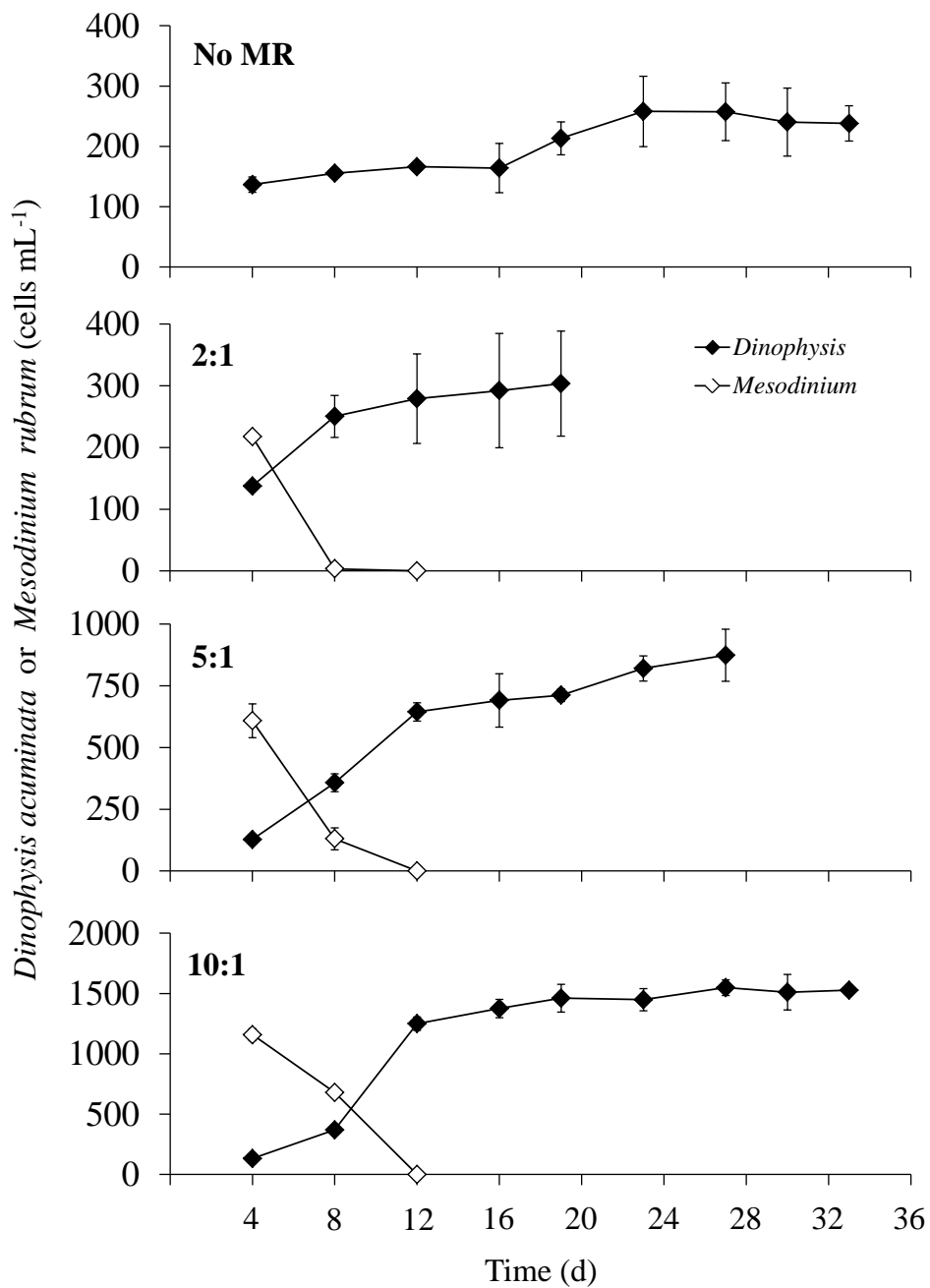
Figure 1. Abundance of *Dinophysis acuminata* (cells mL^{-1}) in cultures fed varying *Mesodinium rubrum* ratios (no MR, 2:1, 5:1 and 10:1). Points are means while error bars represent standard deviation of triplicate measurements.
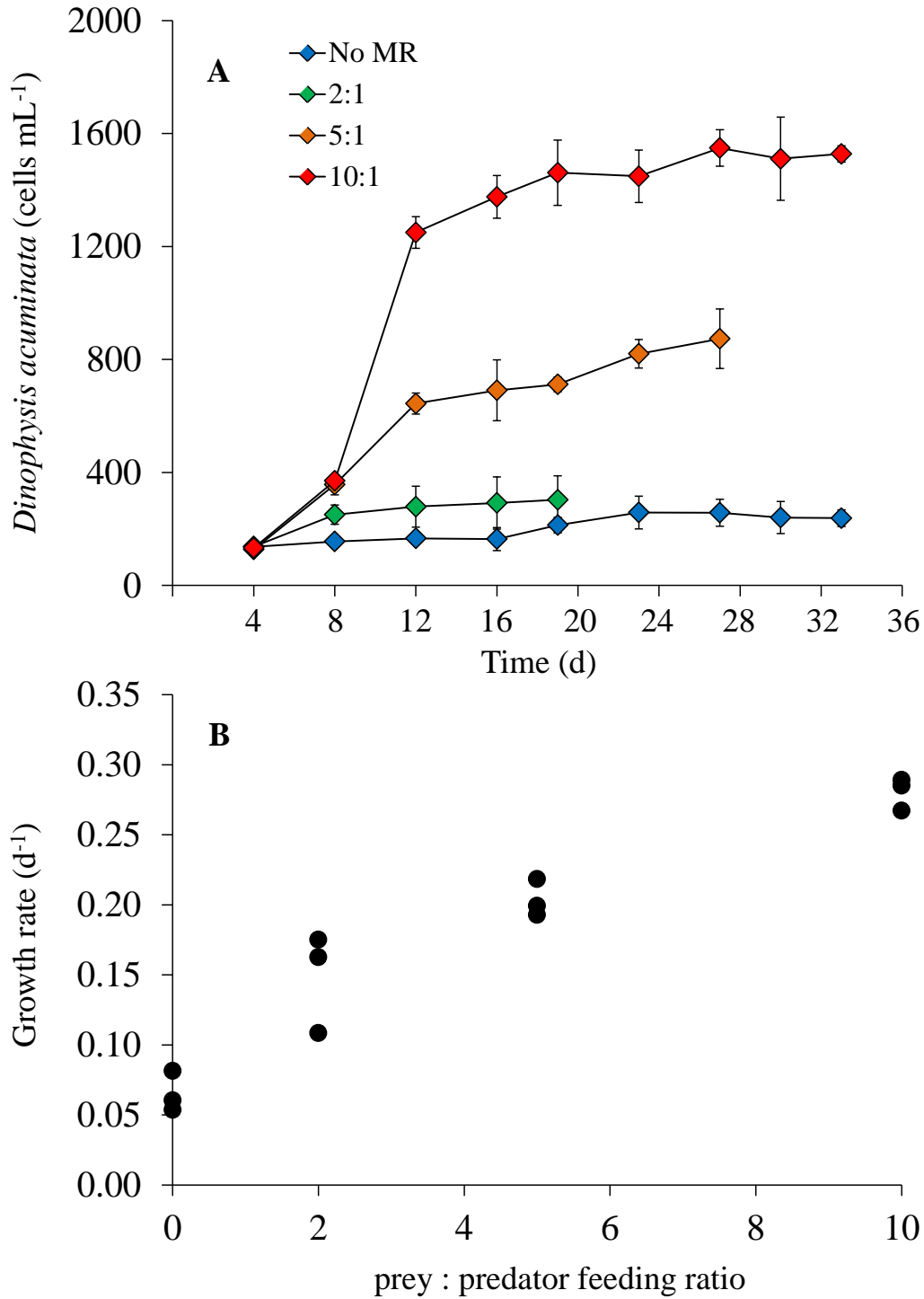
Figure 2. (A) Abundance of *Dinophysis acuminata* (cells mL^{-1}) in cultures fed varying *Mesodinium rubrum* prey:predator ratios (no MR, 2:1, 5:1 and 10:1). Points are means while error bars represent standard deviation of triplicate measurements. (B) *Dinophysis acuminata* growth rates (d^{-1}) as a function of *Mesodinium rubrum* feeding ratios.
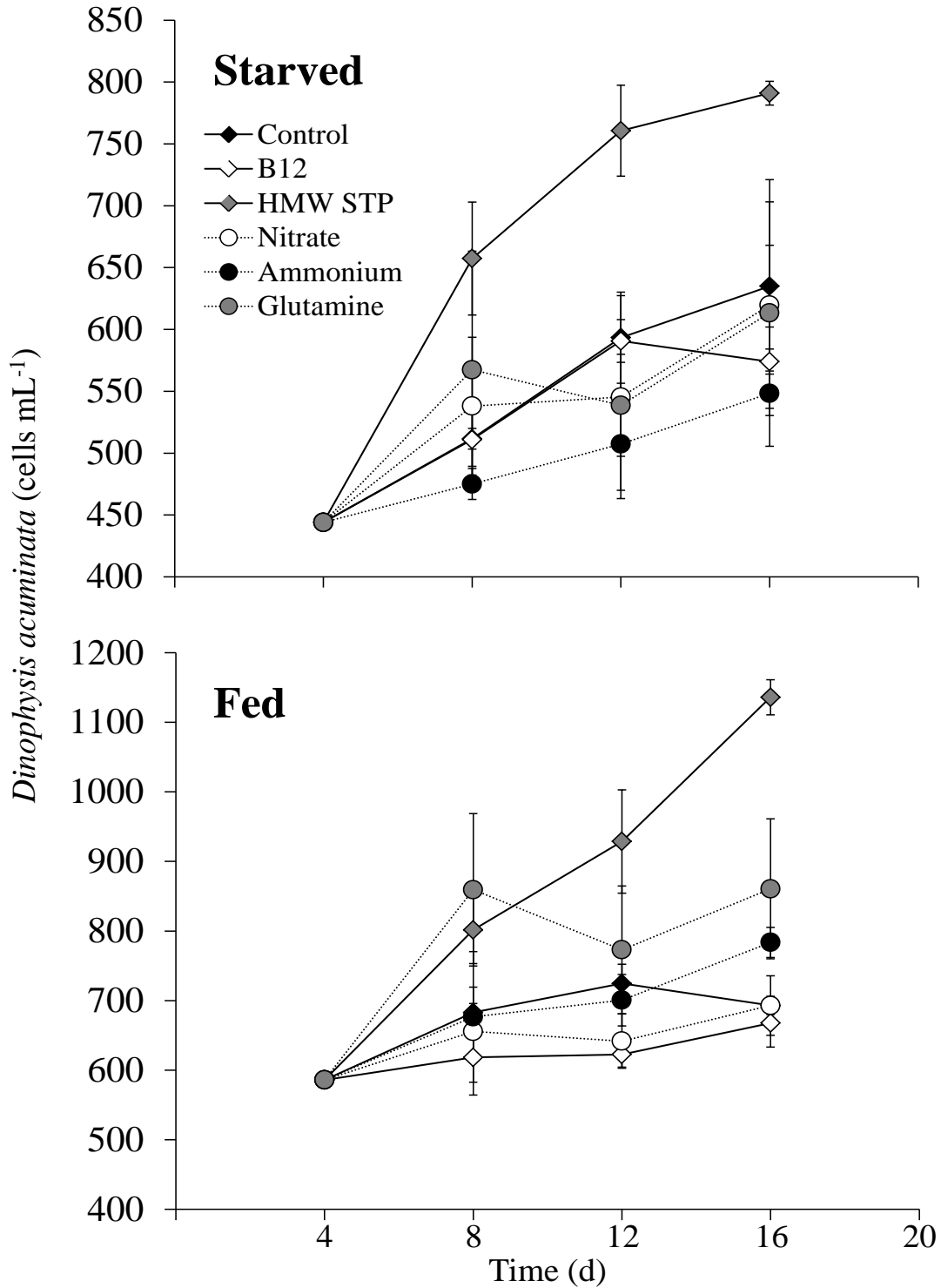
Figure 3. The effects of nutrients on *Dinophysis acuminata* densities (cells mL^{-1}) in cultures that were preconditioned as starved or fed over a 12 day time series. Points are means while error bars represent standard deviation of triplicate measurements. HMW STP= high molecular weight sewage treatment plant effluent.

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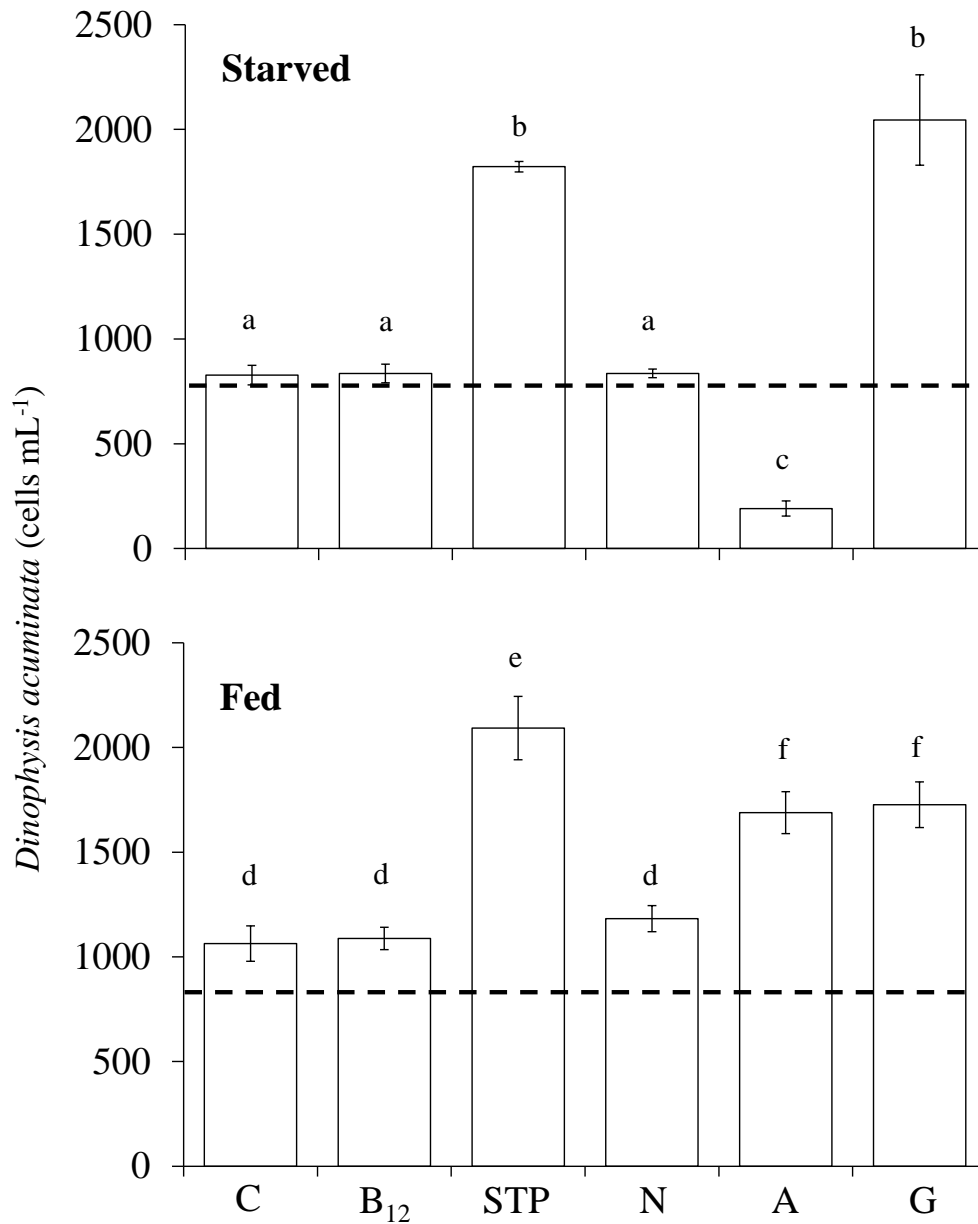
Figure 4. The effects of nutrients on *Dinophysis acuminata* densities (cells mL^{-1}) in cultures that were preconditioned as starved or fed. Bars are means while error bars represent standard deviation of triplicate measurements. The dotted line denotes starting densities for both cultures. Letters indicate Tukey multiple comparisons results (p<0.05). C= Control, B12= vitamin B12, STP= high molecular weight sewage treatment plant effluent, N= Nitrate, A= Ammonium, and G= Glutamine.
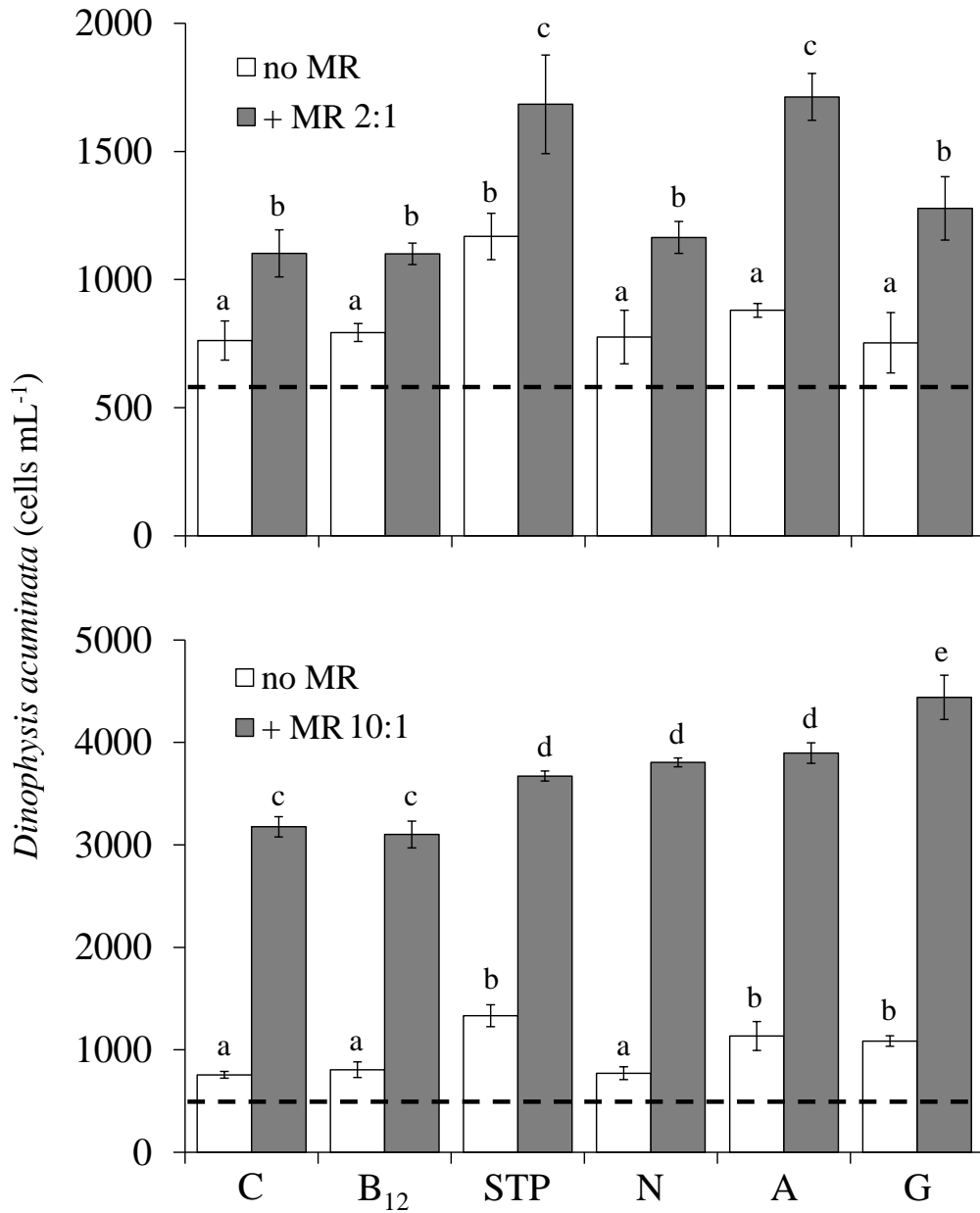
Figure 5. *Dinophysis acuminata* densities (cells mL^{-1}) from experiments comparing the effects of varying *Mesodinium rubrum* feeding ratios (2:1 and 10:1) and nutrients on *D. acuminata* cultures. Bars are means while error bars represent standard deviation of triplicate measurements. The dotted line denotes starting densities for both experiments. Letters indicate Tukey multiple comparisons (p<0.05). Treatments as in Figure 4.
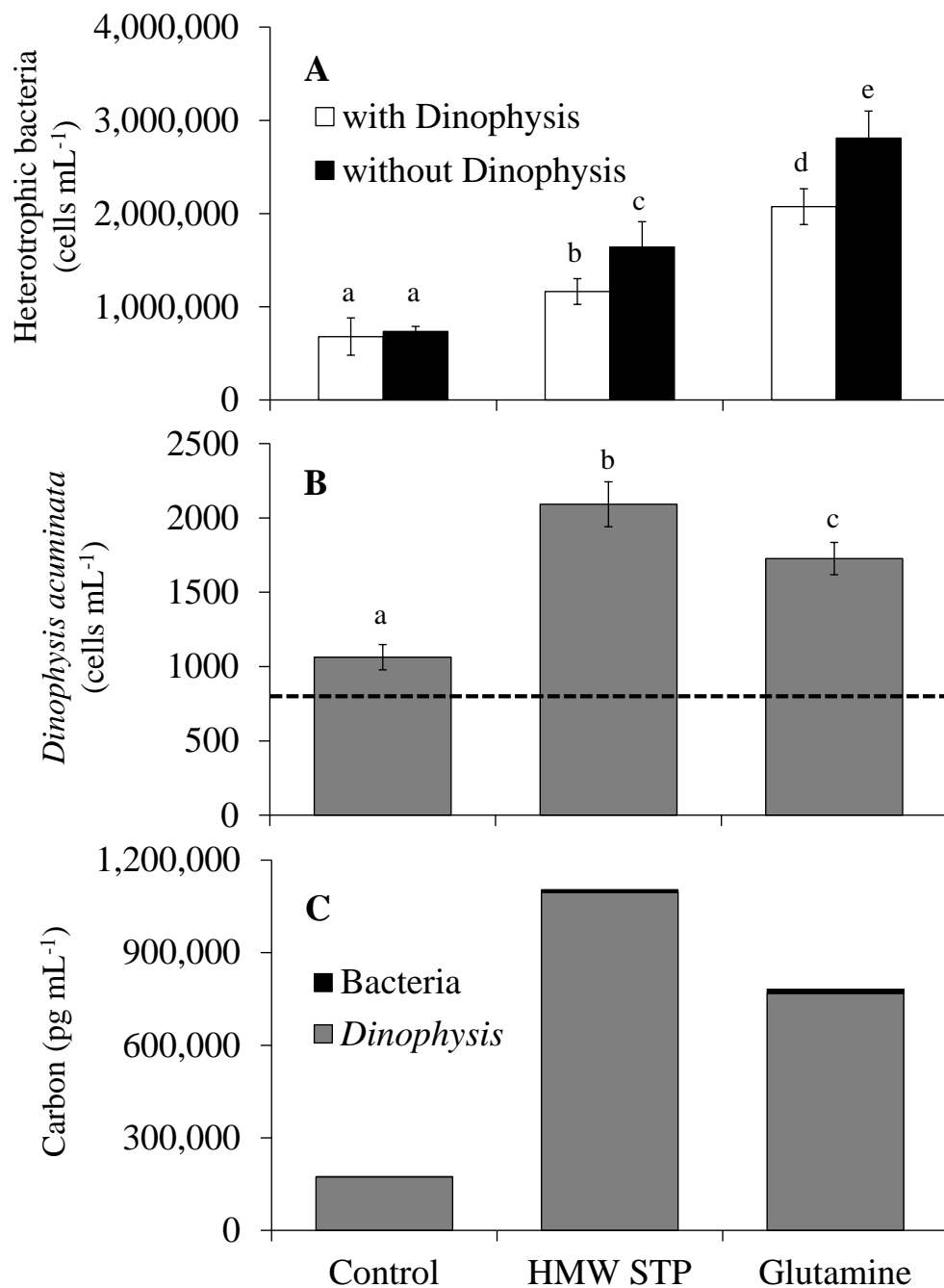
Figure 6. (A) The effects of nutrients and the presence of *D. acuminata* on heterotrophic bacterial densities (cells mL^{-1}). (B) The effects of nutrients on *D. acuminata* densities (cell mL^{-1}). The dotted line denotes starting densities. (C) Carbon-specific biomass (pg C mL^{-1}) of the reduction in heterotrophic bacteria in the presence of *Dinophysis* and increase in *Dinophysis* during experiments. Bars are means while error bars represent standard deviation.

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Table 1. Nutrient concentrations (μM) in experimental controls (stock cultures). Experiments labeled same as in corresponding figures and tables. Data was not available for the time series experiment. DON = dissolved organic nitrogen.

		Nutrients in (μM)		
		Nitrate	Ammonium	DON
Short term	Starved	1.2 (0.3)	0.5 (0.3)	7.1 (0.4)
	Fed	2.5 (0.5)	1.3 (0.8)	8.9 (1.2)
M. rubrum 2:1	Without prey	0.7 (0.1)	2.0 (0.2)	n/a
	With prey	0.5 (0.1)	3.2 (0.3)	n/a
M. rubrum 10:1	Without prey	0.7 (0.1)	3.8 (0.7)	14.0 (1.9)
	With prey	0.3 (0.03)	2.1 (0.3)	16.7 (0.9)

Table 2. Growth rates of *Dinophysis acuminata* from experiments comparing the effects of varying *Mesodinium rubrum* prey:predator ratios and nutrients. Data are means (SD). Asterisks indicate growth rates with *M. rubrum* added that were significantly different from their without prey counterpart (p<0.05, Tukey) whereas those in bold indicate treatments that are significantly different from their respective control.

| | Mean growth rates (μ d^{-1}) | | | |
| | *M. rubrum* 2:1 | | *M. rubrum* 10:1 | |
	Without prey	With prey	Without prey	With prey
Control	0.05 (0.02)	0.10 (0.01)*	0.07 (0.01)	0.31 (0.01)*
B12	0.05 (0.01)	0.10 (0.01)*	0.08 (0.02)	0.30 (0.01)*
HMW STP	**0.12 (0.01)**	**0.17 (0.02)***	**0.16 (0.01)**	0.33 (0.002)*
Nitrate	0.05 (0.02)	0.11 (0.01)*	0.07 (0.01)	**0.34 (0.002)***
Ammonium	0.07 (0.01)	**0.17 (0.01)***	**0.14 (0.02)**	**0.34 (0.004)***
Glutamine	0.04 (0.03)	0.12 (0.02)*	**0.13 (0.01)**	**0.36 (0.01)***

Chapter 7

Dissertation summary and conclusions

The spatial and temporal expansion and increased intensity of HABs is a globally recognized phenomenon (Hallegraeff, 1993; Glibert et al., 2005). Dinoflagellates can cause harmful algal blooms (HABs) associated with human health syndromes such as paralytic shellfish poisoning (PSP) and diarrhetic shellfish poisoning (DSP) and these HABs are a growing human health and economic concern in many coastal regions (Anderson et al., 2008; Anderson et al., 2012). Documenting the factors affecting the growth and maintenance of HABs is of critical importance as this is the time period during which vegetative, toxin-containing cells accumulate in shellfish. My dissertation explored the factors promoting this critical period during toxic dinoflagellate blooms in estuarine ecosystems. Northport Bay, NY, which has experienced chronic PSP-induced shellfish closures due to the toxic dinoflagellate *Alexandrium fundyense* and blooms of the DSP-producer, *Dinophysis acuminata*, was used as a natural system to explore these factors while multiple strains of *A. fundyense* and a newly established *D. acuminata* culture isolated from North America were investigated in controlled, laboratory experiments.

While prior research demonstrated that nutrients and physical factors are important contributing factors to *Alexandrium* blooms (Hattenrath et al., 2010), this dissertation demonstrates the importance of additional chemical factors as well as biological processes (Chapter 2 and 3). The role of allelopathy in bloom formation by the PSP-producing dinoflagellate, *Alexandrium fundyense,* was examined using five strains isolated from across the latitudinal PSP-toxicity gradient found along the North American East Coast. I specifically present bi-algal laboratory experiments, field experiments using cultured *A. fundyense* and natural phytoplankton communities, and the temporal dynamics of plankton assemblages during *A. fundyense* blooms within Northport Bay, NY, USA. Culture experiments, field experiments,

and field observations demonstrated that North American strains of *Alexandrium* were capable of allelopathically inhibiting competing autotrophic nanoflagellates and diatoms. This allelopathic effect was density dependent for both donor (*Alexandrium*) and target species. Further, this effect was not due to saxitoxin production as the NY strain which had one of the lowest per cell toxin content had the largest allelopathic effect, followed by strains from Maine, Canada and Connecticut. Collectively, these findings suggest that allelopathy may play an important role in the maintenance of *Alexandrium* blooms (Chapter 2; Hattenrath-Lehmann and Gobler, 2011). Next, I investigated the effects of coastal acidification on the growth and toxicity of *Alexandrium*, and suggest that allelopathic inhibition contributed to elevated organic matter production, bacterial respiration, and pCO_2 levels during *Alexandrium* blooms (Chapter 3). Chapter 3 demonstrated that levels of pCO_2 recorded in Northport Bay (~1700μatm) were higher than those predicted for the open ocean in the next century and highest in regions with the greatest *Alexandrium* abundances. Additionally, culture and field experiments demonstrated that elevated pCO_2 (~1700μatm) enhanced *Alexandrium* growth rates and, in some cases, toxicity, suggesting that *Alexandrium* blooms may be promoted by coastal ocean acidification.

Over the course of a four-year study, toxic *Dinophysis* blooms exceeding 10^4 cells L^{-1} were observed in New York waters and were positively identified as blooms of *Dinophysis acuminata* using electron microscopy and genetic sequencing (*cox1* gene; Chapter 4). A *Dinophysis acuminata* bloom exceeding one million cells L^{-1} was documented for the first time anywhere during my dissertation, and in several cases DSP toxins in shellfish (1245 ng g^{-1} total OA in shellfish tissue) far exceeded the USFDA action level (160 ng g^{-1} of shellfish tissue; Chapter 4; Hattenrath-Lehmann et al. 2013). Chapter 4 also documented species-specific toxin profiles in shellfish collected from Northport Bay, NY, and emphasizes the importance of

analyzing for esterified toxins which in NY were 74 – 98 % of the total DSP toxins present, all of which is important information for management agencies. In chapter 5, a four-year field investigation of *D. acuminata* blooms revealed that abundances were significantly correlated with ammonium, phosphate and total dissolved phosphorus concentrations and were inversely correlated with nitrate levels. Additionally, nutrient amendment experiments conducted with bloom water over a three-year period demonstrated that inorganic and organic nutrients significantly and consistently enhanced *D. acuminata* densities, and on occasion toxicity. Specifically, ammonium consistently enhanced the growth of *Dinophysis* while glutamine consistently enhanced its toxicity. However, it was unclear whether these higher densities were directly (nutrient stimulation of *Dinophysis*) or indirectly (nutrient stimulation of prey) caused by the nutrients given that during experiments there were also significant correlations with several members of the planktonic community (flagellates and *Mesodinium*). In chapter 6, however, the establishment of a *Dinophysis* culture allowed me to demonstrate that both inorganic (ammonium, nitrate) and organic (glutamine and HMW STP effluent) nutrients were capable of significantly enhancing *Dinophysis* growth and that these enhancements were due to a direct effect (i.e. due to true mixotrophy) rather than being due to an indirect effect (i.e. nutrient use by *M. rubrum* prey and subsequent consumption by *Dinophysis;* i.e. phagotrophy). Hence, this dissertation provides the first evidence that blooms of this ubiquitous, toxic dinoflagellate can be directly promoted by nutrient loading.

New Insights

Collectively, my dissertation research provides new perspective regarding HABs caused by toxic dinoflagellates. Prior to this dissertation almost 40 years of field observations labeled typical *Dinophysis* blooms as low density events ($10^2 > 10^5$ cells L^{-1}) with high density events

being a rarity (Reguera et al., 2012). This dissertation, however, has demonstrated that high density *Dinophysis* blooms are not uncommon in New York with densities exceeding 10^6 cells L^{-1} twice in six years. Moreover, while DSP events are a common occurrence in Europe, South America and Asia (Hallegraeff, 1993; Van Dolah, 2000; Reguera et al., 2012), this dissertation (Hattenrath-Lehmann et al., 2013), along with other recent studies (Campbell et al., 2010; Deeds et al., 2010; Swanson et al., 2010; Trainer et al., 2013), demonstrated that DSP is an emerging issue in the United States. Specifically, data collected during this dissertation (see below) identified multiple instances when shellfish beds in New York should have been closed due to DSP toxins in shellfish exceeding the USFDA regulatory limit. Additionally, while the role of nutrients in the occurrence of *Dinophysis* blooms has been poorly researched and long debated since it is an obligate mixotroph (Reguera et al., 2012) that steals and uses (klepto)plastids from the ciliate, *Mesodinium rubrum* (Minnhagen and Janson, 2006; Minnhagen et al., 2008; Minnhagen et al., 2011), my dissertation research provided the very first unambiguous evidence that blooms of *Dinophysis* are directly promoted by nutrient loading.

Research conducted during this dissertation (Chapter 3) contributes to the mounting evidence that HABs are promoted by ocean acidification (increased pCO_2, decreased pH; reviewed by Fu et al., 2012). Specifically, Chapter 3 demonstrated that the growth, and in some cases, the toxicity, of North American strains of *Alexandrium fundyense* were enhanced by increased pCO_2, overall suggesting that blooms of this dinoflagellate may be promoted by coastal ocean acidification. Additionally, Chapter 3 demonstrated that levels of pCO_2 recorded in Northport Bay (~1700μatm), a eutrophic embayment, were higher than those predicted for the open ocean in the next century (~1000ppm; I.P.C.C., 2007), further substantiating recent research suggesting that eutrophication enhances the acidification of coastal systems (Borges and

Gypens, 2010; Cai et al., 2011; Melzner et al., 2013). This research also provided evidence to suggest that *Alexandrium* can directly or indirectly exacerbate acidification by promoting the production of organic matter (via allelopathy), which can in turn enhance bacterial respiration and pCO_2 levels which may ultimately increase the growth and toxicity of *Alexandrium*. This potential positive feedback system is a previously unrecognized negative impact that HABs can have on an ecosystem and warrants further investigation.

Currently, there is some debate regarding the role of anthropogenic nutrient loading in the occurrence of HABs, with multiple US scientists having put forth the hypothesis that anthropogenic nutrient loading promotes HABs (Anderson et al., 2008; Heisler et al., 2008) and some scientists from the UK arguing that there is not enough evidence to conclusively indicate that anthropogenic nutrient loading is the driving force of the increased incidences of HABs (Davidson et al., 2012; Gowen et al., 2012). The research I have performed during this dissertation (Chapters 3, 5, 6) demonstrates that enhancing nutrient loading can directly and indirectly enhance the growth and toxicity of *Alexandrium* and *Dinophysis*, findings consistent with my prior research on *Alexandrium* blooms in NY estuaries (Hattenrath et al., 2010). Furthermore, later in this chapter I will discuss long term changes in *Alexandrium* and *Dinophysis* densities that further link these events in NY to nutrient loading. As is the case in many scientific debates, there exists data to support multiple hypotheses and with regard to the current issue, it seems important to evaluate HABs on a case-by-case basis, even for a given species. For example, while *Alexandrium* blooms are intense within eutrophied regions of NY estuaries, they can also occur in the Gulf of Maine where nutrient loading rates are lower and physical circulation dominates blooms dynamics. In this case, however, bloom densities are typically several orders of magnitude lower than estuarine blooms, suggesting that perhaps

physical circulation initiates these blooms, but nutrient loads control their intensity. Regardless, some HABs can occur in low nutrient environments while others, such as *Alexandrium* and *Dinophysis* in NY, are promoted by anthropogenic nutrient loading.

Limitations

While there were a series of 'firsts' among my doctoral dissertation findings, this research also had some limitations. Many of my chapters included culture experiments (Chapter 2, 3, 6) in attempts to isolate the effects of these toxic dinoflagellates (*Alexandrium*) on the rest of the planktonic community (Chapter 2), and the effects of chemical factors (pCO_2, nutrients) on the toxic dinoflagellates themselves (*Alexandrium* and *Dinophysis*; Chapter 3, 6). Given the vast differences observed among strains of phytoplankton and the continued need for strain-specific information (Burkholder and Glibert, 2009), for the most part (Chapters 2, 3), culture investigations in my dissertation included multiple geographically distinct isolates of *Alexandrium fundyense*. In light of the discovery of intra-population clonal variability, in both PSP and allelochemical potency, by Tillmann et al. (2009), testing multiple isolates from the same field population would have also been ideal. Intra-population variability was, in part, why I combined all viable isolates (2) of *Dinophysis* for experiments conducted in Chapter 6 and why I used field populations in experiments conducted in Chapter 5. Still, an expanded investigation of intra-population clonal variability in culture studies would have strengthened the robustness of my conclusions.

Regarding my field research, I did not account for vertical differences in abundances of these toxic dinoflagellates due to vertical migration or tides, factors that may affect dinoflagellate abundances and distributions (Lai and Yin, 2014). Constraints such as time, the hours of operation of the dock, sampling equipment, and other logistical restraints prevented me from

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addressing these factors in this study. Fortunately, all of the systems I studied were shallow (1 –

4 m) and mixed with regard to temperature and salinity and all of my sampling was performed at

the same time of day suggesting that any diel vertically migrating population would be sampled

in the same manner during each trip. Still, the vertical distribution of *Alexandrium* and

Dinophysis in NY estuaries remains an open question.

Synthesis of dissertation ecosystem observations

During my doctoral dissertation work, I collected seven years of field data from

numerous systems across Long Island. While some of that data is represented within my

individual chapters, still other data was not utilized but represents a rich resource from which

additional information regarding the PSP-producing blooms of *Alexandrium fundyense* and DSP-

producing blooms of *Dinophysis acuminata* can be gleaned. Northport Bay and Meetinghouse

Creek have hosted annual, high density, toxic blooms of both *Alexandrium fundyense* and

Dinophysis acuminata for more than five years (Fig. 1 and 2). In 2008, the largest *Alexandrium*

bloom ($>10^6$ cells L^{-1}) recorded anywhere was observed in Northport Bay (Hattenrath et al.,

2010) with smaller blooms ($<10^5$ cells L^{-1}) occurring in successive years (Fig. 1). While

Meetinghouse Creek hosted chronic blooms of *Alexandrium*, these were often less dense and

generally shorter in duration than those in Northport Bay (Fig. 1). In 2008, an initial *Dinophysis*

bloom in Northport Bay spurred my continued monitoring of this toxic dinoflagellate, with

typical densities of ~10^4 cells L^{-1} documented in the subsequent years and an unprecedented

bloom in 2011 (10^6 cells L^{-1}; Reguera et al., 2012, Fig. 2). In 2012, the largest observed

Dinophysis bloom occurred in Meetinghouse Creek, superseding the 2011 Northport Bay bloom

(Fig. 2). Contrary to most blooms in Meetinghouse Creek, the 2012 *Dinophysis* bloom lasted for

~2 months, reached >2 million cells L^{-1} and sustained densities over 10^4 cells L^{-1} for ~ 1 month

(Fig. 2). Moreover, a smaller *Dinophysis* bloom (63,000 cells L^{-1}) occurred in the adjacent embayment, Reeves Bay (data not shown), highlighting the capability of these blooms to spread from tributaries that are typically closed to shellfish harvest to areas that are opened to harvest year round.

During my dissertation, I found that shellfish accumulate PSP- and DSP-toxins to levels exceeding USFDA toxin limits, whereas toxic shellfish were not observed 30 years ago (Schrey et al., 1984; Freudenthal and Jijina, 1988). Furthermore, the annual blooms of *Alexandrium* and *Dinophysis* in Northport Bay and Meetinghouse Creek are significantly denser than those observed during comprehensive surveys of Long Island estuaries ~30 years ago (Schrey et al., 1984; Freudenthal and Jijina, 1988). For example, Freudenthal and Jijina (1988) reported on the analysis of nearly 1,500 samples collected from 1971 and 1986 using the same methods I used and they never observed cell densities exceeding 1.3 x 10^4 cells L^{-1}, a threshold surpassed annually today. Concurrently, the human population on Long Island has increased by 500,000 since 1970 (US Census Bureau, 2010) and concentrations of nitrogen in Upper Glacial Aquifer groundwater have increased by 40% since the 1980s (SCDHS, 2010). The ability of nitrogen to directly and indirectly promote the growth of *Alexandrium* and *Dinophysis*, the changes in nitrogen loading from groundwater since the 1980s, and the concurrent human population increase during these past decades, collectively evidence the intensification of HABs due to accelerated anthropogenic nutrient loading.

During this study, blooms of *Alexandrium* and *Dinophysis* occurred both concurrently and in succession (Chapter 4; Hattenrath-Lehmann et al., 2013). Even in earlier sampling years when this was not clearly observed, this was partly due to an abbreviated sampling scheme as *Dinophysis* was not a primary focus of sampling until 2010. The timing of the co-occurrence of

Alexandrium and *Dinophysis* blooms were likely not driven solely by nutrients or temperature as there was no difference in mean nutrient concentrations, nutrient ratios or temperature between years where blooms were concurrent (2008, 2009) versus in succession (2010, 2011, 2012; Table 1), but rather were likely due to a combination of factors. Since *Alexandrium* allelopathically inhibits populations of autotrophic nanoflagellates (Chapter 2; Hattenrath-Lehmann and Gobler, 2011), the concurrence of *Alexandrium* and *Dinophysis* blooms almost seems counterintuitive since cryptophytes, the food source of *Mesodinium*, which is in turn the food source of *Dinophysis*, would be considered part of this population. However, during 2009 when *Alexandrium* and *Dinophysis* peaks co-occurred (Chapter 4; Hattenrath-Lehmann et al., 2013), *Alexandrium* inhibited autotrophic nanoflagellate populations but did not eliminate (~1000 cells mL^{-1}) them from the community (Chapter 2; Hattenrath-Lehmann and Gobler, 2011). Therefore if cryptophytes were part of this population they too may not have been completely eliminated, although species-specific data on cryptophytes to support this theory is not available. An alternative scenario that allows *Dinophysis* and *Alexandrium* blooms to co-occur is the possibility that cryptophytes have already been grazed down by *Mesodinium rubrum* before the *Alexandrium* bloom occurs. I hypothesize that the latter scenario is the case given that *Alexandrium* had no negative effect on *Mesodinium* densities during 2009 (Chapter 4; Hattenrath-Lehmann et al., 2013) and that Myung et al. (2013) demonstrated that *M. rubrum* can grow for ~6 weeks, and its kleptoplastids remain functional for up to 80 days, without the addition of cryptophyte prey. In addition, grazing and/or allelopathy are also likely to contribute to the succession of the plankton from *Alexandrium* to *Dinophysis*. Clearly, the factors contributing to the co-occurrence of these blooms warrant exploration given the lack of knowledge on the additive effects of multiple marine toxins on human health.

Beyond the two New York estuaries, Northport Bay and Meetinghouse Creek, the field work performed during my dissertation also included extensive, multi-year surveys of both *Alexandrium* and *Dinophysis* in waters across Long Island, NY (Fig. 3 and 4). *Alexandrium* was found at 47 of 63 (75%) sites sampled across Long Island (*n*=792; Fig. 3). Additionally, this first large-scale survey to assess the presence of *Dinophysis* since the 1980s (Freudenthal and Jijina, 1988) found that *Dinophysis* was present at almost every station sampled (41 of 42 sites, *n*=622), and 17% of those sites had higher densities than those reported ~30 years ago (13,000 cells L^{-1}; Freudenthal and Jijina, 1988, Fig. 4). Overall, this survey demonstrates that *Alexandrium* and *Dinophysis* are ubiquitous within Long Island coastal waters. Given that my research has demonstrated that enhanced nitrogen loading increases the intensity of blooms of these species, efforts to curb nitrogen discharge to coastal waters may reduce the intensity of these bloom events in the future. Alternatively, processes that increase nutrient loading, such as human population expansion or enhanced agricultural activities, could lead to an intensification of these bloom events in the future.

Over the course of this study, several Long Island locations were closed to shellfish harvest due to the presence of PSP contaminated shellfish (Fig. 5). Since 2006, the Northport-Huntington Bay Complex, specifically, has experienced chronic annual closures (every year except 2007; Fig. 5). During 2011, Shinnecock Bay was closed due to PSP contaminated shellfish for the very first time. In spring 2012, the number of shellfish bed closures expanded from two to four sites (new closures including Mattituck Inlet and Sag Harbor Cove; Fig. 5). Both the Northport-Huntington Bay complex and Shinnecock Bay closures occurred weeks earlier than previous year's closures possibly due to the unusually warm March 2012. My large-scale monitoring program also detected the presence of elevated *Alexandrium* densities in Sag

Harbor Cove which led to the closure of this embayment for the first time (Fig. 5). In sum, more than 13,000 acres of shellfish beds were closed across Suffolk County due to *Alexandrium* blooms and PSP in 2012 (Fig. 5). Clearly, PSP-induced shellfish bed closures due to the presence of *Alexandrium* have become annual events on Long Island.

While there have been several PSP-induced shellfish bed closures during this study, thus far there has yet to be a DSP-induced closure implemented by NYSDEC or any other local agencies. This is due, in part, to this study being the first to demonstrate the need for monitoring of the DSP-producing dinoflagellate, *Dinophysis acuminata*, as cell densities higher than those observed 30 years ago (Freudenthal and Jijina, 1988) are now a frequent occurrence (Fig 4). Given that DSP levels in shellfish found in Northport Bay (Chapter 4; Hattenrath-Lehmann et al., 2013) during the high density (10^6 cells L^{-1}) 2011 *Dinophysis* bloom were above the USFDA action limit (160 ng g^{-1} total OA of shellfish tissue) in areas that are normally open to shellfish harvest, this bay should have been closed to protect human health. Moreover, recent (2012) time series data of blue mussels harvested from Northport Bay indicated that even lower density *Dinophysis* blooms (10^4 cells L^{-1}) are enough to make mussels toxic and close shellfish beds for two months, which is longer than PSP-induced closures (1 month) in this area (data not shown). Additionally, in 2013, blue mussels collected from a normally uncertified area of Cold Spring Harbor during a low density *Dinophysis* bloom (10^4 cells L^{-1}) were also above the USFDA action level (data not shown). These instances demonstrate the clear need for *Dinophysis*-specific monitoring programs in local waters, especially given that it is now evident that low density blooms also cause elevated shellfish toxicity. Data presented in Chapter 4 (Hattenrath-Lehmann et al., 2013) found that shellfish differentially accumulate DSP toxins and that the accumulation of DSP toxins in blue mussels differs from other native shellfish including *Mya arenaria* and

Geukensia demissa, suggesting blue mussels may not be an ideal indicator species to represent DSP toxicity. More information on the accumulation of DSP toxins in other native shellfish species is necessary to make informed decisions about shellfish closures. From the data gathered thus far, I would strongly recommend shellfish species-specific closures to minimize the strain of closures on local economies.

Climate change is expected in increase global temperatures by up to 4°C this century (I.P.C.C., 2007) and the year 2012 could be considered representative of what global warming may bring in the future. March 2012 was the warmest March on record dating to the 1800s and temperatures during March through June consistently were above average. Concurrently, *Alexandrium* and *Dinophysis* blooms in Northport Bay and Meetinghouse Creek both started, peaked, and declined weeks earlier than observed in prior years. Warmer temperatures occurring earlier (March) in the 2012 season may have played an important role in the early germination of *Alexandrium* cysts across Long Island (Hattenrath et al., 2010). In turn, I hypothesize that the establishment of a vegetative population earlier than normal gave *Alexandrium* more time to grow and outcompete (allelopathically or otherwise) other phytoplankton that may not normally co-occur with this alga, thus causing 13,000 acres of PSP-induced closures around the island. However, given that *Alexandrium* in this system cannot withstand temperatures above 21°C (Hattenrath et al., 2010) these blooms also ended earlier than normal. Future warming will be accompanied by higher pCO_2 levels (Gruber, 2011) that will intensify acidification in Northport Bay currently caused by eutrophication (Chapter 3) and thus may intensify *Alexandrium* blooms due to increased pCO_2 within its optimal temperature window. Regarding *Dinophysis*, the most intense and extensive bloom ever observed occurred in 2012 in Meetinghouse Creek while the *Dinophysis* bloom in Northport started earlier in 2012 than any other year studied and was the

second most intense bloom ever observed there. Hence, temperature seems to be a very important factor for regulating both of these blooms, a conclusion supported by an iterative statistical analysis of all environmental parameters (BEST analysis within Primer 6.0) which found that temperature explained 58% of community composition patterns. Therefore, I hypothesize that global warming may intensify *Alexandrium* blooms due to increases in pCO_2 (see above) while also yielding a seasonal shift in *Alexandrium* and *Dinophysis* blooms causing them to bloom earlier than they do currently.

While this dissertation and prior work (Hattenrath et al., 2010) clearly demonstrated that nutrients are important drivers of these dinoflagellate blooms, specifically in Northport Bay, these blooms are also linked to a multitude of other complex factors. Given that Northport Bay is a eutrophic embayment with nutrient contributions from multiple effluent (i.e. sewage treatment plant) and non-point sources (e.g. septic systems), the interannual variations in bloom intensity and duration over the seven years studied are likely due to a combination of additional factors. As mentioned above, temperature, for example, is an important regulator of the initiation, growth and demise of *Alexandrium* blooms (Hattenrath et al., 2010). Additionally, while the above mentioned comprehensive surveys of these two toxic dinoflagellates found that most of the embayments on Long Island host populations of these dinoflagellates, only a few experience high density blooms, an occurrence likely influenced by the residence time of individual systems. A recent study (Stinnette, 2014) demonstrated that *Alexandrium* abundances within Long Island's south shore estuaries were significantly correlated with residence times of and total nitrogen loads to Moriches, Shinnecock, and Quantuck Bay. While there are no published residence times for Northport Bay, information gathered from this study, specifically Chapter 3, suggests that the residence time is likely long given the salinity gradient and net

heterotrophic nature of the system. Overall, an assessment of residence times of bays across Long Island is needed to quantitatively assess the extent to which flushing modulates the intensity of HABs.

Future directions

The findings of this dissertation provide promising new directions for research on toxic dinoflagellates. As discussed in Chapter 6, beyond prey concentrations, the growth and ingestion rates of *Dinophysis* spp. may also depend on a combination of species/strain specific differences among *Dinophysis* and their prey items (*Mesodinium rubrum* and cryptophytes). To date, it has been demonstrated that *M. rubrum* can sequester and use chloroplasts from cryptophytes of the clade *Teleaulax*/*Plagioselmis*/*Geminigera* (Park et al., 2007; Hansen et al., 2012) and thus far two species within this clade have been used in the three-step *Dinophysis* culturing process (*Teleaulax* and *Geminigera*), which may contribute toward differences seen among culture studies reviewed here. Recently, Tong et al. (2013) presented data demonstrating that larger (~3 times) *M. rubrum* cells from Japan yielded significantly higher *D. acuminata* densities than smaller *M. rubrum* cells from Spain. While it is possible that this could be due to differences in nutritional quality among *M. rubrum* strains, the authors concluded that *M. rubrum* size accounted for *D. acuminata* growth differences. Extrapolating from the work of Tong et al. (2013), the *D. acuminata* strain isolated from NY waters may similarly experience faster growth rates if fed a larger *M. rubrum* isolate from NY rather than the smaller Denmark isolate (~four-fold smaller; personal observation). Tong et al. (2013)'s work brings about several important questions: Is prey (*M. rubrum*) size a function of the number of plastids (i.e. larger prey= more plastids) it retains and subsequently passes on to *Dinophysis*? Are certain strains of *M. rubrum* able to better utilize prey plastids compared to others thereby passing along "healthier" plastids

to *Dinophysis*? Further, given the relative lack of information on the subject (Wilkerson and Grunseich, 1990; Crawford et al., 1997; Herfort et al., 2012), another important question is: what is the role of nutrients in *M. rubrum* dynamics and how does this subsequently effect *Dinophysis* populations and bloom formation? Finally and importantly, there is very little known about *Dinophysis* in North America. Recent investigations on isolates from this region are not ecologically realistic given the temperatures at which they are cultured (4-10°C) are not within the range of those found during DSP-producing blooms (13-24°C in NY; Chapter 5) because the prey used are from polar latitudes (Hackett et al., 2009; Tong et al., 2010; Fux et al., 2011; Tong et al., 2011; Smith et al., 2012). Therefore a reassessment of *Dinophysis* strains from North America is needed using locally established (temperate) prey isolates to better constrain the effects of multiple factors, such as prey concentration, size and light on *D. acuminata* growth and toxicity.

Given that *Dinophysis* cultures were only recently established (Park et al., 2006) there remains a dearth of information in several areas of *Dinophysis* ecology. While this dissertation (Chapter 5 and 6) was the first in depth study on the role of nutrients in *Dinophysis* growth, additional studies of other species and strains of *Dinophysis* are needed to better understand the nutritional ecology of this dinoflagellate. Of particular importance would be establishing nitrogen uptake rates and growth kinetics (various nutrients at various concentrations), as this has only been established, in part, for a *D. acuminata* dominated community from a ^{15}N tracer experiment (Seeyave et al., 2009). Additionally, establishing the relationship between *Dinophysis* and bacteria and potential links to requirements for B-vitamins may help explain the prevalence of these blooms in areas influenced by sewage effluent (Northport and Meetinghouse Creek) as B-vitamins may be enriched in these bacterial laden waters. More specifically,

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screening *D. acuminata* for genes encoding proteins that required B-vitamins to be synthesized could help further resolve this association. Another important area of study that is currently lacking (Reguera et al., 2012) is cyst formation by *Dinophysis*, a field of study that may quickly advance given the recent establishment of cultures. Research to date has concluded *Dinophysis* does not form cysts in the field or in culture, but has demonstrated the ability of *Dinophysis* cultures to produced small cells and planozygotes that can revert into large cells (Escalera and Reguera, 2008; Reguera et al., 2012). Since my observations demonstrate that *Dinophysis* blooms are seasonal (spring/summer) and do not occur in succession to *Mesodinium* blooms that occur in the fall, I hypothesize that this dinoflagellate either produces cysts or has some other dormant form (perhaps, small cells) during the fall/winter months. Equally as important are investigations of zooplankton grazing on *Dinophysis* and of competitive interactions (allelopathy, nutrient competition, etc.) between *Dinophysis* and other plankton, as these processes ultimately affect bloom density, shellfish toxicity, and, most importantly, human health.

Collectively, this dissertation combined with my Master's Thesis (Hattenrath et al., 2010) demonstrates that multiple biological, chemical and physical factors can act and interact to promote dinoflagellate growth and toxicity which, in turn, can affect human health and local economies. These findings provide some of the information needed to develop management practices that mitigate blooms and/or protect human health. However, from data presented in this dissertation it is clear that management strategies should not only be species specific, as there are important differences between the ecology of different HABs, but also location specific as the physical characteristics of each embayment (i.e. flushing time) can be an important driver of bloom intensity, and thus shellfish toxicity. While factors such as competition, nutrients, and

acidification were investigated here, additional factors that strongly affect dinoflagellate blooms obviously warrant future exploration.

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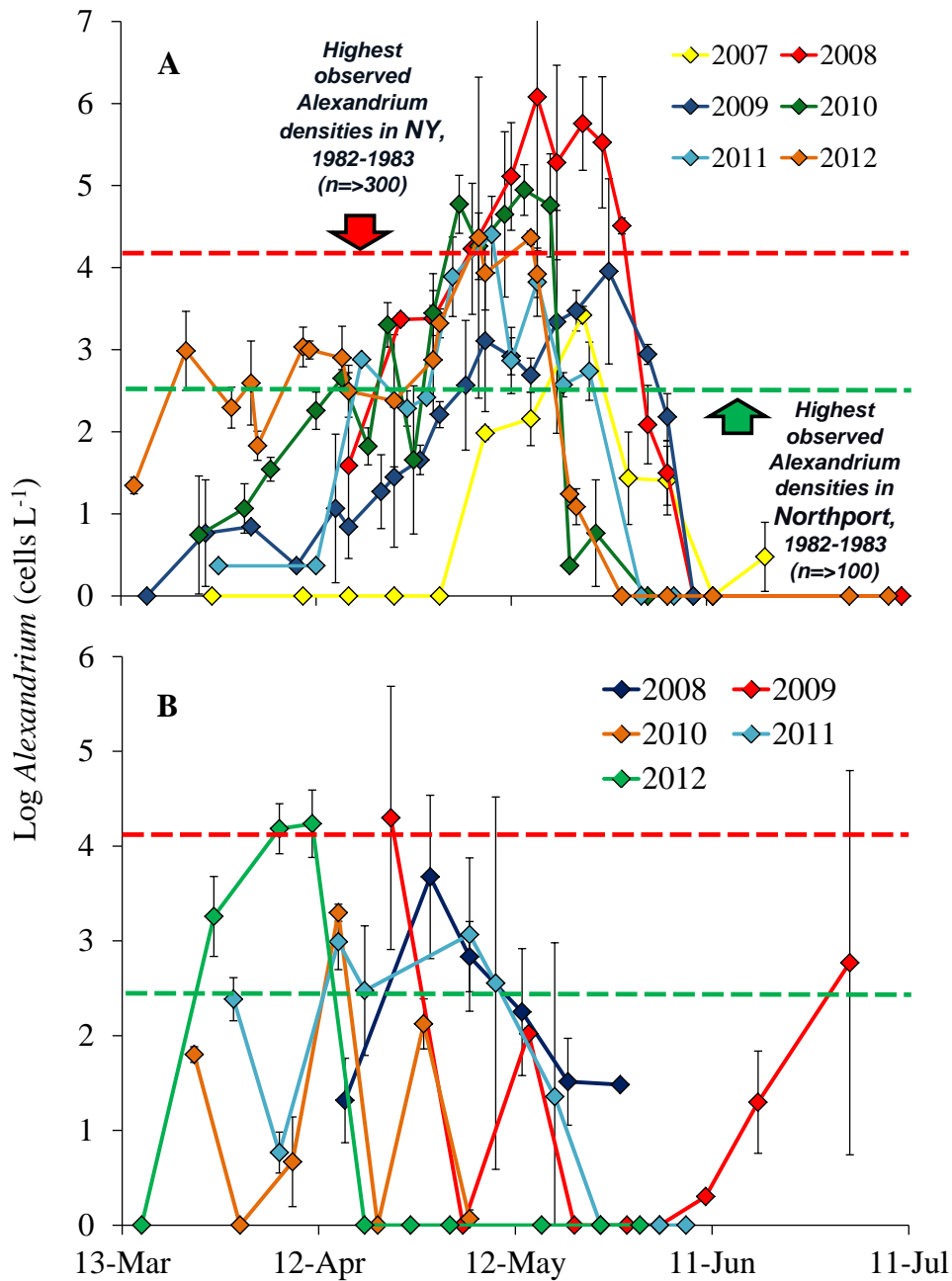
Figure 1. A) *Alexandrium fundyense* (cells L^{-1}) from 2007 to 2012 in Northport Bay, NY. B) *Alexandrium fundyense* (cells L^{-1}) from 2008 to 2012 in Meetinghouse Creek, NY. The red dotted line indicates the highest observed *Alexandrium* densities measured in multiple sites across Long Island, NY in the early 1980s, while the green dotted line represents *Alexandrium* densities measured specifically in Northport Bay, NY.
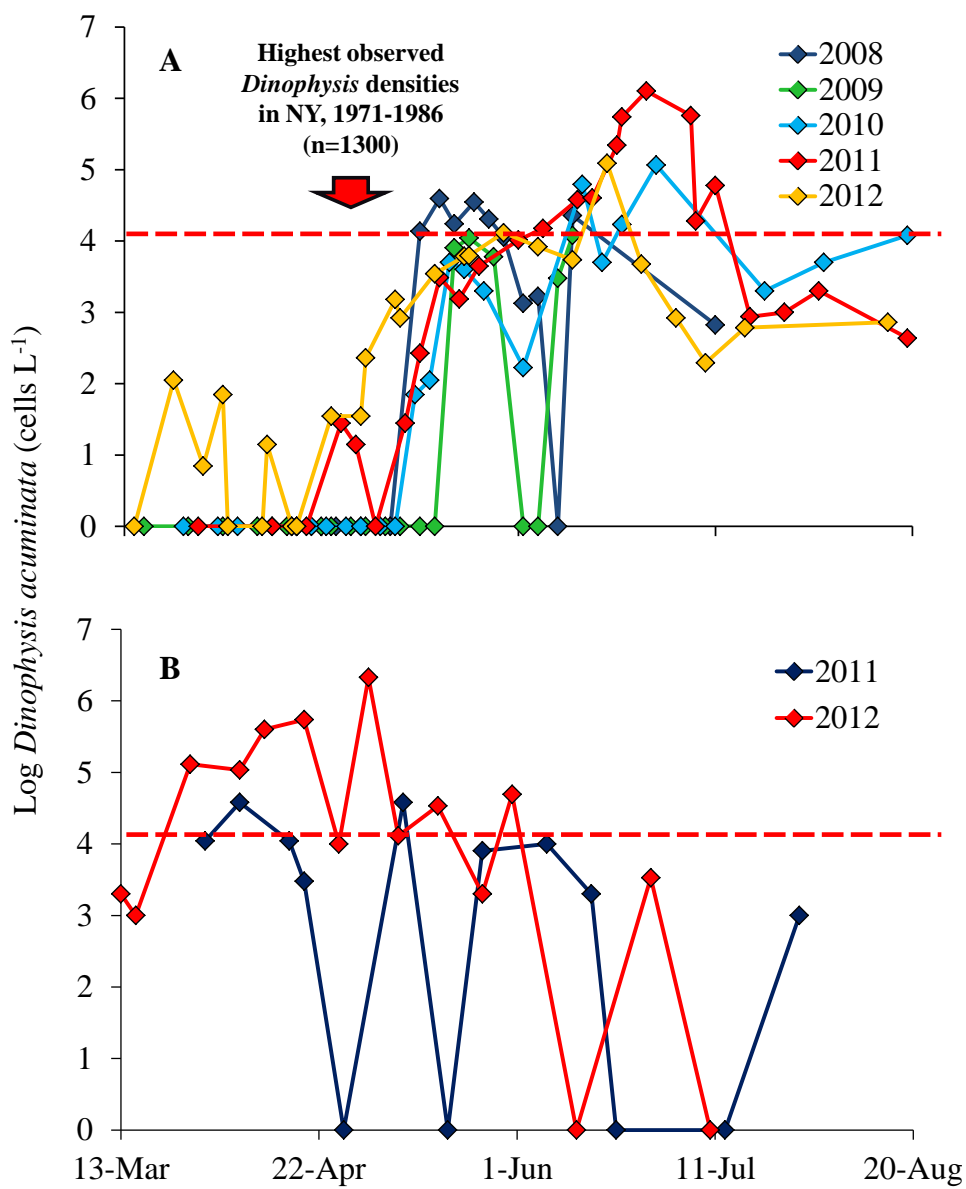
Figure 2. A) *Dinophysis acuminata* (cells L^{-1}) from 2008 to 2012 in Northport Bay, NY. B) *Dinophysis acuminata* (cells L^{-1}) from 2011 to 2012 in Meetinghouse Creek, NY. The red dotted line indicates the highest observed *Dinophysis* densities measured in multiple sites across Long Island, NY during the 1970s and 80s.
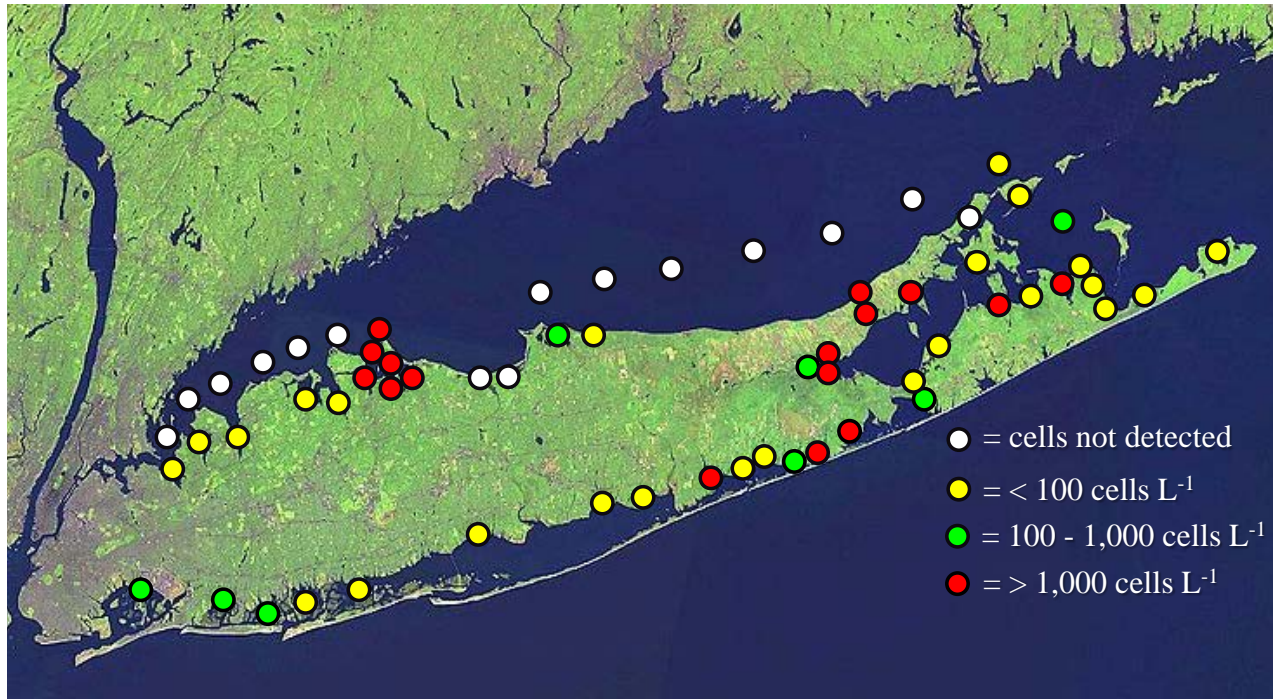
Figure 3. The distribution of PSP-producing *Alexandrium fundyense* in Long Island embayments.   Circles indicate the highest observed densities of *Alexandrium* (cells L^{-1}) found at each site during 2007 – 2013 (n= 792).
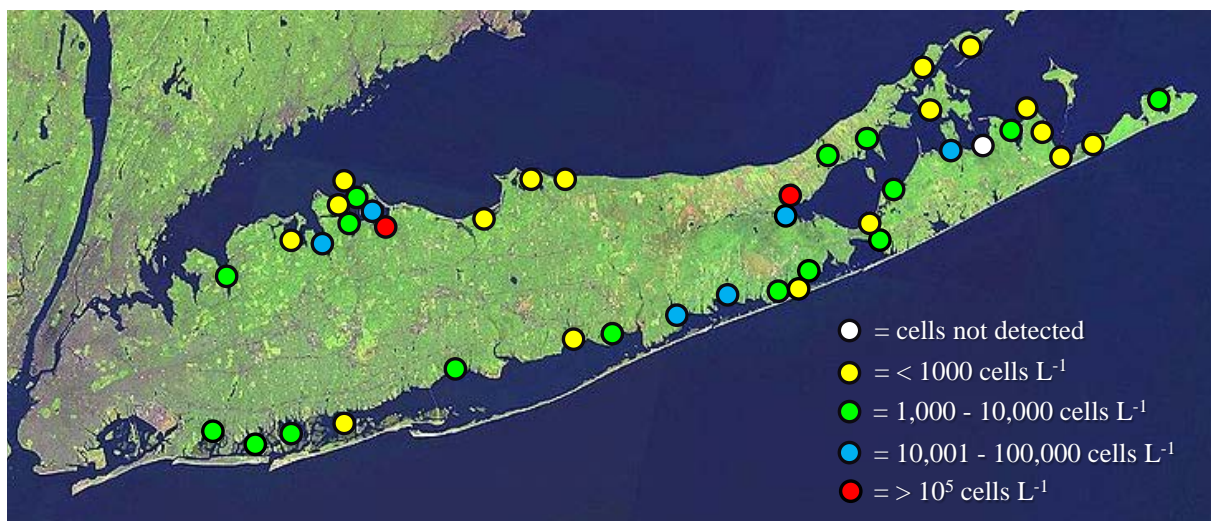
Figure 4. The distribution of DSP-producing *Dinophysis acuminata* in Long Island embayments. Circles indicate the highest observed densities of *Dinophysis* (cells L^{-1}) found at each site during 2008 – 2013 (n=622).
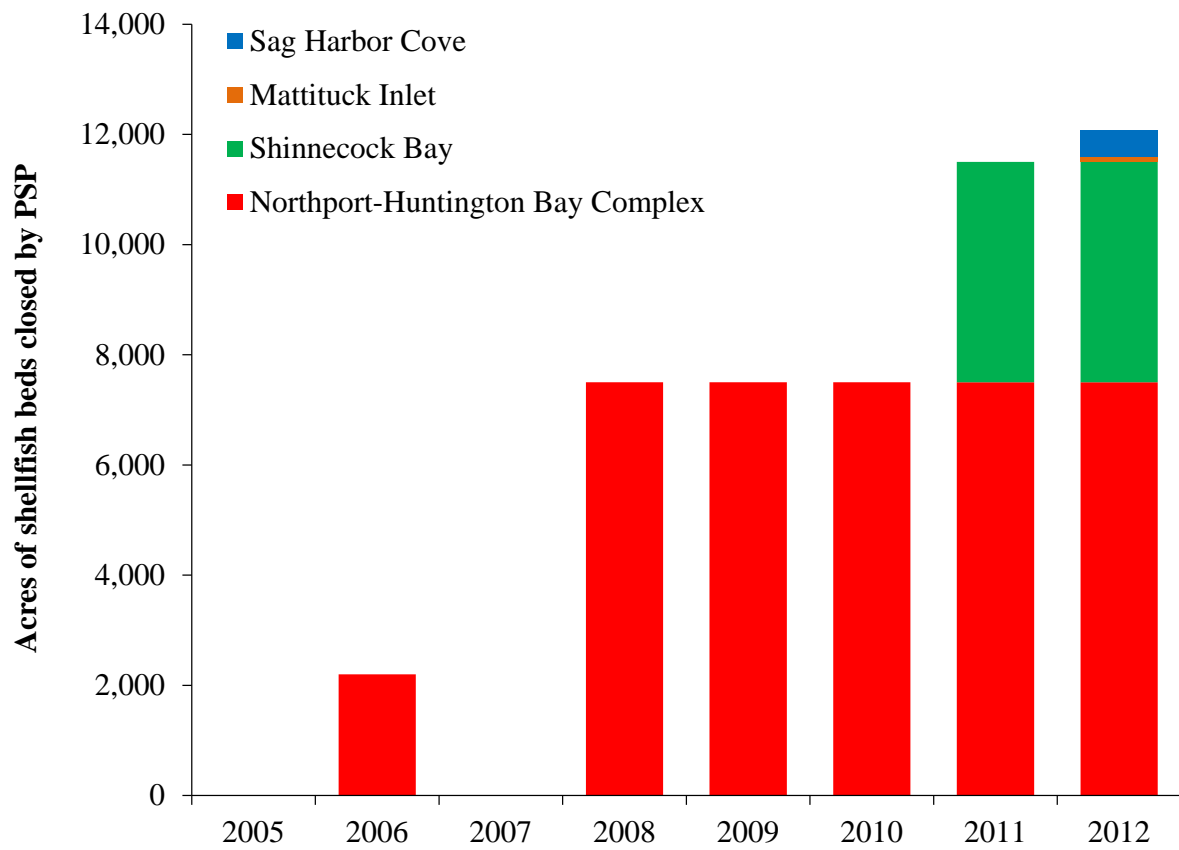
Figure 5. The expansion of PSP- induced shellfish bed closures (in acres) on Long Island from 2005 – 2012.

Table 1. Inorganic nutrients (ammonium, nitrate, phosphate, silicate; μM), total dissolved and organic nutrients (μM) as well as nutrient ratios found when both *Alexandrium* and *Dinophysis* are present in the water column in Northport Bay, NY from 2008 – 2012. Samples were averaged across the respective inclusive dates with standard errors indicated in parentheses.

	2008	2009	2010	2011	2012
Inclusive dates	5/12 - 6/5	5/19 - 6/5	5/11 - 5/25	4/26 - 5/24	3/23 - 5/22
Ammonium (μM)	2.05 (1.05)	8.00 (1.84)	5.17 (1.50)	3.61 (1.39)	2.39 (0.57)
Nitrate (μM)	4.44 (1.63)	15.1 (2.59)	26.7 (3.72)	14.5 (2.16)	17.8 (3.72)
Phosphate (μM)	0.50 (0.09)	0.52 (0.11)	0.61 (0.11)	0.91 (0.19)	0.77 (0.11)
Silicate (μM)	21.1 (3.17)	23.7 (1.08)	33.9 (2.34)	22.8 (3.38)	27.5 (4.02)
TDN (μM)	23.5 (2.96)	36.2 (2.69)	42.8 (7.78)	34.8 (3.55)	41.0 (3.47)
DON (μM)	17.0 (0.93)	13.1 (2.85)	10.9 (3.29)	16.7 (0.98)	20.8 (1.39)
TDP (μM)	0.97 (0.16)	1.61 (0.12)	1.34 (0.25)	1.79 (0.19)	1.27 (0.09)
DOP (μM)	0.47 (0.14)	1.09 (0.06)	0.73 (0.27)	0.87 (0.09)	0.50 (0.06)
N:P	11.7 (2.89)	51.9 (13.9)	55.6 (5.45)	26.5 (7.34)	31.1 (6.22)
N:Si	0.28 (0.08)	0.99 (0.19)	0.92 (0.09)	0.81 (0.09)	0.75 (0.07)
DON:DOP	54.9 (11.1)	12.2 (2.82)	38.5 (22.8)	20.5 (2.28)	47.8 (7.75)
DON:Si	0.96 (0.18)	0.55 (0.12)	0.31 (0.09)	0.87 (0.14)	0.91 (0.13)
TDN: TDP	27.2 (3.58)	22.9 (2.43)	34.8 (7.93)	20.0 (1.25)	33.1 (2.87)
TDN:Si	1.25 (0.17)	1.54 (0.12)	1.23 (0.17)	1.68 (0.18)	1.66 (0.17)
temperature	16.6 (1.18)	18.4 (0.84)	15.6 (1.03)	14.9 (0.67)	13.1 (0.69)

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