

NITROGEN ISOTOPE FRACTIONATION AND TOXIN PRODUCTION DURING THE
UPTAKE OF MICROMOLAR CONCENTRATIONS OF NITRATE, AMMONIUM, AND
UREA BY A MARINE DINOFLAGELLATE

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ABSTRACT

Despite an increased global interest in harmful algal bloom (HAB) species and eutrophication, the relationship between nutrient sources and changes in species composition or toxicity remains unclear. Stable isotopes are routinely used to identify and track nitrogen (N) sources to water bodies, as sources can be differentiated based on stable isotope values. While literature is available describing N fractionation by diatoms and coccolithophores, data are lacking regarding isotope fractionation by dinoflagellates. Here we investigate the fractionation of N isotopes by saxitoxin-producing *Alexandrium fundyense*, to validate the use of the $\delta^{15}\text{N}$ of particulate organic matter and identify the N source fueling a dinoflagellate bloom and its toxicity. The effects of N chemical form on isotope fractionation (ϵ), toxin content, and toxicity, were investigated using isolates in single-N and mixed-N experiments. Growth on NO_3^- , NH_4^+ , or urea, resulted in isotope fractionation (ϵ) of $2.76 \pm 1.48\%$, $29.01 \pm 9.32\%$, or $0.34 \pm 0.19\%$, respectively, with the lowest cellular toxicity and toxin quotas reported during urea utilization. Toxin composition and growth rates, however, remained constant across all N treatments, showing no effects of NO_3^- , NH_4^+ , or urea utilization. *Alexandrium fundyense* was then preconditioned to either NO_3^- , NH_4^+ , or urea, and abruptly inoculated into mixed-N medium containing all three chemical forms. All treatments initially utilized NH_4^+ and urea upon inoculation into mixed medium, suggesting no effect of preconditioning. Cells only began utilizing NO_3^- after NH_4^+ decreased below 2-4 μM in the medium. During the inhibition of NO_3^- uptake by NH_4^+ utilization, the cellular $\delta^{15}\text{N}$ was at its lowest (-5‰), and through the course of the experiment, the $\delta^{15}\text{N}$ continuously changed to mimic the isotope value of the most recent N source(s) being utilized. When utilizing multiple sources, the isotope signature of the cells fell between the signal of the two N sources. Together this suggests that in NO_3^- and urea rich environments, the $\delta^{15}\text{N}_{\text{POM}}$ would reliably look like the source or sources of N utilized, but that

caution should be taken in NH_4^+ rich environments where the large ε value could lead to misinterpretation of the signal. Nutrients are only one factor influencing bloom dynamics, but information about the relative importance of natural or anthropogenic nutrients in the development and toxicity of bloom events is necessary to predict future shifts in phytoplankton species composition, density, and toxicity.

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INTRODUCTION

NUTRIENTS AND HARMFUL ALGAL BLOOMS

Microscopic algae are the base of marine food webs and play an important role in the cycling of nutrients in coastal habitats. Yet some algae produce endogenous toxins or cause harm to the environment through their sheer biomass, e.g., through low food quality, interference to grazing, or low dissolved oxygen during decomposition (Glibert et al. 2005). Harmful algal blooms (HABs) have increased in frequency, intensity, and geographical range over the past 40 years, creating dire consequences for public health, aquatic ecosystems, fishery services, and local economies (Anderson et al. 2008). Coastline development has been identified as a contributor to this deterioration of ecosystem health through its connection to increased nutrient loading via groundwater flow, sewage effluent, atmospheric deposition, aquaculture, and agricultural runoff (Anderson et al. 2002; Backer & McGillicuddy 2006).

In the marine environment, biogeochemical cycling typically leads to surface waters depleted of bioavailable, inorganic N and an increase in concentration with depth (Dagenais-Bellefeuille and Morse 2013). Coastal algal blooms, therefore, rely on the upwelling of nutrients from the deep waters, adaptations like vertical migration and co-occurrence with N-fixing bacteria, or nutrients from atmospheric and riverine inputs. These processes are exacerbated in nearshore areas, where human activity has led to accelerated nutrient input from point sources, e.g., sewage overflows, and/or non-point sources, e.g. agricultural runoff (Dagenais-Bellefeuille and Morse 2013). The success of some nearshore blooms in embayments, estuaries, and sounds have been linked to the type (point vs nonpoint sources) and concentration of anthropogenic nutrients. In particular, this relationship has been defined in sites with reduced mixing and flushing rates, where nutrients accumulate and are readily available for algal growth (Anderson et al. 2002; Anderson et al. 2008).

The influx of anthropogenic nutrients can affect estuarine and marine phytoplankton species by altering both the chemical composition of the nutrients (e.g. organic vs inorganic, NO_3^- vs NH_4^+) and the concentration of the nutrients available for growth. For instance, striped bass aquaculture ponds on the Maokin River, a tributary of Chesapeake Bay, have disproportionately altered phytoplankton succession (Glibert and Terlizzi 1999). Finfish aquaculture facilities tend to cause high nutrient concentrations from routine fertilizer and feed additions, which can lead to large algal blooms of both favorable and harmful species (Hargreaves 1998). Glibert and Terlizzi (1999) sampled near the aquaculture ponds to investigate a correlation between nutrient type and HAB occurrence. Although the NO_3^- and NH_4^+ concentrations exceeded $50 \mu\text{M}$ on several occasions and chlorophyll α remained higher than $100 \mu\text{g liter}^{-1}$ during the summer sampling period, it was when urea concentrations were above $1.5 \mu\text{M-N}$ that dinoflagellate blooms were observed (Glibert & Terlizzi 1999). In a more recent example, Xu et al. (2017) documented a correlation between anthropogenic phosphorous and nitrogen and annual mean chlorophyll a levels, identifying a link between a rise in cyanobacterial blooms and eutrophication in Lake Taihu, China between 1992-2012. Ultimately, these case studies demonstrate how nutrient anomalies can alter toxin production and community structure by selecting for algal species that are capable of capitalizing on the chemical form (Anderson et al. 2008). The link between anthropogenic nutrient loading and HABs, however, is site- and organism- specific (Davidson et al. 2014). Reliable prediction of HAB formation in complex marine systems, based on nutrient types and concentrations, remains a challenge (Anderson et al. 2002). Nutrients are only one factor influencing bloom dynamics, but the relative importance of natural or anthropogenic nutrients in the development of a specific toxic bloom is necessary to predict future shifts in phytoplankton species composition and toxicity.

SAXITOXINS AND PSP

Many HAB species are capable of producing bioactive compounds that can pose human health threats through exposure via drinking water, occupational or recreational contact, bioaccumulation in seafood products, and/or aerosolization during wave action (Carmichael 2001; Backer and McGillicuddy 2006). One of the most prominent HAB-associated syndromes worldwide is paralytic shellfish poisoning (PSP), caused by a group of potent algal neurotoxins called saxitoxins (STXs, Figure 1). Saxitoxins are produced by species of freshwater, prokaryotic cyanobacteria and marine eukaryotes predominately from three genera of dinoflagellates: *Alexandrium*, *Gymnodinium* and *Pyrodinium*. Exposure to STXs typically occurs through the human consumption of filter-feeding organisms like mussels, clams and oysters, which have previously accumulated the toxins or algae in their edible tissue. Although initially documented in temperate regions of North America, Japan, and Europe, STX-producing marine dinoflagellates can now be found throughout the Pacific and Atlantic coasts (Figure 2; Anderson 1989; Lilly et al. 2007; Anderson et al. 2012). This perceived expansion in geographical range is likely due to increased monitoring and detection of the species and toxin, transportation in ballast water and shellfish seeding, immigration with increased sea surface temperatures and water current, and an increase in nutrient loading with coastal development (Anderson et al. 2008). The abundance, frequency of occurrence, and diversity of STX-producing species over a variety of habitats and regions, emphasizes the seriousness of PSP as a human health concern.

Saxitoxins, including the parent molecule (saxitoxin) and its 57 congeners, are a group of small molecules with a molecular weight of 280-450 Daltons, which have the same tricyclic core, but differ structurally at four side group positions (Figure 1). The four positions may be hydroxylated, sulfated, or carbamoylated, affecting the chemical properties and potency of the

compound (EFSA 2009). A single algal cell can contain have multiple STX congeners, comprising the strain's toxin profile (i.e., percent composition of congeners). The most common paralytic shellfish toxins can be classified into three major groups at a neutral pH of 7: saxitoxin (STX) with a net charge of +2, gonyautoxins (GTXs) with a net charge of +1, and C-toxins with a net charge of zero (Rossini 2014). Saxitoxin, the non-sulfonated congener with a molecular formula of $C_{10}H_{17}N_7O_4$, falls into the most toxic carbamate group, followed by the mono-sulfonated, GTX and C-toxins. The toxins are relatively heat stable but highly sensitive to strong alkali, having different rates of thermal degradation and temperature thresholds based on the congener. For instance, when exposed to a low pH (3 to 4), C toxins decline rapidly while STXs increase, and GTX 1/4 toxins degrade at a high temperature and elevated pH (Figure 3) (Indrasena and Gill 2000).

This variability in the chemical properties between STX congeners leads to selective retention and biotransformation during bivalve metabolism. Samsur et al. (2006) found that the toxin profile of the producer, *Alexandrium catenella*, changed immediately upon filter-feeding by short-necked clams, and that saxitoxins continued to biotransform until only 1% of the supplied toxin was detectable at 168h (Samsur et al. 2006). When attempting to detoxify toxin-laden bivalve meat via heat (e.g., by steaming, boiling, or canning), the least toxic congeners biotransform to create a more toxic product before the toxins eventually degrade after ~180 min at 100°C (Nagashima et al. 1991). This poses a serious risk for shellfish industries who have no effective strategy to treat STX-contaminated shellfish. The biotransformation of toxins and the variation in metabolic and detoxification rates between commercially harvested species, leads to the need for different monitoring and management strategies of PSP producing algal blooms between regions (Bricelj & Shumway 1998).

When ingested by humans, STXs are absorbed by the gastrointestinal tract and transferred through the mucous membranes. In the membranes of the neurons and muscle cells, the compounds bind to the neurotoxin receptor site 1 of the sodium channels (Anderson & Wexler 2005). Instead of altering the kinetics of the gates, the toxin physically lodges itself in the ion conducting pores, prohibiting synaptic transmission and reducing nerve conduction velocities. In addition to being a highly potent blocker of sodium channels, STXs have been documented to block calcium channels and prolong the gating of potassium channels in heart muscle cells (Henderson et al. 1973; Wang et al. 2003; Su et al. 2004). The clinical effects of STX develop almost immediately, around 30 minutes to three hours after consumption of the toxin. The first symptoms are tingling and numbness of the lips and face, followed by gastrointestinal distress and muscle paralysis. At an ingested dose of around 9-10 mg toxin·kg⁻¹ body weight, the biotoxin causes death by respiratory failure and cardiovascular shock (Anderson & Wexler 2005). There is no known antidote for PSP, but treatment includes administering fluids and artificial respiration until toxins are excreted.

Saxitoxins are thus actively monitored and regulated in seafood by shellfish and phytoplankton monitoring programs, human health officials, and resource managers as a mechanism for protecting human health and seafood safety. Since the 1930's, a regulatory limit of 80 µg toxin·100 g⁻¹ edible shellfish meat has been recognized by most countries (Wekell et al. 2004). Even with the regulatory limit reflecting a 10-fold uncertainty factor, more susceptible populations such as the elderly, children under the age of 20, or persons in underdeveloped countries without regular STX testing capabilities, likely remain susceptible to PSP (Dolah et al. 2001; Wekell et al. 2004).

The potential toxicity of seafood is dependent upon bloom density and duration, filtering adaptations of the organism, and the relative toxicity of the algae. The relative toxicity can change based on the amount of toxin per cell (toxin content or quota) or the array of STX congeners (toxin profile or percent composition) present within a single algal cell. This is a management concern, as the relative toxicity of a bloom is not immediately apparent to lay persons, health officials, or resource managers. Management agencies currently use the mouse bioassay or high performance liquid chromatography coupled with fluorescence detection (HPLC-FLD) to quantify STXs in edible meat (Anderson et al. 2000; Burrell et al. 2016), methods that require days of analyses before enforcement, e.g., shellfish bed closure. The utilization of HPLC-FLD data by managers requires the conversion of molar concentrations to toxicity or potency (saxitoxin equivalents per cell) using toxicity equivalent factors (TEFs). The TEFs represent the relative amount of each congener that is equivalent in potency to the parent compound, saxitoxin (Figure 1;EFSA 2009). Alternatively, some regions employ a Jellett test, a mixture of antibodies which can successfully detect most STX congeners and requires little training, with minimal cost. It is an effective tool for a first-step in monitoring as it achieves rapid screening (within 35 min) of either tissue or phytoplankton during suspected threats and is designed for lay persons (Jellett et al. 2002). Approved methods, e.g., the post-column HPLC-FLD method (AOAC 2011.02) or mouse bioassay (APHA 1970), should be done subsequently to confirm toxicity and monitor the threat in accordance with the National Shellfish Sanitation Program (NSSP 2015).

ALEXANDRIUM FUNDYENSE BLOOMS

The active management of PSP to protect human health, has led to serious economic loss for shellfish industries, tourism, and state agencies. A study in the 1990's estimated an average

economic impact of over \$49 million each year in the United States due to HABs (Anderson et al. 2000). During 2005, an extensive bloom occurred in New England that closed the coastline along Maine, Massachusetts, and New Hampshire to shellfishing from April to August due to elevated levels of saxitoxin in edible meat. The price of this event may be as high as \$18 million in direct costs to the Massachusetts shellfish landings and \$2.5 million to the mussel and softshell crab industry in Maine (Anderson et al. 2005; Jin et al. 2007). New England is now well known for its almost annual shellfish bed closures due to STX-producing blooms of *Alexandrium fundyense* (McGillicuddy et al. 2005; Hattenrath et al. 2010; Crespo et al. 2011).

Alexandrium fundyense was recently separated from *Alexandrium tamarense*, using molecular and microscopy methods, which resulted in the description of five genetically distinct groups of *Alexandrium* (John et al. 2014). *Alexandrium fundyense* (and other strains in the *Alexandrium tamarense* complex) can be found in the Arctic and throughout the Pacific and Atlantic coasts, but gets its name from the Bay of Fundy, where it was first identified and predominately studied (Anderson et al. 2012; John et al. 2014). *Alexandrium fundyense* cells can, in general, be identified by their lack of chains, a length that equals the width of the dinoflagellate cell, golden brown elongated chloroplasts, and consistent lack of a ventral pore (John et al. 2014). *Alexandrium fundyense* is not found throughout the year in the northeast United States, but blooms between late March to June when the water temperatures are between 5-18°C (Nauset Marsh System), 4-14°C (Gulf of Maine) and 10-20°C (Long Island Sound) (Poulton et al. 2005; Hattenrath et al. 2010; Crespo et al. 2011). Other typical environmental parameters that are associated with *A. fundyense* blooms include salinity ranging from 30-33°C (Nauset Marsh System, Gulf of Maine), stratification, and a sufficient supply of inorganic N (4-7 µM Gulf of Maine, 6.8-21 µM Long Island Sound) and PO₄³⁻ (0.1-1.3 µM Gulf of Maine, 0.5-1.5

µM Long Island Sound) (Poulton et al. 2005; Hattenrath et al. 2010; Crespo et al. 2011). The blooms can co-occur with other algal species and typically contain one of multiple sexual or asexual life stages of *A. fundyense* at a time (Etheridge Degrasse & Roesler 2005; Hattenrath et al. 2010; Toebe et al. 2013). During asexual reproduction cells repeatedly divide, leading to the proliferation of motile vegetative cells. As environmental conditions become less ideal, sexual reproduction commences and gametes fuse together into swimming zygotes. The planozygotes eventually transform into dormant, resting cysts that overwinter in sediments until they germinate in the spring to fuel the next year's bloom. The resting cysts can be viable for decades and are arguably the leading contributor to the spread and annual consistency of *Alexandrium* blooms throughout the world (Anderson 1998; Anderson et al. 2002).

Two types of *A. fundyense* blooms are dominate in the Northeast U.S.: large regional open-water blooms, influenced by an oceanic supply of inorganic nitrogen, and localized coastal outbreaks in estuaries, where flushing rates are low and there are high N inputs from point (sewage overflows, wastewater treatment plants, etc) and nonpoint sources (atmospheric deposition, fertilizers, etc.) (Anderson et al., 2008; Hattenrath, Anderson, and Gobler, 2010). Together these observations demonstrate that *A. fundyense* subpopulations possess high plasticity allowing them to dominate and persist in a variety of environmental conditions (Dyhrman and Anderson 2003). Knowledge of the environmental divers, including nutrients, that lead to the intensification and expansion of STX-producing algae and their toxicity, is necessary to mitigate PSP events.

NITROGEN UTILIZATION BY *ALEXANDRIUM*

Nitrogen pollution in coastal marine systems can have a strong effect on the dominant species, growth, and toxicity of a phytoplankton bloom. Nitrogen (N), the most common limiting

nutrient in coastal waters, is of particular interest because areas with elevated concentrations of reduced N, i.e., NH_4^+ and urea, are often correlated with HABs comprised of toxic dinoflagellates (Dagenais-Bellefeuille and Morse 2013; Lehman et al. 2015). Even though urea and other forms of dissolved organic N (DON) may be associated with toxic dinoflagellates (Glibert & Terlizzi 1999; Kudela et al. 2008), most uptake studies and coastal monitoring programs have largely ignored dissolved urea concentrations. This large gap in information is disconcerting as urea is quickly becoming the leading N component of fertilizer and is used in animal feed and manufacturing products (Glibert et al. 2006).

In addition to increasing the number of algal cells, anthropogenic loading has the potential to alter the toxicity of cells. Studies have found toxin synthesis to be influenced by cellular N levels and growth phase, e.g., exponential vs. stationary growth (Anderson et al. 1990; Taroncher-oldenburg et al. 1997; Etheridge & Roesler 2005). Toxin within a single cell is produced for the first 8-10 hours until reaching a constant value, at which time the cell is thought to divide the toxin between two daughter cells (Taroncher-oldenburg et al. 1997). This leads to a step-wise increase of total toxin in cultures with algal growth. Saxitoxin-producing dinoflagellates, like *Alexandrium fundyense*, alter their growth rate and toxicity in response to varying N environments as well; this is not surprising given that STXs are N-rich molecules (Leong et al. 2004). Nitrogen limitation can result in a decrease in toxin content and toxin production by *A. fundyense* as STX synthesis competes with other metabolic processes for N (Anderson et al. 1990; John and Flynn 2000; Dagenais-Bellefeuille and Morse 2013). Nitrogen enrichment, likewise, significantly increases toxin content (Anderson et al. 1990; John & Flynn 1999; Han et al. 2016). Toxin content can also be altered by the chemical form of nitrogen being utilized for growth; an axenic strain of *A. fundyense* showed the highest STX per cell when

grown on NO_3 , followed by NH_4^+ and then urea (Dyhrman and Anderson 2003; Rossini 2014). Together these studies suggest that bloom toxicity can fluctuate based on the chemical form *and* concentration of the N source utilized by the cells. As such, bloom density alone is an insufficient predictor of STX concentrations in shellfish or the corresponding risk for PSP in humans. To truly be able to evaluate risk, the concentration(s), chemical form(s), and fluctuations in N supply, should all be monitored within systems to evaluate effects on algal species composition, bloom toxicity, and shellfish contamination.

STABLE ISOTOPES IN NUTRIENT STUDIES

One method to track the source of N pollution in a waterbody is through the use of stable isotopes. Many processes and reactions alter the ratio of heavy and light isotopes, ^{15}N : ^{14}N , or “fractionate” stable isotopes, resulting in a product with a different isotopic value than the reactants (Needoba et al., 2003). There are two major types of isotope fractionation processes: kinetic and equilibrium. Equilibrium fractionation processes are reversible processes where the heavier isotope accumulates in the compound with the stronger bonds. Kinetic fractionation is associated with incomplete processes and occurs based on the motility of the different isotopes, with the heavier isotope having a reduced velocity and reaction rate (Ryabenko 2013). Stable N isotope values are typically reported as $\delta^{15}\text{N}$ values, which are parts per thousand (per mil, ‰) differences between the ratio of ^{15}N : ^{14}N found in the sample relative to the standard: atmospheric N_2 gas. The ability to use N stable isotopes to track N sources relies upon the measured difference in isotope ratios between N derived from human or animal waste, which tends to be ^{15}N enriched (i.e., $\delta^{15}\text{N}$ of nitrate ranging from 10-30‰), and N derived from natural sources (i.e., $\delta^{15}\text{N}$ of nitrate ranging from 2-8‰) (Kendall & McDonnell 1998). In practice, one can identify the main sources of available N by measuring the isotopic signature of one or many

components, e.g., particulate organic matter, NO_3^- , and/or specific compounds, within the waterbody of concern.

Particulate organic matter (POM) is any organic matter greater than $0.45 \mu\text{m}$ and less than 2 mm in size. The ratio of ^{15}N : ^{14}N of POM ($\delta^{15}\text{N}_{\text{POM}}$) has been successfully applied to compare and evaluate N sources in aquatic systems (Doi et al. 2004; Savage et al. 2005; Dailer et al. 2010; Hattenrath et al. 2010; Zvab Rozic et al. 2015). The isotopic value of POM is a useful diagnostic when the goal is to identify the N source supporting phytoplankton, as the method provides a value for bulk particulates that corresponds to the isotope value of the N source. Additionally, $\delta^{15}\text{N}_{\text{POM}}$ can be useful in understanding N sources fueling HABs if the blooms are largely monospecific; Hattenrath and Gobler (2010) found a direct correlation between high $\delta^{15}\text{N}_{\text{POM}}$ values, increased STX concentration, and elevated *A. fundyense* cell densities during a bloom in Northport-Huntington Bay, New York in 2008. The study demonstrated support of the dense algal bloom by anthropogenic N from a nearby wastewater treatment plant. The isotopic signature of POM, however, becomes a less useful tool for tracing the N source driving HABs when environmental factors and N availability or chemical forms are changing over time, leading to altered fractionation in phytoplankton.

Although all autotrophs preferentially assimilate ^{14}N over ^{15}N , dinoflagellates generally demonstrate relatively less isotopic fractionation (1-3‰) compared to other species of algae: diatoms, cyanobacteria, and coccolithophores (Montoya and McCarthy 1995; Needoba et al. 2003). This suggests the $\delta^{15}\text{N}$ value of dinoflagellates will more closely resemble the $\delta^{15}\text{N}$ of their source, making them ideal for N pollution identification. The strength of this diagnostic, however, varies with environmental variables, as N chemical form, pH, light, salinity, and temperature have been shown to significantly affect N isotope fractionation in other taxonomic

groups: diatoms and coccolithophores (Cifuentes et al. 1988; Arthur et al. 1994; Needoba & Harrison 2004; Wang et al. 2006; Lara et al. 2010). How this translates to N isotope fractionation in dinoflagellates is unknown, but ***I hypothesize that variations in one of these environmental parameters (N chemical form) can affect enzymatic processes within dinoflagellates, and more specifically A. fundyense, leading to large changes in $\delta^{15}N_{POM}$ values relative to the $\delta^{15}N$ of the source.***

Nitrogen exists in a variety of oxidation states that are bioavailable to auto- and heterotrophs, leading to numerous chemical forms and biogeochemical pathways for N in the ocean (Altabet 2006). Based on research with other autotrophs, *A. fundyense* cells likely convert the more oxidized forms of N, such as NO_3^- , into reduced NH_4^+ upon uptake by cells (Kendall et al. 2007); more specifically, reductase enzymes convert NO_3^- to NO_2^- and then to NH_4^+ . Modes of NH_4^+ uptake are likely concentration dependent, whereby at high concentrations, NH_4^+ is taken into cells via passive diffusion, exhibiting equilibrium fractionation (a reversible process). At low concentrations, NH_4^+ instead undergoes active transport, demonstrating kinetic fractionation (a unidirectional reaction). There are then multiple pathways by which this chemical form can be incorporated into the biomass of *A. fundyense*, e.g., NH_4^+ can be used to form glutamate via the enzyme glutamate dehydrogenase which is a reversible reaction, or unidirectionally assimilated using the enzyme glutamine synthetase (Fogel & Cifuentes 1993).

Many studies have documented a concentration dependency on N isotope fractionation when diatoms are utilizing NH_4^+ that coincides with this switch in assimilation mechanisms (Fogel & Cifuentes 1993; Pennock et al. 1996; Waser, Yim, et al. 1998). At high concentrations of NH_4^+ , the fractionation corresponding with glutamate dehydrogenase is large. At lower concentrations (<10 μM - NH_4^+), almost all NH_4^+ is assimilated (i.e., little to none is lost to

diffusion out of the cell), leading to no isotope fractionation during incorporation into organic matter (i.e., the isotope signature of the NH_4^+ in the water and within the cell is the same).

Overall, the variation in biochemical pathways can lead to different levels of isotopic fractionation based on the N chemical form or ambient concentration (Kendall et al. 2007). The subsequent complex reactions that then incorporate N into nucleotides, proteins, and ureides, can lead to further fractionation.

The effect of N chemical form and concentrations is especially important in dynamic estuarine systems with mixed N sources and seasonal inputs. As such, for stable isotopes to be used as a tracer of N sources that fuel *A. fundyense*, the fractionation of isotopes over the different uptake and assimilation pathways must be considered.

OBJECTIVES

I propose that the N-isotope value of A. fundyense can provide information about the primary N sources and chemical forms that initiate a specific bloom and control its toxicity.

Changes in $\delta^{15}\text{N}_{\text{POM}}$ that are associated with uptake and assimilation pathways during the utilization of different N chemical forms need to be characterized before this tool can be applied *in situ* (Kendall et al. 2007). The goal of my research was to use controlled laboratory experiments to identify and characterize how N chemical forms affect growth, toxicity, and N isotope fractionation during N assimilation within a marine dinoflagellate. Ultimately, the results generated here will allow the N isotopic signature of POM to be used more reliably as a tracer of N source fueling toxic blooms of *A. fundyense*. When paired with new knowledge elucidating the effect of N chemical form on growth and toxicity, managers will be better able to predict the resulting magnitude and toxicity of the bloom. This study also produces novel information regarding N isotope fractionation during NH_4^+ and urea utilization in dinoflagellates.

To identify N isotope fractionation (i.e., ϵ values), growth, and toxicity associated with different N chemical forms, isolates of the toxic dinoflagellate, *A. fundyense*, were grown on micromolar concentrations of NH_4^+ , NO_3^- , or urea, under N-sufficient conditions. As systems typically receive a combination of natural and point and non-point sources, composed of a variety of N chemical forms, N isotope fraction and growth were also studied in an experiment with mixed-N medium. Through this second experiment, I investigated the preferential uptake and variation in the $\delta^{15}\text{N}_{\text{POM}}$ during growth on mixed N sources and any effect of preconditioning to a particular N form. As such, the objectives of this study were three-fold, to determine 1) N isotopic fractionation (i.e., ϵ values) by dinoflagellates during growth on micromolar concentrations of either NO_3^- , NH_4^+ , or urea, 2) N isotopic fractionation in a mixed-N environment when preconditioned to a N form, and 3) variation in toxin content and composition in *A. fundyense* when grown on either NO_3^- , NH_4^+ , or urea.

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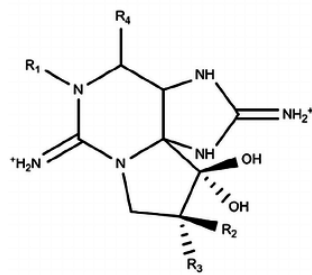
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FIGURES








Toxin	R ₁	R ₂	R ₃	R ₄	Toxicity Equivalency Factors	
					Oshima	EFSA
STX	H	H	H		1.00	1.00
GTX2	H	H	OSO ₃ ⁻		0.36	0.40
GTX3	H	OSO ₃ ⁻	H		0.64	0.60
NEO	OH	H	H		0.92	1.00
GTX1	OH	H	OSO ₃ ⁻		0.99	1.00
GTX4	OH	OSO ₃ ⁻	H		0.73	0.70
M2	H	OH	H		-	-
M4	H	OH	OH		-	-
GTX5	H	H	H		0.06	0.10
C1	H	H	OSO ₃ ⁻		0.01	-
C2	H	OSO ₃ ⁻	H		0.10	0.10
C3	OH	H	OSO ₃ ⁻		0.01	-
C4	OH	OSO ₃ ⁻	H		0.06	0.10
GTX6	OH	H	H		0.06	0.10
M1	H	OH	H		-	-
M3	H	OH	OH		-	-
dcSTX	H	H	H		0.51	1.00
dcGTX2	H	H	OSO ₃ ⁻		0.15	0.20
dcGTX3	H	OSO ₃ ⁻	H	-OH	0.38	0.40
dcNEO	OH	H	H		-	0.40
dcGTX1	OH	H	OSO ₃ ⁻		-	-
dcGTX4	OH	OSO ₃ ⁻	H		-	-

Figure 1. Chemical structure of major congeners of saxitoxin and corresponding toxicity equivalency factors of the depicted by Burrell et al. (2016) from values reported by Oshima et al. (1989) and European Food Safety Authority (EFSA) (2009).

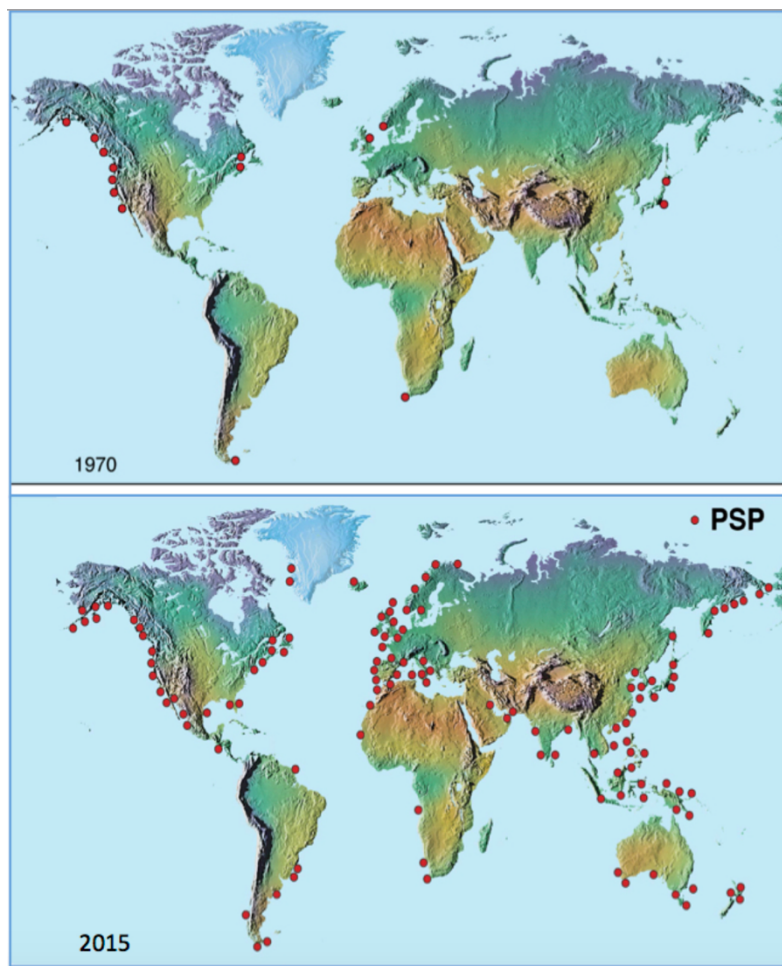


Figure 2. Global distribution of saxitoxins quantified in seafood prior to 1970 and 2015 documented and illustrated by the U.S. National Office for Harmful Algal blooms (www.whoi.edu/redtide).

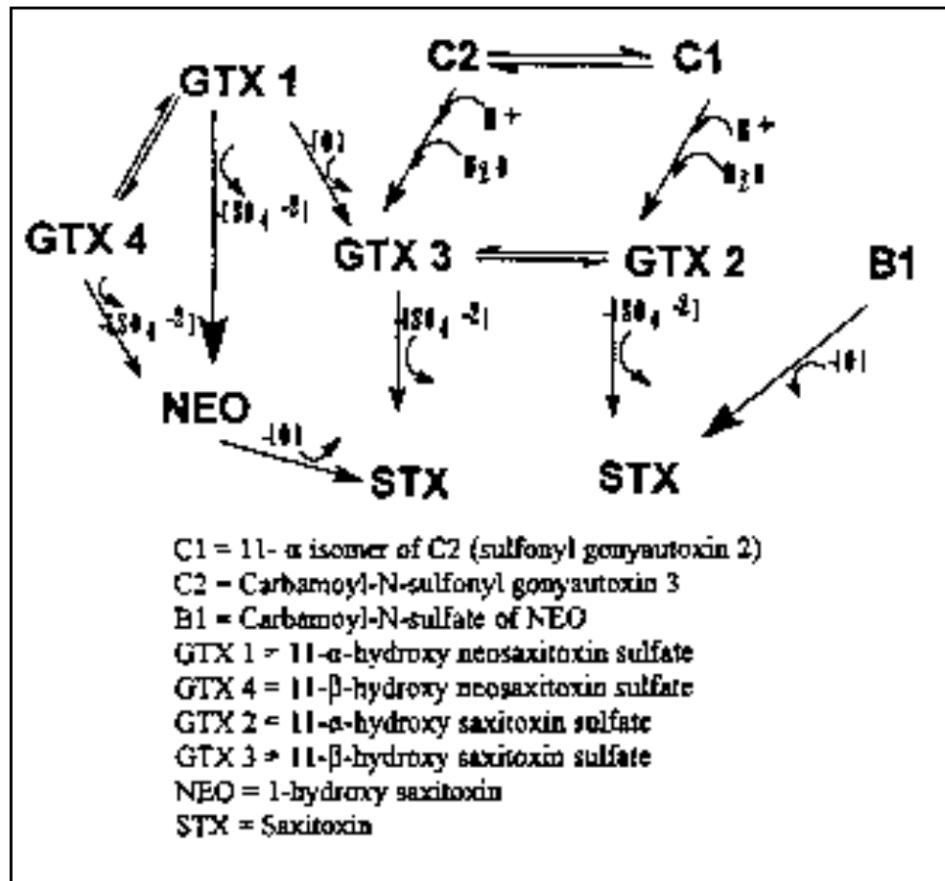


Figure 3. Proposed conversion of saxitoxin congeners during heating. Illustration by Indrasena and Gill (2000) with data from Shimizu (1984) and Laycock et al (1995).

CHAPTER 1

NITROGEN ISOTOPE FRACTIONATION AND TOXIN PRODUCTION DURING THE UPTAKE OF MICROMOLAR CONCENTRATIONS OF NITRATE, AMMONIUM, AND UREA BY A MARINE DINOFLAGELLATE

INTRODUCTION

Harmful algal blooms (HABs) have increased in frequency, intensity, and geographical range over the past 40 years, creating dire consequences for public health, aquatic ecosystems, fishery services, and local economies (Anderson et al. 2008). Coastline development has been identified as a contributor to this deterioration of ecosystem health through its connection to increased nutrient loading via groundwater flow, sewage effluent, atmospheric deposition, and agricultural and aquaculture runoff (Anderson et al. 2002; Backer and McGillicuddy 2006). Nitrogen (N) is of particular interest because areas of high N, in particular the reduced forms of N such as NH_4^+ and urea, are often correlated with HABs comprised of toxic dinoflagellates (Dagenais-Bellefeuille and Morse 2013; Lehman et al. 2015). However, the link between anthropogenic N loading and HABs is site- and organism- specific. The concentration of N, as well as the chemical form (e.g., organic vs inorganic, NO_3^- vs NH_4^+), can alter the growth, toxicity, and dominance of a specific algal species (Anderson et al. 2008). As such, reliable prediction of the growth and toxicity of specific HAB species in complex marine systems based on nutrient types and concentrations available, remains a challenge (Anderson et al. 2002).

One of the most prominent HAB-associated syndromes worldwide is paralytic shellfish poisoning (PSP), caused by the accumulation of potent algal neurotoxins, saxitoxins (STXs), in seafood products and subsequent intoxication of human consumers (Anderson & Wexler 2005). The clinical effects of STX develop almost immediately, causing tingling and numbness of the lips and face, followed by muscle paralysis, and if the dose is high enough, the biotoxin causes death by respiratory failure and cardiovascular shock (Andrinolo et al. 1999; DeGrasse et al. 2014). The parent compound, saxitoxin, and its 57 congeners, are produced by species of both freshwater cyanobacteria and marine eukaryotic dinoflagellates: *Alexandrium*, *Gymnodinium* and *Pyrodinium* (Hackett et al. 2012). Saxitoxin producing algae can have different toxicities based

on the toxin content and toxin profile of the algal cell. The 57 congeners all have the same tricyclic core, but differ structurally at four side group positions, which can be either hydroxylated, sulfated, or carbamoylated. The structural differences affect the chemical properties and potency of the compound, with STX, NEO and GTX4 being the most potent, and C toxins being the least toxic (Rossini 2014; Tan and Ransangan 2015; EFSA 2009). A saxitoxin-producing algal strain can produce multiple congeners, making each bloom within a system a unique management concern (Wang et al. 2006).

Saxitoxins are produced by two types of *Alexandrium fundyense* blooms in the northeast US: large regional open-water blooms, influenced by oceanic supply of inorganic nitrogen, and localized coastal outbreaks in estuaries, where flushing rates are low and there are high N inputs from point (e.g., sewage overflows, wastewater effluent) and nonpoint sources (e.g., atmospheric deposition, surface runoff of fertilizers) (Anderson et al., 2008; Hattenrath, Anderson, and Gobler, 2010). *Alexandrium fundyense* is capable of utilizing the three major N chemical forms (i.e., N species) found in seawater: nitrate (NO_3^-), ammonium (NH_4^+), and urea as the sole N source, with similar growth rates and maximum cell densities across all forms (Dyhrman and Anderson 2003; Leong et al. 2004). The N chemical form, however, can influence toxin production; an axenic batch culture of *A. fundyense* showed elevated toxicity (STX equivalents per cell) during stationary phase after cells were grown on NO_3^- , relative to growth on NH_4^+ or urea (Dyhrman and Anderson 2003; Rossini 2014). As N chemical form can clearly play a role in bloom toxicity, more information is needed for other saxitoxin-producing strains, in the presence of microbial community, and under N-sufficiency. Knowledge of the nutrients and their source, e.g., anthropogenic or natural, that contribute to the intensification and expansion of *A.*

fundyense, as well as those that alter toxin quota, profile or production rate, is necessary to mitigate, predict and/or prevent blooms and subsequent outbreaks.

The isotope ratio of ^{15}N : ^{14}N ($\delta^{15}\text{N}$) of particulate organic matter (POM), $\delta^{15}\text{N}_{\text{POM}}$, has been successfully used to characterize multiple N sources as they are delivered to an aquatic system with the goal of understanding relative inputs and developing management strategies that minimize loads from the most impactful N contributors (Doi et al. 2004; Savage et al. 2005; Dailer et al. 2010; Hattenrath et al. 2010; Zvab Rozic et al. 2015). Many processes and reactions alter the ratio of heavy and light isotopes, or “fractionate” stable isotopes, resulting in a product with a changed isotopic composition (Petersen & Fry 1987). Relying on the knowledge that N derived from human or animal waste tends to be ^{15}N enriched ($\delta^{15}\text{N}$ of nitrate = 10-30‰) and natural sources are ^{15}N depleted (2-8‰), one can differentiate between anthropogenic and natural sources and their extension into the waterbody (Kendall & McDonnell, 1998). Although phytoplankton preferentially assimilate ^{14}N over ^{15}N , dinoflagellates may demonstrate relatively weaker isotopic fractionation (1-3‰) compared to other species of algae: diatoms (2-6‰), cyanobacteria (5‰), and coccolithophores (3-5‰) (Montoya & Mccarthy 1995; Waser, Yim, et al. 1998; Needoba et al. 2003). This means the $\delta^{15}\text{N}$ value of dinoflagellates will more closely resemble the $\delta^{15}\text{N}$ of their source, making the taxonomic group an ideal tool towards the identification of N-pollution for toxic dinoflagellate blooms and the overall system. Hattenrath and Gobler (2010) found a direct correlation between high $\delta^{15}\text{N}_{\text{POM}}$ values, increased STX concentration, and elevated *A. fundyense* cell densities during a bloom in Northport-Huntington Bay, NY, in 2008. This study demonstrated support of the dense algal bloom by anthropogenic N from a nearby wastewater treatment plant.

The isotopic value of POM, however, becomes a less useful tool for tracing N source (s) when N availability or chemical forms change over time or when N sources have overlapping $\delta^{15}\text{N}$ values. Variability in N availability or chemical forms can lead to altered fractionation by the phytoplankton and/or changes to the phytoplankton community, resulting in the misinterpretation of or inability to interpret the $\delta^{15}\text{N}_{\text{POM}}$ and responsible N source. I propose that the utility of the tool can be preserved, however, if this change in ambient N, variation in species composition making up the POM, and fractionation patterns by the dominant species can be well characterized. Nitrogen isotope fractionation in diatoms and coccolithophores is well documented (Pennock et al. 1996; Waser, Harrison, et al. 1998; Waser, Yim, et al. 1998); the fractionation factors (ϵ) for the diatom *Thalassiosira pseudonana* were $5.2\pm 0.2\text{‰}$, $0.9\pm 0.6\text{‰}$, $20\pm 1\text{‰}$, $-0.8\pm 0.6\text{‰}$, during the utilization of NO_3^- , NO_2^- , NH_4^+ , and urea, respectively. Similarly, the fractionation factor (ϵ) for the coccolithophore *Emiliania huxleyi* was 4‰ , 16‰ , and 0‰ , for the uptake of NO_3^- , NH_4^+ , and urea, respectively. Data, however, are lacking regarding N isotope fractionation by dinoflagellates when grown on different chemical forms, presented alone or in mixed media.

Here we investigated how the isotopic signature of the dinoflagellate, *A. fundyense*, changes when cells are utilizing micromolar concentrations of NO_3^- , NH_4^+ , or urea, or a combination of the three, following pre-conditioning to a specific form. Pre-conditioning was an important element of the study as it provided novel information regarding how a population may respond to the introduction or removal of a N source consisting of a different chemical form, e.g., storm event or mediation of point-source pollution. Ultimately, we expect these results will aid in the interpretation of $\delta^{15}\text{N}_{\text{POM}}$ in a system with N contributions from multiple sources (i.e., mixed N sources) or where sources change rapidly. To better understand how different N

chemical forms and source switching would affect potential toxicity of the occurring dinoflagellate blooms, we also investigated the effect of these sources on toxin content and composition (i.e., toxin profile) in multiple strains of *A. fundyense*. As such, the objectives of this study were three-fold, to determine: 1) N isotopic fractionation (i.e., ϵ values) by dinoflagellates during growth on micromolar concentrations of either NO_3^- , NH_4^+ , or urea, 2) N isotopic fractionation in a mixed-N environment, and 3) variation in toxin content and composition in *A. fundyense* when grown on either NO_3^- , NH_4^+ , or urea.

MATERIALS AND METHODS

Chemicals. Reagents used for analytical chemistry include Optima LC-MS (liquid chromatography coupled to mass spectrometry) additive grade formic acid (99-100%), Fluka Analytical LC-MS additive grade acetonitrile, methanol, and ammonium hydroxide (>25% in H₂O; PCode: 101612155). During extraction and culturing efforts, HPLC (high performance liquid chromatography) reagent grade glacial acetic acid, ACS certified ammonium chloride, urea, and sodium chloride (Fisher Scientific, Loughborough, UK), and ACS Reagent grade sodium nitrate (99%) (Alfa Aesar) were utilized. Reference toxin standards, STX, NEO, GTX1, GTX2, GTX3, GTX4, GTX5, dcNEO, dcSTX, dcGTX2/3, C1, and C2 (National Research Council, NRC, Halifax, Nova Scotia, Canada) were used during saxitoxin quantification.

Culture Maintenance. The saxitoxin producing dinoflagellate *Alexandrium fundyense* clones GTCA-28 (Western Gulf of Maine, 1985, single cell from cyst germination) and ATSP7-D9 (Nauset Marsh System, Cape Cod, MA, 2009, single vegetative cell) were obtained from the laboratory of D. Anderson at Woods Hole Oceanographic Institution (Vladar et al. 2015).

Cultures were grown in pre-filtered (pore size 0.22 μm) seawater from Wachapreague, Virginia brought to a pH of 8.3, salinity of 35 ppt, and amended with sterile f/6 nutrients excepting SiO₂, and NO₃⁻ (36.2 μM PO₂⁻, trace metals and vitamins; Guillard 1975). The cultures were illuminated at saturating light intensities (~60 μE•m⁻²•s⁻¹) on a 14:10h L:D cycle, and kept at a set temperature (15°C). Cells were grown for at least three generations, i.e., pre-conditioned, with reduced concentrations of N (25-150 μM-N) in the form of NO₃⁻, NH₄⁺, or urea (amended with 100 nM NiSO₄), prior to use in the experiment. Nickel was added with urea because *Alexandrium* species use the nickel-dependent urease enzyme to hydrolyze urea into NH₄⁺ (Dyhrman and Anderson, 2003). The cells were grown on a lower concentration of N to better

mimic concentrations found in the field and to avoid NH_4^+ toxicity at *ca.* 50 μM . When bulking-up culture volume in preparations for the experiments, 5 ml of maintenance culture was transferred to 150 ml f/6 media containing 50 μM -N of either NO_3^- , NH_4^+ , or urea. Twenty-five μM of the respective N reagent in Milli-Q water was added every three days to keep N from becoming depleted. After two weeks, 50 ml of culture was transferred to 350 ml of fresh media. Additional fresh media, 600 ml, was added to each flask to further increase volume once cell concentrations reached $\sim 5,000 \text{ cells}\cdot\text{ml}^{-1}$.

Experimental Conditions. Two experiments were conducted: (1) A single-source study where N, in the forms of NO_3^- , NH_4^+ , or urea with 100 nM nickel (NiSO_4), was repeatedly replenished to avoid N limitation and end-point measurements of $\delta^{15}\text{N}_{\text{POM}}$ and toxin were obtained, and (2) a mixed-source study where batch cultures were supplied with a combination of NO_3^- , NH_4^+ , and urea at inoculation and $\delta^{15}\text{N}_{\text{POM}}$ and nutrient samples were collected over batch culture. The initial $\delta^{15}\text{N}$ value of the NO_3^- , NH_4^+ , and urea stock reagents were measured at $12.7\pm 0.8\text{‰}$, $2.1\pm 0.4\text{‰}$, $-0.3\pm 0.4\text{‰}$ ($n=3$), providing sufficient disparity between N oxidation states. In both experiments, all samples were collected during exponential growth to maintain consistency as toxin production, toxin profile, and fractionation can vary over growth stage (Anderson et al. 1990; Persson et al. 2012). During the experiments, cultures were grown in 0.22- μM filtered, autoclaved seawater from Wachapreague, VA, brought to a pH of 8.3, salinity of 35 ppt, and amended with sterile f/6 nutrients, excluding SiO_2 , and NO_3^- (i.e., 36.2 μM PO_4^{3-} , trace metals and vitamins; modified from Guillard 1975). The experimental cultures were illuminated at $60 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ on a 14:10h L:D cycle, and kept at a set temperature (15°C) in a Percival incubator. On a daily basis, flasks were randomly arranged in the incubator to control for potential discrepancies in light levels. The cultures were not bubbled as to reduce N gas exchange.

Single N Source Experiment. In preparation for the single-N source experiment, two strains of *A. fundyense*, GTCA-28 and ATSP7-D9, were used to inoculate triplicate experimental flasks of each treatment (NO_3^- , NH_4^+ , or urea), at a concentration of $500 \text{ cells}\cdot\text{ml}^{-1}$ that were pre-conditioned for three generations. To avoid N limitation and minimize effects of recycled N, N was added throughout the experiment to all three treatments. The NH_4^+ concentration was specifically monitored in real-time, using a $\text{NH}_3/\text{NH}_4^+$ test kit with a limit of detection of 0-8 ppm (Aquarium Pharmaceuticals), to avoid reaching toxic levels during N additions; the other two chemical forms were added to their respective treatments assuming a similar uptake rate. Nitrogen was added daily to all treatments up to day 5, after which the frequency of N amendment was increased to every 6 hours to account for the increase in culture biomass, maintaining N at a concentration of over $10 \mu\text{M-N}$. Culture medium was collected, filtered, and frozen for the independent quantification of dissolved concentrations of NO_3^- , NH_4^+ , and urea at the start of the experiment and every two days thereafter. Additionally, 1-ml samples were withdrawn aseptically, at the same time every day, and preserved with Lugol's iodine solution for cell enumeration using a Sedgewick-Rafter (S-R) counting cell and light microscopy. Growth rate per day (U) was calculated using the cell counts, where N1 and N2 are the concentrations of cells ($\text{cells}\cdot\text{ml}^{-1}$) at time 1 (t1) and time 2 (t2), i.e. day (d) in equation 1 (Leong et al, 2004).

$$U = \frac{\ln\left(\frac{N2}{N1}\right)}{(t2 - t1)}$$

(Equation 1)

Six hours before the end of the experiment, culture medium was collected to determine the $\delta^{15}\text{N}$ of the dissolved NO_3^- or NH_4^+ ($\delta^{15}\text{N}_{\text{NO}_3^-}$ and $\delta^{15}\text{N}_{\text{NH}_4^+}$) after the last N addition, allowing for source characterization most relevant to the end $\delta^{15}\text{N}_{\text{POM}}$ measurement. To end the experiment,

i.e., when cell concentrations reached $\sim 3,000 \text{ cells}\cdot\text{ml}^{-1}$ during exponential growth, endpoint samples were collected for measurement of the isotopic composition ($\delta^{15}\text{N}$) of particulate nitrogen ($\delta^{15}\text{N}_{\text{POM}}$) and media $\delta^{15}\text{N}_{\text{NO}_3^-}$ and $\delta^{15}\text{N}_{\text{NH}_4^+}$. An extra 15-ml of culture was also collected in a centrifuge tube for STX extraction and quantification.

Mixed N Source Experiment. In preparation for the mixed-N source experiment, *A. fundyense*, strain ATSP7-D9, was pre-conditioned to grow on a single source of NO_3^- , NH_4^+ , or urea (25-50 $\mu\text{M-N}$) for three generations. Only one strain, ATSP7-D9, was used in this more complex time-course experiment as the results of the previous, single-N experiment showed similar fractionation between strains. To mark the beginning of the experiment, nine 3-L Fernbach flasks per treatment were inoculated with pre-conditioned cultures in exponential growth at an initial concentration of $\sim 100 \text{ cells}\cdot\text{ml}^{-1}$. Experimental flasks began with a mix of NO_3^- , NH_4^+ , and urea at concentrations of 40 $\mu\text{M-N}$ each, or a total of 120 $\mu\text{M-N}$ (plus 100 nM NiSO_4). As opposed to the first experiment, N was not replenished during the experiment to allow for the determination of nutrient selectivity and drawdown thresholds. Triplicate cell samples from each of the three flasks were enumerated daily with the use of Lugol's preservation, a S-R counting chamber, and light microscopy to produce growth rate estimates (Eq 1). Samples for N medium concentrations, $\delta^{15}\text{N}_{\text{POM}}$, and media $\delta^{15}\text{N}_{\text{NO}_3^-}$ and $\delta^{15}\text{N}_{\text{NH}_4^+}$ were taken from triplicate flasks every 12 hours over a 6-7 day experimental period. Culture volume depleted due to repeated sampling, and therefore, at 60 hours and 96 hours, our experimental design allowed for subsequent sampling from the next set of triplicate flasks.

Dissolved Nutrient Analysis. Dissolved nutrient samples were filtered through a glass fiber filter (25-mm diameter, Whatman GF/F, CAT No. 1825-025). The filtrate was collected in acid-washed 20-ml scintillation vials for nutrient analysis and duplicate 15-ml falcon tubes for urea

quantification, and then frozen at -20°C until analysis. A SEAL AA3 four-channel segmented flow analyzer (Nutrient Chemical Facility, Woods Hole Oceanographic Institution, WHOI) was used to quantify NH_4^+ , $\text{NO}_3^-/\text{NO}_2^-$, PO_4^{3-} , and total dissolved N in the GF/F-filtered medium using standard methods. Urea was quantified at VIMS using the manual diacetyl monoxime thiosemicarbazide technique method adapted from Rahmatullah and Boyde (1980), modified by Price and Harrison (1987). Duplicate samples were diluted to fall within the standard curve (0-10 $\mu\text{M-N}$, 6 points x 3 replicates) and run on a spectrophotometer equipped with a 10-cm cell (525 nm, Solorzano, 1969).

Toxin Extraction and Analysis. The toxin samples were immediately pelleted by centrifugation at 3,000 rcf for 15 minutes at 10°C , aspirated to remove overlying seawater, and the pellets frozen at -20°C until STX extraction. To promote cell lysis, toxin extraction, and compound stability, pellets were resuspended in 0.5 ml of 1% acetic acid (1:99 v/v, acetic acid:water), subjected to freeze/thaw x3, and probe sonicated 1 minute at 40W on ice (Branson Digital Sonifier-450, Danbury, Connecticut). Subsequently, samples were centrifuged (3,000 rcf, 10°C , 15 minutes) to remove cell debris. The supernatant was pushed through a $0.22\ \mu\text{m}$ 13-mm filter syringe (Millex-GV, Millipore, durapore), diluted 1:1 with ACN in an autosampler vial, and frozen (-20°C) for up to two weeks before quantification.

Quantification was performed by hydrophilic interaction chromatography using an Acquity UPLC (ultra-performance liquid chromatography) coupled to a Xevo TQD triple quadrupole (Waters, Milford, MA) tandem mass spectrometer (HILIC-MS/MS). Separation was achieved using a Waters BEH Amide $1.7\ \mu\text{m}$ column (2.1 mm x 100 mm; P/N: 186004801) with a Waters BEH Amide $1.7\ \mu\text{m}$ guard column attached (P/N:186004799). The autosampler and columns were maintained at 10°C and 60°C , respectively. Modified from Boundy et al (2015),

mobile phases were A: water/formic acid/NH₄OH (500:0.075:0.3 v/v/v) and B: acetonitrile/water/formic acid (700:300:0.1 v/v/v). Initial conditions of 95% B were held at 0.4ml/min for 1.83 min, followed by a gradient from 95% to 50% B over 2.34 min and held for 1 min before returning to 95% B using a linear gradient over 0.2 min. For re-equilibration before the next injection, mobile phase remained at 95% B and flow at 0.4ml/min for an additional 1.13 min before increasing flow to 0.8ml/min using a linear gradient over 0.3min. The conditions were held for 0.5 min, returned to 0.4ml/min linearly in 0.3 min, and then held for 1.4 min. This resulted in a total run time of 9 min per injection (1.3 µl) (Boundy et al. 2015).

Both positive (ESI+) and negative (ESI-) electrospray ionization modes were employed and the following transitions were monitored using their protonated precursors [M + H]⁺ in the same run: dcSTX, *m/z* 257.1>126.1, dcNEO, *m/z* 273.1>255.1, STX, *m/z* 300.1>204.1, NEO, *m/z* 316.1>298.1, GTX5, *m/z* 380.1>300.1, dcGTX3, *m/z* 353.1>255.1, dcGTX2, *m/z* 353.1>273.1, GTX3/C2, *m/z* 396.1>298.1, C1, *m/z* 396.1>316.1, GTX4, *m/z* 412.1>314.1 in positive mode; and dcGTX2, *m/z* 351.1>164, GTX2, *m/z* 394.1>351.1, GTX1, *m/z* 410.1>367.1 in negative mode. Toxin quantification was calculated using a 6 level calibration curve (3 times per run) made with STX reference solutions: STX, NEO, GTX1, GTX2, GTX3, GTX4, GTX5, dcNEO, dcSTX, dcGTX2/3, C1, and C2 (NRC, Halifax, Nova Scotia, Canada) ranging from a 1:2000 to 1:10 dilution of the original standard solution. The precision of toxin measurements, i.e., the percent relative standard deviation (RSD = standard deviation/mean*100) based on the peak area of six injections, was less than 6% for C2, GTX3, and GTX4. The relative cellular toxicities (fmol STX equivalents/cell) of the congeners were calculated in terms of the parent toxin, saxitoxin, based on toxicity equivalent factors (TEFs) given by the European Food Safety Authority (2009).

Stable Isotope Processing and Analysis. All filters, glassware, and glass vials used for isotope analysis were pre-combusted for 4 hours at 450°C and plastic bottles were acid washed in a 10% HCl bath overnight, to eliminate any crossover N. In preparation for stable isotope analysis, culture was filtered onto a glass fiber filter (25-mm diameter, Whatman GF/D, Cat #: 1823010) to separate particulate (POM) and dissolved fractions (NO_3^- and NH_4^+ of medium). Filters were transferred into 20-ml glass vials (Fisher, catalog #05-719-117) and frozen at -20°C until isotope analysis of POM ($\delta^{15}\text{N}_{\text{POM}}$). Filtrate, 200 ml, for isotope analysis of dissolved NH_4^+ ($\delta^{15}\text{N}_{\text{NH}_4^+}$) was processed using a modified version of the NH_4^+ diffusion method of Holmes (1998), whereby polypropylene membrane filters (25 mm, lot #: 151579, Sterlitech) replaced Teflon filters (Hannon and Bohlke 2008, see Appendix I for more detail). Isotope analysis of NH_4^+ and POM was performed on a Finnigan-MAT DeltaPlus Isotope Ratio Monitoring Mass Spectrometer coupled with a Carlo Erba NC 2500 Elemental Analyzer (Model 1108) (Organic Mass Spectrometry Facility, WHOI). Depending on the expected amount of N per sample, the instrument was configured for the typical range of detection, 0.5 to 5 $\mu\text{moles N}$, or modified at the EA combustion furnace to reach a lower detection limit, 0.15 $\mu\text{moles N}$ (Houghton et al. 2000, Holtvoeth et al. 2005, 2006, York et al. 2007). The precision of $\delta^{15}\text{N}$ measurements on this instrument was 0.17‰. Filtrate, 20 ml for isotope analysis of dissolved nitrate ($\delta^{15}\text{N}_{\text{NO}_3^-}$) was transferred into duplicate 30-ml HDPE bottles and analyzed by bacteria denitrification assay using ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope-ratio mass spectrometer (Bremen, Germany) (UC Davis Stable Isotope Facility). Methods for measuring the isotopic composition of DON compounds such as urea and amino acids are more complicated and less routine, and therefore, not quantified in this study.

The N isotope ratios, $\delta^{15}\text{N}$, of the samples are reported here as a δ value (‰ = per mil) with respect to the established standard, atmospheric N_2 gas in air (~ 0.004) (Petersen & Fry 1987):

$$\delta^{15}\text{N} = \left(\frac{\frac{^{15}\text{N}}{^{14}\text{N}}_{\text{sample}}}{\frac{^{15}\text{N}}{^{14}\text{N}}_{\text{air}}} - 1 \right) \times 1000$$

(Equation 2)

The difference between N isotope values of the dissolved NH_4^+ or NO_3^- and the POM are defined by the enrichment factor or epsilon value (ϵ) using the following equation (Petersen & Fry 1987):

$$\epsilon = \left(\frac{\frac{^{15}\text{N}}{^{14}\text{N}}_{\text{medium}}}{\frac{^{15}\text{N}}{^{14}\text{N}}_{\text{POM}}} - 1 \right) \times 1000$$

(Equation 3)

In equation 2 and 3, $^{15}\text{N}/^{14}\text{N}$ is the absolute number of heavy to light atoms. Since the laboratory experiments represent closed systems, the isotopic signature of the medium is changing as N is processed by the algae. Assuming ϵ is constant during the consumption of the medium, ϵ can also be calculated using the accumulated product equation (Mariotti et al. 1981).

$$\delta^{15}\text{N}_{\text{POM}} = \delta^{15}\text{N}_{\text{medium}} - \epsilon \times \left[\frac{f}{(1-f)} \right] \times \ln f$$

(Equation 4)

where f is the fraction of unreacted substrate in the medium at any time during exponential growth.

Statistical Analysis. A Shapiro-Wilk test was used to test for normality in the data and Bartlett's test to determine for equal variances among populations. Two-way analysis of variance (ANOVA) was used to assess the combination effect of strain and N source for each parameter. If either or both normality or equal variance assumptions were not met, nonparametric Kruskal-Wallis was used to test the null hypothesis. A post-hoc analysis, Tukey's HSD test, was performed to test for differences between strains and N sources. Repeated measures ANOVAs were performed on the data generated in the mixed-N experiment. Each of the nine flasks was considered an independent treatment as flask sets were changed at 60 and 96 hours (n=9). Statistics and graphs were generated using the R statistical program (version 0.99.879; 2016) and α was set at 0.05.

RESULTS

Single N Experiment.

Growth Rates. The cultures demonstrated a two-day lag period where cell concentrations remained constant or declined before cultures transitioned into exponential growth. Overall, exponential growth rates were similar between the two strains and single-N treatments, i.e., NH_4^+ , NO_3^- , or urea, and the experiment ended prior to stationary phase (Figures 1A and 1B). No difference in growth rate was detected between *A. fundyense* strains (Kuskal Wallis ANOVA: $\chi^2=0.29$, $df=1$, $p=0.588$), with ATSP7-D9 (Figure 1A) demonstrating a growth rate (μ) of 0.34-0.37 d^{-1} and GTCA-28 (Figure 1B) a μ of 0.26-0.29 d^{-1} during exponential growth. Similarly, an ANOVA did not detect an effect of nitrogen treatment, i.e., chemical form, on growth rate.

Nitrogen Utilization. The initial seawater, pre-amendment, contained concentrations of 0.6 μM NO_3^- , 1.97 μM NH_4^+ , and 0.14 μM -N urea. These N chemical forms remained under 2.5 μM for the remainder of the experiment with the exception of the specific N chemical form added every 6-24 hours. During the last 6 hours of the experiment for the strain ATSP7-D9, the average uptake rate of NO_3^- was $0.024 \pm 0.003 \text{ N} \cdot \text{cell}^{-1} \cdot \text{d}^{-1}$, NH_4^+ was $0.01 \pm 0.007 \text{ N} \cdot \text{cell}^{-1} \cdot \text{d}^{-1}$, and urea was $0.003 \pm 0.003 \text{ N} \cdot \text{cell}^{-1} \cdot \text{d}^{-1}$. The strain GTCA-28 had a similar uptake rate for NO_3^- of $0.031 \pm 0.021 \text{ N} \cdot \text{cell}^{-1} \cdot \text{d}^{-1}$, and NH_4^+ of $0.009 \pm 0.0018 \text{ N} \cdot \text{cell}^{-1} \cdot \text{d}^{-1}$. Urea uptake rates were not calculated for GTCA-28 because instrument error was excessive at such high concentrations.

Isotope Fractionation. Both strains were sampled for endpoint isotope measurements while cultures were still undergoing exponential growth, but had reached a minimum cell concentration of $\sim 2,800 \text{ cells} \cdot \text{ml}^{-1}$. This cell concentration occurred on day 7 for the NO_3^- , NH_4^+ , and urea treatments with both strains, with the exception of the NO_3^- treatment for strain GTCA-28, which required an additional day. The degree of fractionation, reported as the enrichment factor, ϵ ,

represents the difference between the $\delta^{15}\text{N}_{\text{POM}}$ at the end of the experiment and the isotope value of the medium ($\delta^{15}\text{N}_{\text{medium}}$) immediately after the final N addition, i.e., 6 hours prior to the end of the experiment (Table 2). The only exception was for urea, where the ϵ value was calculated based on the $\delta^{15}\text{N}$ value of the urea stock reagent prior to dissolving into seawater (Eq. 2). The degree of N fractionation by *A. fundyense* varied significantly between N treatments ($\chi^2=14.3$, $\text{df}=2$, $p<0.01$), with the largest difference in isotopic ratios measured between cells and source N during the uptake and assimilation of NH_4^+ ($\epsilon = 23.5\pm 7.2\text{‰}$), followed by NO_3^- ($\epsilon = 2\pm 0.3\text{‰}$), and urea ($\epsilon = 0.34\pm 0.19\text{‰}$) (Table 2). The degree of source-mediated isotopic fractionation, however, was similar between stains, suggesting consistent uptake processes across the species (Table 2; Kuskal Wallis ANOVA: $\chi^2=0.6$, $\text{df}=1$). For this reason, strain data were grouped by N treatment prior to additional statistical analyses, improving replication to six flasks for each N source ($n=6$). Calculating the fractionation factor based on the F value (i.e., the amount of nitrogen still in the medium at a time point, divided by the initial medium; Eq. 4), resulted in similar ϵ values (NH_4^+ : $30.8\pm 8.9\text{‰}$, NO_3^- : $2.8\pm 0.2\text{‰}$, Urea: $0.6\pm 0.02\text{‰}$) to the ϵ values calculated from the 6 hours prior to the end of the experiment, suggesting that the calculated ϵ values are accurate.

The $\delta^{15}\text{N}_{\text{NH}_4^+}$ value of the medium increased from 2.1‰ to 24.8‰ in the ATSP7-D9 experiments over time, and to 15.5‰ in the GTCA-28 experiments (Table 2). The change in the $\delta^{15}\text{N}_{\text{NO}_3^-}$ value of the medium was minimal over the course of the experiment, increasing only from 12.7‰ to 13‰. We do not expect that the observed enrichment in ^{15}N in the NH_4^+ medium was due to recycling or remineralization of N by the dinoflagellate or microbial community as there was no measured increase in NO_3^- , NH_4^+ , urea, TDN, or DON through the course of the experiment.

Mixed N Experiment.

Growth Rates. Similar to the single-N experiment, all cultures in the mixed-N experiment showed a lag and then exponential growth, with μ ranging from 0.51-0.54 d⁻¹ (Figure 2). No effect of N pre-conditioning was detected on growth rate or between growth curves of the three treatments (Figures 3A, 4A, and 5A). All three treatments of the mixed-N experiment presented a higher growth rate (average μ of 0.51-0.54 d⁻¹) than ATSP7-D9 cells grown on a single-N source (average μ of 0.34-0.37 d⁻¹) (Figures 1B and 2). Slight variations in cell concentration were observed between replicates during flask changes at 60 and 96 hours, i.e., when subsequent measurements were then taken from another set of triplicate flasks inoculated at the same time. When comparing the nine flasks over a treatment, however, there was no difference detected between flask-specific growth rates (Repeated Measures ANOVA: F-value=3.345, df=2).

Nitrogen Utilization. During the mixed-N source experiment, there was no indication that preconditioning *A. fundyense* cells to any of the three N forms affected the overall pattern of NH₄⁺ utilization (ANOVA: F=3.1, df=1), NO₃⁻ utilization (ANOVA: F=71.31, df=1), or urea utilization (ANOVA: F=2.943, df=1) over the course of the experiment. As such, a consistent pattern of N selectivity holds across treatments, whereby *A. fundyense* cells took up NH₄⁺ and urea initially, and only started assimilating NO₃⁻ after NH₄⁺ was drawn down below 2-4 μ M and total other N (NH₄⁺ + urea) was below 20 μ M (Figures 3A, 4A, 5A). In comparison, urea concentrations ranged from 5-30 μ M-N at the onset of NO₃⁻ utilization, and continued to be utilized at the same time as NO₃⁻ (Figures 3A, 4A, 5A). The maximum N uptake rates varied between the N chemical forms; with the highest uptake on NH₄⁺ (0.036 N•cell⁻¹•d⁻¹), followed by NO₃⁻ (0.031 N•cell⁻¹•d⁻¹), and then urea (0.025 N•cell⁻¹•d⁻¹). Along with the depletion of N

during the experiment, phosphorous decreased by day 6 (i.e., decreased from ~14 μM initially to ~2 μM by Day 6).

Isotope Fractionation. The $\delta^{15}\text{N}_{\text{POM}}$ was at its lowest at around 48 hours (averaging -5‰) during co-utilization of NH_4^+ and urea, and then increased by 84 hours (averaging 0.9‰) during the uptake of NO_3^- and urea (Figures 3B, 4B, 5B). This pattern was consistent between treatments, demonstrating that preconditioning cells to NO_3^- , NH_4^+ , or urea did not affect the fractionation processes within cells (repeated measures ANOVA: $F=136.8$, $df=1$, $p=0.054$). As in the single-N source experiment, the $\delta^{15}\text{N}$ of the dissolved NH_4^+ , and to a lesser extent NO_3^- , increased over time in the medium. The $\delta^{15}\text{N}$ of the dissolved NH_4^+ increased from $2.6\pm 0.7\text{‰}$ to $19.5\pm 2.4\text{‰}$ by 48 hours and NO_3^- increased from $12.6\pm 0.2\text{‰}$ to $13.5\pm 0.2\text{‰}$ by 132 hours (Figure 6). As in the first set of experiments, we do not expect that the observed enrichment in ^{15}N in the NH_4^+ medium was due to recycling or remineralization of N by the dinoflagellate or microbial community as there was no measured increase in NO_3^- , NH_4^+ , urea, TDN, or DON through the course of the experiment.

Toxicity, Quotas and Toxin Profile.

The toxin profile (i.e., percent composition of each STX congener quantified within the cell) was consistent within a strain, across all N treatments in the single-N source experiment, NH_4^+ , NO_3^- , or urea (Figure 7). Profiles, however, varied between strains, with ATSP-D9 being dominated by C2 and GTX4, and GTCA-28 dominated by C2 and GTX3. This difference in toxin profile, however, did not lead to one strain being *more toxic* based on cellular toxicity calculations (i.e., toxicity estimated using toxicity equivalency factors, reported as saxitoxin equivalents per cell, Figure 8) (ANOVA: $F=2.17$, $df=1$, $p=0.16$). When averaged over all N

treatments, the total toxicity of ATSP7-D9 and GTCA-28 were 57.7 ± 13.6 and 46.3 ± 19.1 fmol STX eq. · cell⁻¹, respectively.

When comparing between N treatments (2-way ANOVA: $F=18.33$, $df=2$, $p<0.01$), total toxicity ($F=34.53$, $df=1$, $p<0.01$) (Figure 6) and total toxin content ($F=13.61$, $df=3$, $p<0.01$) (Figure 9) showed similar trends. For both strains, the NH_4^+ -grown culture was significantly more toxic than the urea-grown culture. The NO_3^- -grown cultures, however varied in toxin content; where strain ATSP7-D9 had a lower toxin content that was similar to urea-grown culture while strain GTCA-28 had a higher toxin content similar to ammonium-grown culture. When comparing across both strains and all N-treatments, toxicity was greatest under NH_4^+ utilization by strain ATSP7-D9 (mean: 73.5 ± 4.2 fmol STX eq. · cell⁻¹), and was the lowest when strain GTCA-28 was taking up urea (mean: 24 ± 2.4 fmol STX eq. · cell⁻¹, Figure 8). Total cellular N was positively correlated with total toxin content ($y = 3.25x + 21.24$; $r^2=0.81$; $p<0.01$; Figure 10).

DISCUSSION

Nutrient availability can affect bloom species composition, duration, concentration, and toxicity (Glibert & Terlizzi 1999; Anderson et al. 2002; Anderson et al. 2008; Hattenrath et al. 2010). As such, more information is needed regarding cellular toxicity, growth, and nutrient preference in dinoflagellates when grown on different chemical forms of nitrogen (N), under different nutrient regimes if we are to improve predictive and management capabilities. Stable isotopes provide a method by which to identify the sources and chemical forms of N assimilated by algae in a system, and thus, better understand the connection between nutrients and HABs. Here we investigate the fractionation of nitrogen isotopes by saxitoxin-producing *Alexandrium fundyense*, to validate the use of the $\delta^{15}\text{N}$ of particulate organic matter and identify the nitrogen source fueling a dinoflagellate bloom and its toxicity. Although dinoflagellates are arguably the most cosmopolitan and prolific of the HAB groups, minimal work has studied isotopic fractionation by this taxonomic group, and no research to date, has specifically investigated fractionation during NH_4^+ or urea assimilation by dinoflagellates. Here we document fractionation during NO_3^- , NH_4^+ or urea assimilation by two strains (ATSP7-D9 and GTCA-28) of *Alexandrium fundyense* and the changes in isotope fractionation with simultaneous growth on NO_3^- , NH_4^+ and urea. Furthermore, we report how toxicity is affected by N chemical form and describe inconsistencies in the response between strains of the same species.

Under N-sufficient conditions, *Alexandrium fundyense* cells can utilize NO_3^- , NH_4^+ , or urea for growth and toxin production, but are also capable of taking up organic and inorganic forms simultaneously to support these processes (Figures 3, 4, 5, 6). *Alexandrium fundyense* preferentially assimilated urea and NH_4^+ , while NO_3^- was only utilized when NH_4^+ was below a threshold of 4 μM (Figure 5). This inhibition of NO_3^- by NH_4^+ is already well-documented in species of diatoms and dinoflagellates, but can vary between species, N availability, and

concentrations of N (Dortch 1990; Dortch et al. 1991; Waser, Yim, et al. 1998; Maguer et al. 2007; Shankar et al. 2014). This ability to simultaneously utilize organic and inorganic N, likely provides many toxic dinoflagellates a competitive advantage over other algal species (Glibert & Terlizzi 1999; Collos et al. 2007). For instance, Glibert and Terlizzi (1999) reported toxic dinoflagellate blooms near hybrid striped bass aquaculture ponds with urea levels greater than 1.5 $\mu\text{M-N}$, and Hattenrath and co-authors (2010) found that when NH_4^+ was added to bloom water from Northport Harbor, toxin concentration and cell density increased. This preferential assimilation of urea paired with the co-utilization of multiple inorganic forms is a concern for the management of *A. fundyense* and PSP, especially as urea-based fertilizers now comprise over half of the market (Anderson et al. 2002; Glibert et al. 2006).

The growth rates of *A. fundyense* were similar when grown under N-sufficient conditions of NO_3^- , NH_4^+ , or urea (Figure 1A and 1B), supporting previous research using another strain grown under higher, but depleting, concentrations of N in axenic batch culture (Dyhrman and Anderson 2003). Interestingly, we found the growth rate of *A. fundyense* was significantly faster in the mixed-N source (0.51-0.54 d^{-1}) relative to the single-N source experiment (0.34-0.37 d^{-1}). This increase in growth rate could be linked to a physiological response to the combination of inorganic and organic forms of N that leads to a greater growth rate during simultaneous utilization. The difference in growth between experiments, however, is not due to the amount of total N available, as concentrations were similar between the treatments: mixed-N cultures (max 121 $\mu\text{M-N}$) and single-N cultures (max 195 $\mu\text{M-NO}_3^-$).

Despite similar growth rates, toxin quotas (toxin per cell) and toxicity (calculated from TEFs) of *A. fundyense* varied with N chemical form. For both strains of *A. fundyense*, toxin quotas and toxicity were significantly lower in cells grown on urea relative to cells grown on

NH_4^+ (Figures 8, 9). Other researchers have found a similar reduction in toxin content under urea utilization, including STX-producing *Alexandrium tamarense* and *Alexandrium fundyense* and a pinnatoxin G-producing *Vulcanodinium rugosum* (Dyhrman and Anderson 2003; Leong et al. 2004; Abadie et al. 2015). Leong et al (2004) suggest that utilizing urea for the synthesis of toxins requires different mechanisms than during NO_3^- and NH_4^+ utilization. Interestingly, observed changes in toxicity in response to N chemical form were not due to changes in toxin profile (i.e., percent composition of congeners, Figure 7), as the relative percent of each congener remained essentially constant within strains across N chemical forms. Instead, toxicity differences were due to changes in the amount of toxin per cell (toxin quotas or content) between N treatments. This is in contrast to a previous study with an axenic strain of *A. fundyense* that demonstrated a change in profile, i.e., a relative decrease in C1,2 and increase in GTX2,3, that lead to increased toxicity under urea utilization (Dyhrman and Anderson 2003). Together these growth and toxicity studies suggest that the size of an *A. fundyense* bloom is not restricted by the N chemical form available, but that systems with mixed N forms may support larger blooms and ammonium-fueled blooms may be relatively more toxic due to increased toxin per cell.

While *A. fundyense* was consistently less toxic during urea utilization than during NH_4^+ utilization, a discernable pattern was not evident between NO_3^- and NH_4^+ . Strain ATSP7-D9 was more toxic and had higher toxin content when using NH_4^+ than when utilizing NO_3^- and strain GTCA-28 had relatively the same toxicity on NO_3^- and NH_4^+ in exponential growth (Figure 8 and 9). When grown on a similar concentration (50 μM) of NO_3^- or NH_4^+ , the axenic strain CB301A in stationary phase had similar toxin quotas between treatments, but toxin content significantly increased with N availability (883 μM - NO_3^- , Dyhrman and Anderson 2003).

Toxin content was directly correlated to cellular N in the single-N experiment (Figure 10; $r^2=0.81$, $p<0.01$). This finding is in agreement with early studies showing that N limitation leads to reduced production rates of N-rich saxitoxin and lower overall toxin content in *A. fundyense* (Anderson et al. 1990). The strong correlation between cellular N and toxin content during exponential growth suggests a concurrent allocation of N between important metabolic processes and toxin synthesis (Anderson et al. 1990). During the single-N experiment, the NH_4^+ grown cultures consistently had more cellular N and toxicity.

Also in question was how *A. fundyense* might respond to rapid changes in N source, e.g., during a storm event or after N remediation. To begin to address this concern, cultures of *A. fundyense* were pre-conditioned to three different chemical forms before being abruptly inoculated into a mixed-N medium. The fact that N preference, rates of uptake or utilization, and NH_4^+ and NO_3^- thresholds were consistent across all three pre-conditioning treatments (Figures 3A, 4A and 5A), suggests that past environmental N conditions likely do not affect future bloom responsiveness and that *A. fundyense* can rapidly proliferate when new anthropogenic sources of urea and NH_4^+ are introduced. It is notable that this species' ability for N switching and co-utilization may provide a competitive advantage over other phytoplankton species during variable N conditions, as Dortch et al (1991) found preconditioning the diatom, *Thalassiosira pseudonana*, to particular N forms affected uptake rates, preference, and the inhibition of other N forms.

With the larger goal of determining the utility of N stable isotopes in linking N source to dinoflagellate blooms, we evaluated N isotope fractionation by *A. fundyense* cells grown on NH_4^+ , NO_3^- , or urea. There was a significant difference in N fractionation between the three sources, i.e., N chemical forms ($\chi^2=14.3$, $df=2$, $p<0.01$), but little difference in isotopic

fractionation between the two strains: GTCA-28 and ATSP7-D9 (Table 2; Kuskal Wallis ANOVA: $\chi^2=0.6$, $df=1$, $p=0.44$). Growth on urea and NO_3^- led to an average N isotope ratio in the cells, $\delta^{15}\text{N}_{\text{POM}}$, that was reliably lower than the source, with an average ϵ value of 0.34‰ and 2.37‰ (average of two strains), respectively (Table 2). The fractionation observed during NO_3^- utilization was comparable to the results of Smith and Erdner (2011) who demonstrated that during stationary growth, *A. fundyense* cells were 1.5‰ lower than the NO_3^- source. Similarly, an ϵ value of 1-3‰ for other dinoflagellates grown on NO_3^- , has been reported (Needoba et al 2003). While no data have been reported previously for dinoflagellate fractionation when grown on urea, the average ϵ values reported here (0.34‰) is similar to those reported for marine diatoms, 0.8‰ (Waser, Harrison, et al. 1998). Although we were unable to generate a $\delta^{15}\text{N}$ of the dissolved urea 6 hours prior to the end of the experiment for comparison with the $\delta^{15}\text{N}_{\text{POM}}$ (due to method limitations), the small ϵ value, calculated using the initial stock reagent and endpoint POM, suggests a minimal change in $\delta^{15}\text{N}$ of the dissolved urea source over the course of the entire experiment. Together these results confirm the reliability of the $\delta^{15}\text{N}$ tool in NO_3^- and urea-rich environments as subpopulations are expected to respond similarly (as we found no detectable difference between strain response), and the $\delta^{15}\text{N}_{\text{POM}}$ will closely resemble the $\delta^{15}\text{N}$ of the source. When applied, $\delta^{15}\text{N}_{\text{POM}}$ will allow discrimination between the contribution of anthropogenic vs natural sources of the same chemical form or between different chemical forms that fuel dinoflagellate blooms. The latter will be dependent upon the user's ability to characterize the signals of the various endmembers to the system.

Fractionation, ϵ value of 29‰, was larger when cells were directly utilizing NH_4^+ as opposed to NO_3^- or urea utilization (Table 2). The medium became enriched in ^{15}N over time in the NH_4^+ treatment, i.e., the $\delta^{15}\text{N}$ of the dissolved NH_4^+ increased, likely due to relatively rapid

NH_4^+ uptake rates and enhanced discrimination against ^{15}N in NH_4^+ uptake mechanisms as compared to during NO_3^- and urea utilization (Petersen & Fry 1987). This large ϵ could complicate the use of $\delta^{15}\text{N}_{\text{POM}}$ as a tracer in NH_4^+ rich environments, as the $\delta^{15}\text{N}_{\text{POM}}$ could misleadingly mimic an alternative source. Systems receiving both anthropogenic (e.g., heavier NH_4^+) and natural N inputs (e.g., lighter NO_3^-), for example, may produce a $\delta^{15}\text{N}_{\text{POM}}$ that looks largely of natural influence as the large ϵ value during NH_4^+ utilization and relatively small fractionation on NO_3^- would mask the anthropogenic contribution. In many situations, however, the ϵ value for growth on NH_4^+ is so large, that it could be identifiable by isotopically light cells well below the $\delta^{15}\text{N}$ of any of the identified sources.

The large fractionation observed in dinoflagellates grown in $25\ \mu\text{M-NH}_4^+$ (ϵ value of 29‰) may only be applicable in systems with elevated NH_4^+ concentrations. Other studies have reported similar isotopic fractionation, 27.2‰, in diatoms when NH_4^+ concentrations ranged between 20-50 μM , but found a smaller difference between source and diatoms, 7.8‰, when NH_4^+ concentrations were between 5-20 $\mu\text{M-NH}_4^+$ (Pennock et al. 1996; Vavilin et al. 2014). A concentration-dependent flip in enzymatic processes during N transport was proposed as the driver of this variability in fractionation. If dinoflagellates follow the same pattern as diatoms, then the $\delta^{15}\text{N}_{\text{POM}}$ of dinoflagellate blooms will more effectively mimic the source in low concentrations of NH_4^+ and the utility of $\delta^{15}\text{N}$ will be retained in the presence of anthropogenic NH_4^+ .

To investigate the utility of $\delta^{15}\text{N}_{\text{POM}}$ within more complex systems, i.e., with multiple N inputs, we also incubated cells in mixed-N medium of NO_3^- , NH_4^+ and urea. In estuarine and coastal ecosystems, the N isotopic composition of nutrients is constantly fluctuating and multiple N chemical forms are available simultaneously (Kendall et al, 2007). As such, the identification

of the original N source/form via N isotope ratios in the field can be further complicated by the change in N uptake or the co-utilization of multiple N chemical forms. In our mixed-N source experiments, the response rate of $\delta^{15}\text{N}_{\text{POM}}$ was rapid (within 24 hours) to the new source being utilized by the cells (Figure 5). Isotope discrimination between source and POM was large during the first two days ($\epsilon = 23\text{‰}$) and decreased to a small value ($\epsilon = 3.3\text{‰}$) upon exhaustion of NH_4^+ and switch to NO_3^- utilization (Figure 7). Co-utilization of both inorganic and organic N forms was evident in the isotope ratios of POM as 1) the ϵ value was not as large in the first 48 hours as would be expected if the cells had only been utilizing NH_4^+ , confirming urea co-utilization, and 2) after the depletion of NH_4^+ , the isotopic signature of the cells, $\delta^{15}\text{N}_{\text{POM}} = 3.3\text{‰}$, was between the $\delta^{15}\text{N}$ value of urea (-0.7‰) and NO_3^- (12.7‰) in the medium, again confirming co-utilization. Together this suggests that the $\delta^{15}\text{N}_{\text{POM}}$ represents a weighted average of all the N sources being immediately taken up by the cells. This means that a bloom utilizing both urea and NH_4^+ in the field could produce a smaller magnitude of fractionation, as the $\delta^{15}\text{N}_{\text{POM}}$ is indicative of a source that is a mixture of $\delta^{15}\text{N}_{\text{NH}_4^+}$ and $\delta^{15}\text{N}_{\text{urea}}$. A mixed isotope model (Waser, Yim, et al. 1998; Vavilin et al. 2014) could be used in the field when dinoflagellates are utilizing more than one N source. Waser et al (1998b) found that when *Emiliania huxleyi* was given a medium containing NO_3^- , NH_4^+ , and urea, apparent discrimination was initially lower than growth on NH_4^+ would suggest (i.e. $\delta^{15}\text{N}_{\text{POM}}$ was closer in value to $\delta^{15}\text{N}_{\text{SOURCE}}$). They concluded that this was due to the simultaneous growth of the cells on NH_4^+ , with a high ϵ of 17‰ , and urea, with a low ϵ of 0‰ , which led to a ϵ between these values. As such, simultaneous growth of NH_4^+ and urea in the field would lead to smaller changes in $\delta^{15}\text{N}_{\text{POM}}$.

CONCLUSIONS

In summary, this study is the first to show the impact of N chemical form and N preference on N isotope fractionation during uptake by dinoflagellates. Overall, a better understanding of N metabolism within dinoflagellates and the role of N in the synthesis of toxins would enhance prediction and control of HABs. The results of this study demonstrate the ability for $\delta^{15}\text{N}_{\text{POM}}$ to be used as a source tracer in systems rich in either NO_3^- or urea, e.g., septic-contaminated groundwater sites like in the kettle ponds of Nauset Marsh Estuary Systems or Gulf of Maine where NH_4^+ concentrations are minimal and N is primarily in the form of NO_3^- . Toxin content and toxicity were dependent upon the N chemical form being utilized, as well as the *A. fundyense* strain, suggesting that eutrophication and dominating strains can affect cellular toxicity of a bloom. High inputs of NH_4^+ , for example, may lead to large blooms of *A. fundyense* with elevated toxicity per cell.

The novel results presented here for dinoflagellates are consistent with the isotopic fractionation results found for other taxonomic groups, namely diatoms and coccolithophores, and adds new insight into the biochemistry of N utilization and assimilation for toxin production by dinoflagellates, and how preferential uptake affects $\delta^{15}\text{N}$. Causal analysis of nutrient pollution sources via stable isotopes will advance sustainability for the environment, economy, and human health by helping policy makers make informed decisions about point and nonpoint source pollution control in the United States. Nutrients are only one factor influencing bloom dynamics, but the relative importance of natural or anthropogenic nutrients in the development of a specific toxic bloom is necessary to predict future decadal, annual, and compositional shifts in algal blooms.

FUTURE RESEARCH

In order for the isotopic signature of POM to be utilized as a tracer, identifying the original source of N, further research must also identify a method for analyzing the $\delta^{15}\text{N}$ of dissolved urea. Based on the results of this study, *A. fundyense* can utilize organic N, urea, at the same time as inorganic forms of N (NH_4^+ and NO_3^-). In areas with increased land runoff, urea concentrations can be substantial as urea is becoming the leading N component of fertilizer and is used in animal feed and manufacturing products. More specifically, urea-based fertilizers now comprise >50% of the market and worldwide urea usage has increased 100-fold in the past 4 decades, which could translate to an increase in dinoflagellate HABs (Glibert et al. 2006). Although many wastewater treatment plants have updated technology to successfully reduce inorganic N discharge, organic N continues to be introduced to the ocean in the effluent. With a realized method for dissolved urea in seawater, $\delta^{15}\text{N}_{\text{POM}}$ can then be validated in controlled field studies with the goal of identifying when urea-based eutrophication is a driver of potentially less toxic *Alexandrium fundyense* blooms.

Another area for research is in the identification of a species-specific compound for stable-isotope analysis. Compound-specific stable isotope analysis (CSIA) is often used in describing organic matter composition and for distinguishing between the constituents of bulk POM samples. Since organic matter is comprised of multiple substances/organisms that all fractionate differently, a compound specific to an algal species may be a more appropriate tool in identifying the N source fueling HABs and increased toxin production. Saxitoxins (STXs) have the potential to be used as species-specific tracers, as most are only produced by one species in an area at a given time and the toxins are relatively stable, nitrogen-rich compounds (6-7 N atoms per molecule). Smith and Erdner (2011) exposed the dinoflagellate *Alexandrium fundyense* to 883 μM concentrations of nitrate and found the *Alexandrium* cells and saxitoxins

were 1.5‰ and 3.0‰ lighter than the source, respectively, suggesting that STXs mimic the $\delta^{15}\text{N}$ of the N source. Saxitoxins also responded to the N source more rapidly than the bulk cells (Smith and Erdner 2011), indicating $\delta^{15}\text{N}_{\text{STX}}$ may be more appropriate tool than the $\delta^{15}\text{N}$ of particulate organic matter when identifying N sources in more dynamic systems. Experiments are currently being conducted to isolate STX from all other internal and external N compounds and examine the change in $\delta^{15}\text{N}_{\text{STX}}$ under different nutrient regimes to identify the potential for this chemical to be utilized as a species-specific source tracer (Appendix 2). Beyond this project's application in nutrient management and bloom dynamics, the $\delta^{15}\text{N}_{\text{STX}}$ could provide additional information regarding STX synthesis within dinoflagellates.

TABLES

Table 1. Major natural and anthropogenic nitrogen sources to the ocean and corresponding $\delta^{15}\text{N}$ values for dissolved nitrate (NO_3^-).

Source	Form of N	$\delta^{15}\text{N}$ - NO_3^-	Reference
Atmospheric Deposition-Natural (biogenic soil emissions, lightning, biomass burning)	N_2 , NO_3^- , NH_4^+	<0‰	Hoering (1957)
Atmospheric Deposition-Fossil Fuel Combustion	NO_3^- , NH_4^+	Coal combustion: +4.8-9.6‰ Vehicle emissions: -13 to 2‰	Kiga et al. (2000) Heaton (1990)
Wastewater Treatment Plants	Urea, NO_3^- , NH_4^+ , NH_3	10-30‰	Kendall et al (2007)
Fertilizer	Urea, NO_3^- , NH_4^+ , NH_3	-4 to +4‰	Hübner (1986)
Animal Manure	Urea, NO_3^- , NH_4^+ , NH_3	+10 to +20‰	Kreitler (1975, 1979) USDA. Manure Management Information Sheet. 2007.

Table 2: Growth rates (μ), nitrogen isotope ratios ($\delta^{15}\text{N}$) of the medium and *Alexandrium fundyense* cells, i.e., particulate organic matter ($\delta^{15}\text{N}_{\text{POM}}$), and ϵ value based on $\delta^{15}\text{N}$ of the medium in the last 6 hours, $\delta^{15}\text{N}$ of the medium at the end of the experiment, and fraction of the medium taken up by the cells, when grown on one of three nitrogen forms during the single-N source experiment. There was no difference in ϵ value between the two strains ($\chi^2=0.59551$, p-value=0.44) but there are changes in fractionation based on the nitrogen source ($\chi^2=14.31$, p-value<0.01) being taken up by the cells. Samples performed in triplicate.

<i>Alexandrium fundyense</i> strain	ATSP7-D9			GTCA-28		
Nitrogen substrate	Nitrate	Ammonium	Urea	Nitrate	Ammonium	Urea
μ (d^{-1})	0.32±0.02	0.27±0.01	0.29±0.01	0.19±0.01	0.28±0.01	0.29±0.02
$\delta^{15}\text{N}$ medium (‰) - Last 6 hours	12.7±0.1	24.8±5.5	-0.3±0.4*	12.6±0.1	15.5±1.1	-0.3±0.4*
$\delta^{15}\text{N}$ medium (‰) - End of exp.	13.3±0.1	37±4.8		12.6±0	30.1±3.3	
$\delta^{15}\text{N}$ POM (‰) - End of exp.	10.6±0.2	-2.5±1.6	-0.75±0.1	9.2±2.5	-2.3±1.4	-0.57±0.2
ϵ value based on medium (‰)						
Last 6 hours	2.09±0.1	27.3±6.9	0.45±0.1	3.4±2.5	17.9±3.1	0.27±0.2
ϵ value based on medium (‰)						
End of exp.	2.6±0.1	39.49±3.59	na	3.4±2.5	32.45±2	na
ϵ value based on F (‰)	2.7±0.2	34.7±10.2	na	4.9±3.5	23.3±3.1	na

* Indicates that the “medium” nitrogen isotope ratios, $\delta^{15}\text{N}$, in the urea treatments were determined using stock reagent because standard methods do not yet exist for dissolved urea.

Mean ± SD

$$\mu = \ln(\text{concentration}_2 / \text{concentration}_1) / (\text{time}_2 - \text{time}_1)$$

$$\epsilon = (\delta^{15}\text{N}_{\text{medium}} - \delta^{15}\text{N}_{\text{POM}})$$

$$\epsilon = (\delta^{15}\text{N}_{\text{medium}} - \delta^{15}\text{N}_{\text{POM}}) / ((-f / (1-f)) * \ln(f))$$

F= fraction of unreacted medium/ total medium

na=data not available

Table 3: The individual ϵ value, nitrogen (N) concentration, and toxin content of each triplicate flask for two *A. fundyense* strains during the single-N source experiment, i.e., when grown on one of three N sources. Samples for N and toxin quantification were collected at the end of the experiment. Flasks were excluded if samples were lost.

Strain	N substrate	N concentration ($\mu\text{M-N}$)	ϵ value (%)	Toxin content (fmol/cell)
ATSP7-D9	NO_3^-	47.32	2.01	108.72
ATSP7-D9	NO_3^-	65.08	2.12	120.75
ATSP7-D9	NO_3^-	64.68	2.13	106.81
ATSP7-D9	NH_4^+	10.52	21.32	206.36
ATSP7-D9	NH_4^+	24.60	25.73	194.13
ATSP7-D9	NH_4^+	23.32	34.80	183.91
ATSP7-D9	Urea	70.34	0.50	138.33
ATSP7-D9	Urea	83.56	0.40	130.44
GTCA-28	NO_3^-	119.68	2.21	118.01
GTCA-28	NO_3^-	189.20	1.62	121.43
GTCA-28	NO_3^-	195.41	6.26	144.12
GTCA-28	NH_4^+	19.01	20.04	107.53
GTCA-28	NH_4^+	12.77	15.72	74.54
GTCA-28	Urea	82.68	0.00	53.43
GTCA-28	Urea	86.47	0.40	41.05
GTCA-28	Urea	88.8	0.40	47.62

$$\epsilon = (\delta^{15}\text{N}_{\text{medium}} - \delta^{15}\text{N}_{\text{POM}})$$

FIGURES

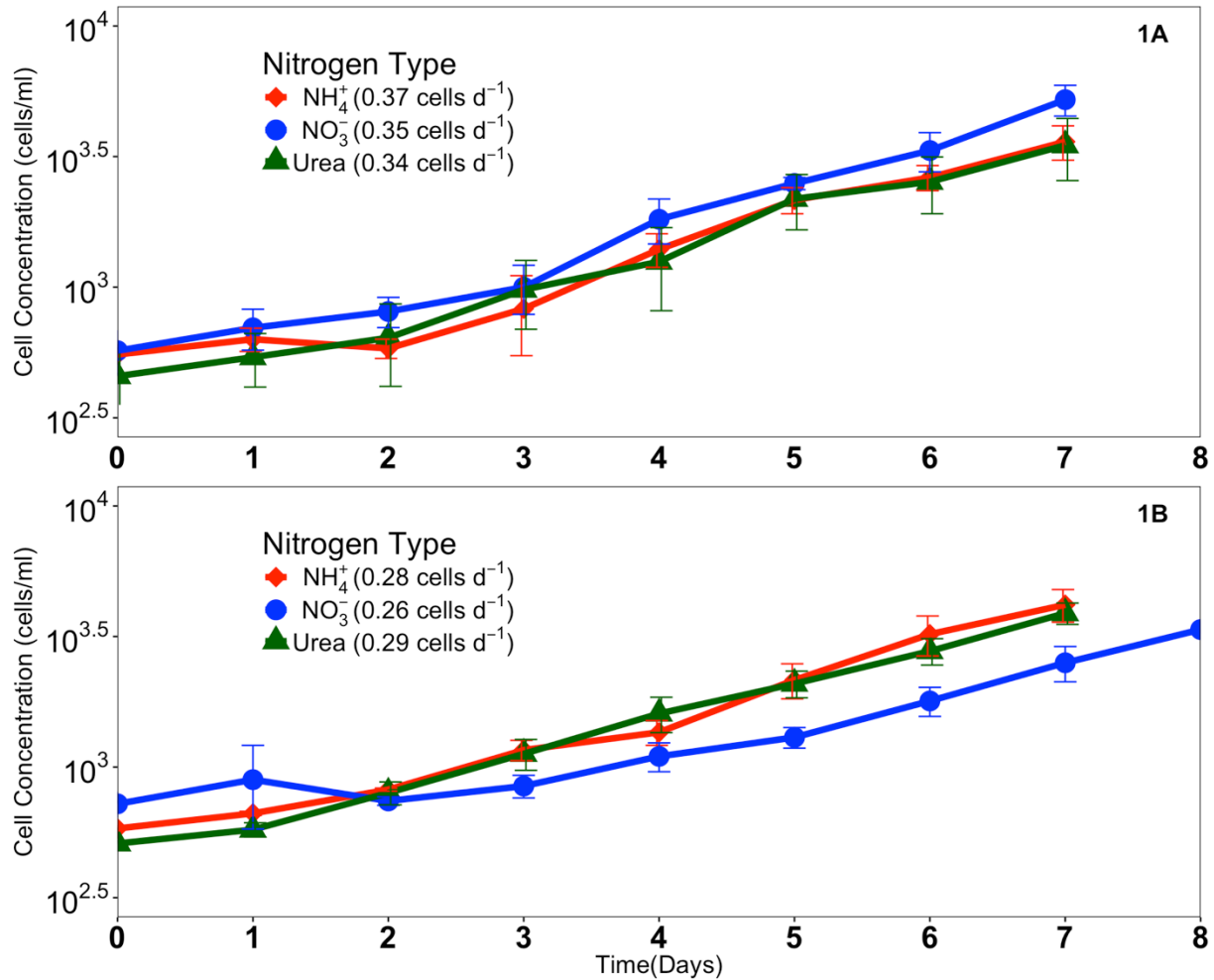


Figure 1. The increase in cell concentration and growth rates for strains ATSP7-D9 (1A) and GTCA-28 (1B) over time when grown separately on ammonium (\blacklozenge), nitrate (\bullet), or urea+Ni (\blacktriangle) during the single-N source experiment. Starting after growth lag, strain ATSP7-D9 cells grew at an average growth rate (μ) of 0.37 cells \cdot d⁻¹ on NH₄⁺, 0.35 cells \cdot d⁻¹ on nitrate, and 0.34 cells \cdot d⁻¹ on urea+Ni and GTCA-28 grew at growth rate (μ) of 0.28 cells \cdot d⁻¹ on NH₄⁺, 0.26 cells \cdot d⁻¹ on nitrate, and 0.29 cells \cdot d⁻¹ on urea+Ni. Statistical analyses performed using log-transformed cell concentrations indicated no significant difference between strains ($p=0.588$) nor N type ($p=0.6953$) (nonparametric Kruskal-Wallis Test) ($n=3$, mean \pm SD).

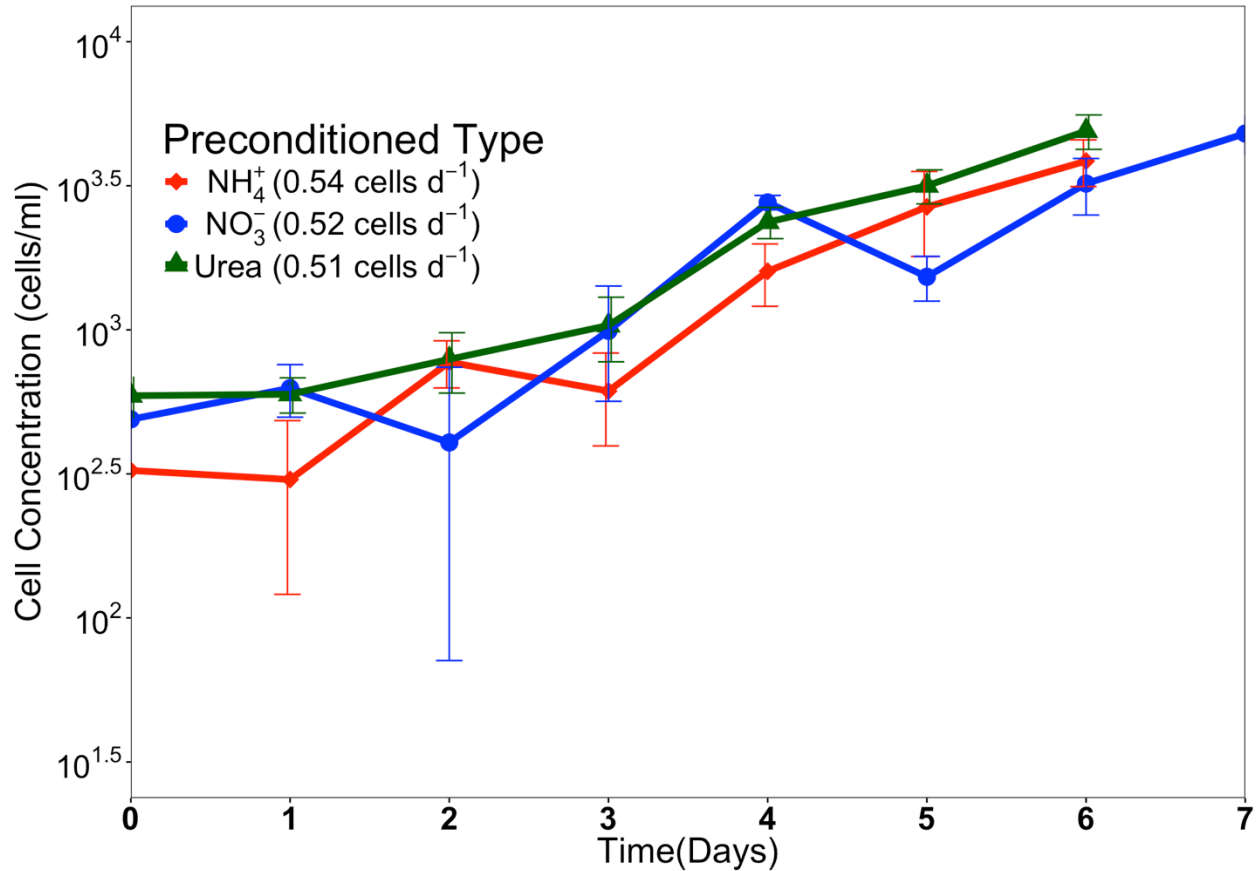


Figure 2. Increase in cell concentration and growth rates for strains ATSP7-D9 over time when preconditioned either on ammonium (♦), nitrate (•), or urea+Ni (▲) and then grown in mixed medium (all three N chemical forms) during the mixed-N source experiment. Starting after growth lag, strain ATSP7-D9 cells grew at an average growth rate (μ) of 0.51-0.54 cells \cdot d⁻¹. Statistical analyses performed using log-transformed cell concentrations indicated no significant difference based on the N type on which the cells were preconditioned (Repeated Measures ANOVA: F-value=3.345, df=2, p=0.172) (n=3, mean \pm SD).

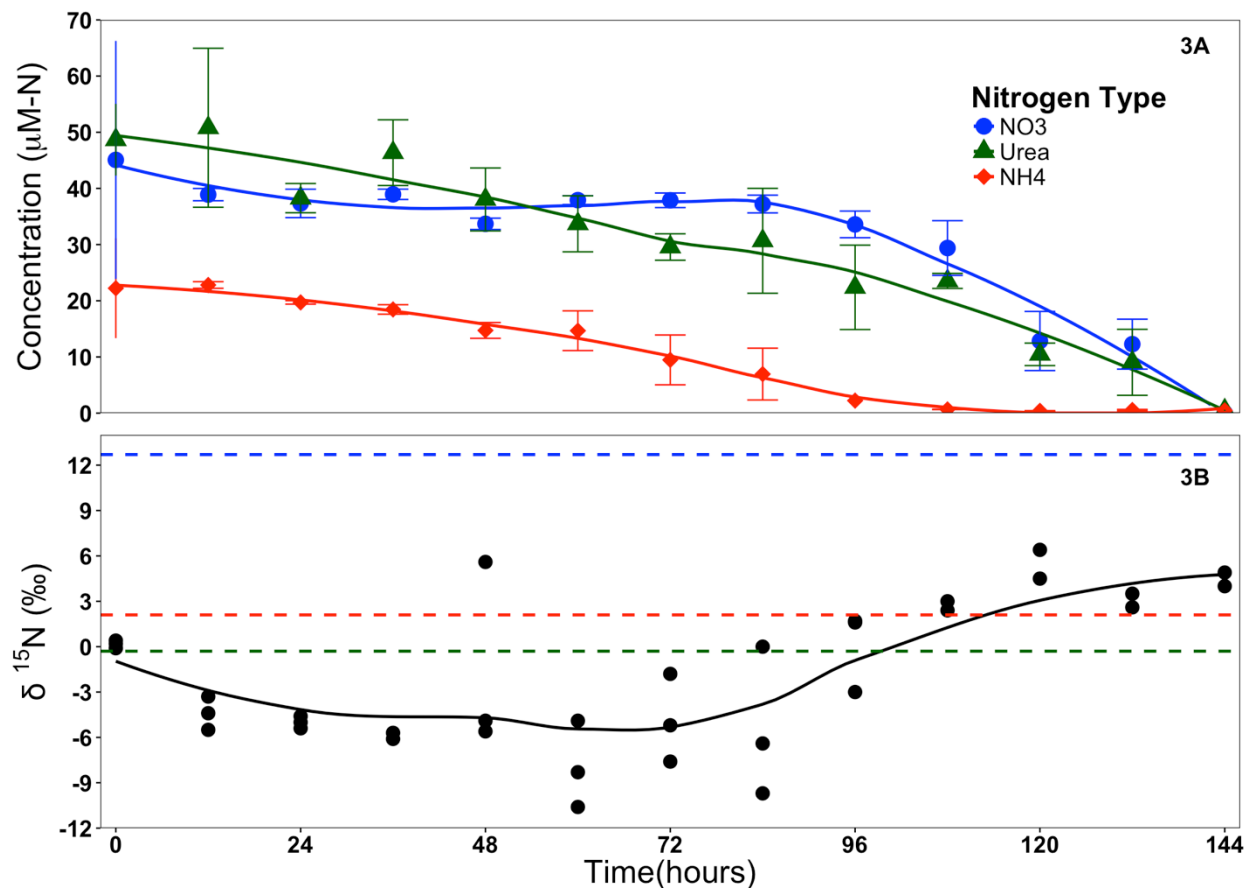


Figure 3. Nitrogen depletion (3A) and associated $\delta^{15}\text{N}$ of particulate organic matter (i.e., cells) (3B) over time for *Alexandrium fundyense* culture preconditioned to ammonium and then grown in mixed medium (all three N chemical forms) during the mixed-N source experiment. Ammonium (♦), nitrate (•), or urea+Ni (▲) concentrations are presented in $\mu\text{M-N}$. The $\delta^{15}\text{N}$ of whole cells are represented in black circles while the dashed lines represent the initial isotope value of nitrate (blue), ammonium (red), and urea (green). (n=3, mean \pm SD).

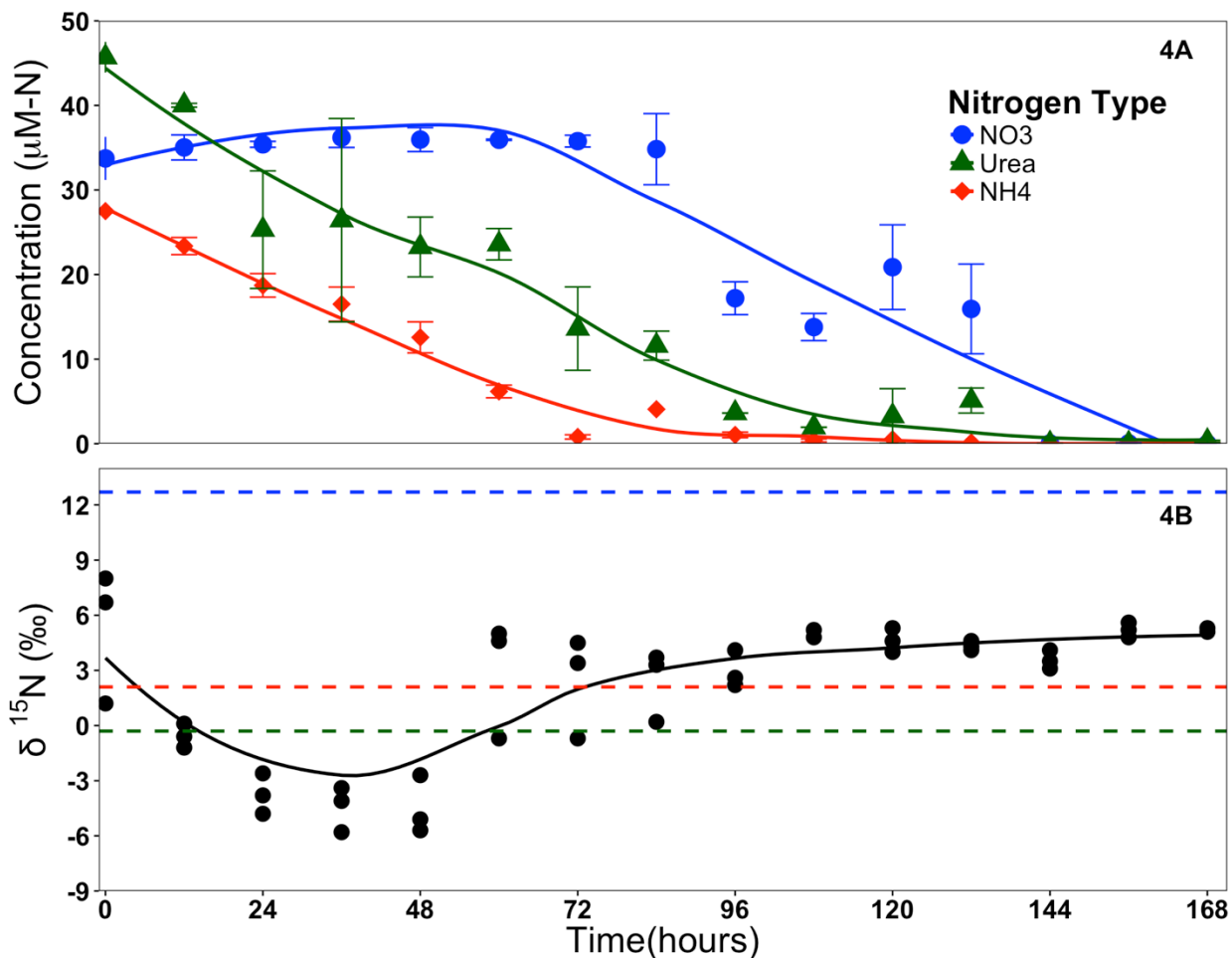


Figure 4. The Nitrogen depletion (4A) and associated $\delta^{15}\text{N}$ of particulate organic matter (i.e., cells) (4B) over time for *Alexandrium fundyense* culture preconditioned to nitrate and then grown in mixed medium (all three N chemical forms) during the mixed-N source experiment. Ammonium (\blacklozenge), nitrate (\bullet), or urea+Ni (\blacktriangle) concentrations are presented in $\mu\text{M-N}$. The $\delta^{15}\text{N}$ of whole cells are represented in black circles while the dashed lines represent the initial isotope value of nitrate (blue), ammonium (red), and urea (green). ($n=3$, mean \pm SD).

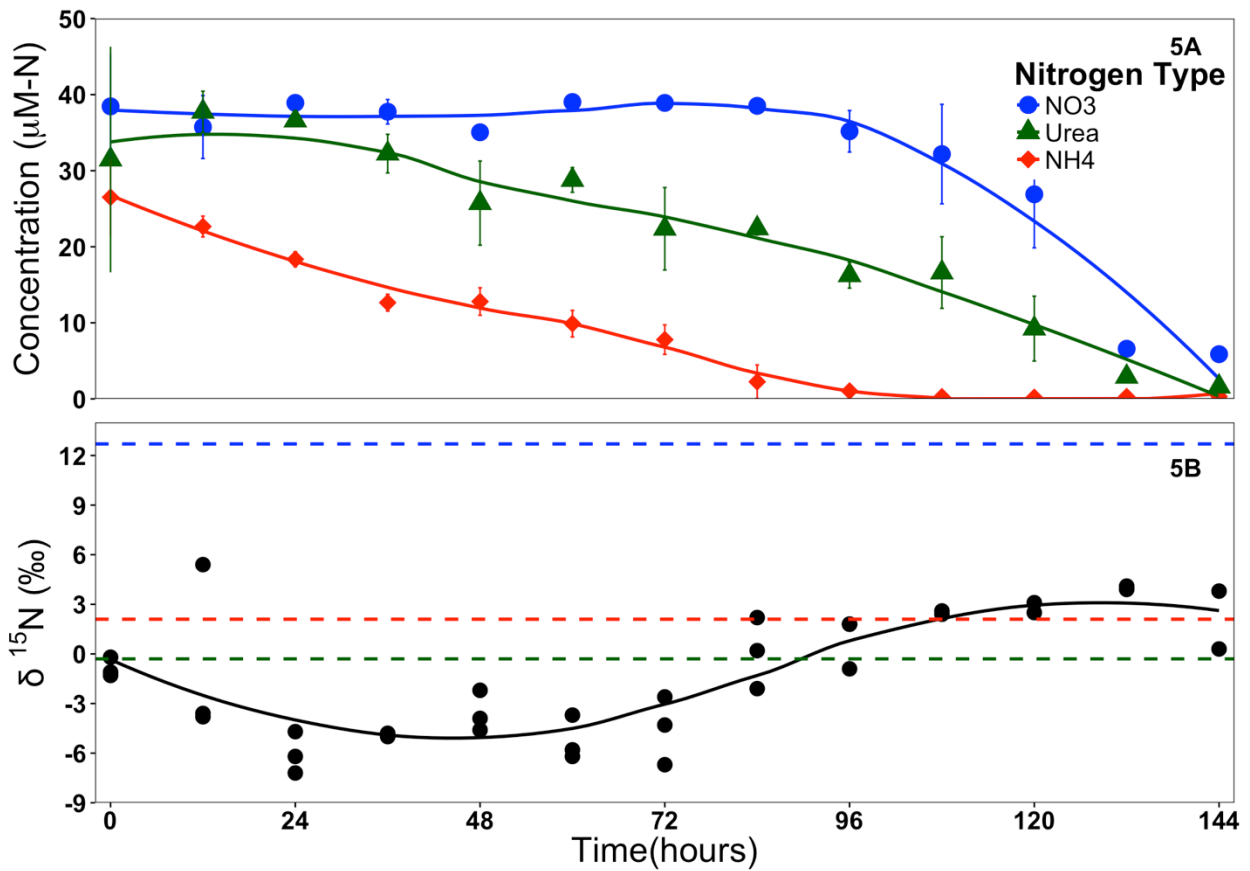


Figure 5. The Nitrogen depletion (5A) and associated $\delta^{15}\text{N}$ of particulate organic matter (i.e., cells) (5B) over time for *Alexandrium fundyense* culture preconditioned to urea and then grown in mixed medium (all three N chemical forms) during the mixed-N source experiment. Ammonium (\blacklozenge), nitrate (\bullet), or urea+Ni (\blacktriangle) concentrations are presented in $\mu\text{M-N}$. The $\delta^{15}\text{N}$ of whole cells are represented in black circles while the dashed lines represent the initial isotope value of nitrate (blue), ammonium (red), and urea (green). (n=3, mean \pm SD)

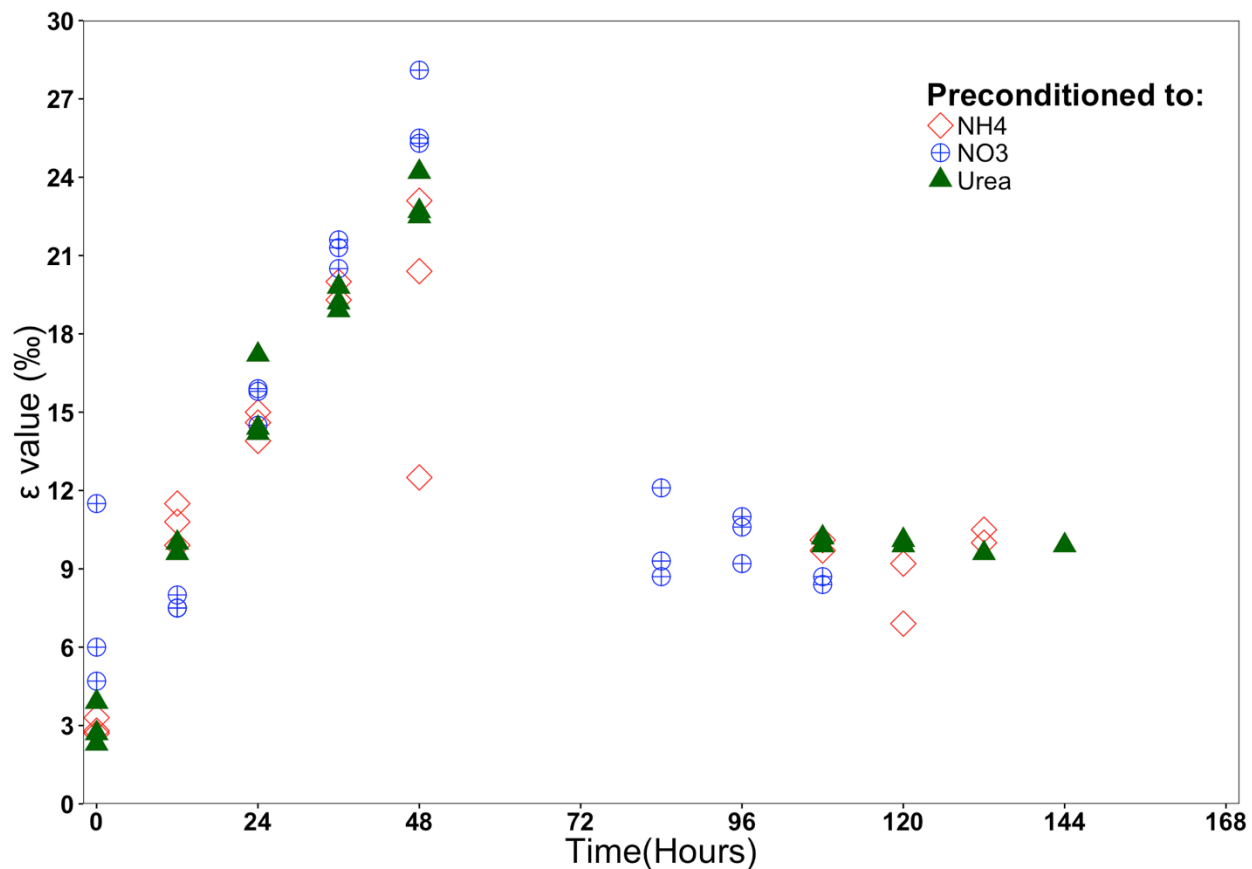


Figure 6. The estimated ϵ value of particulate organic matter (i.e., cells) over time when grown in mixed medium (all three N chemical forms) during the mixed-N source experiment. The ϵ value is calculated as the difference in the $\delta^{15}\text{N}$ of the medium minus the $\delta^{15}\text{N}$ particulate organic matter, assuming cells were only utilizing ammonium the first 48 hours and nitrate the last 54 hours. The ϵ value is presented for *Alexandrium fundyense* cells preconditioned on ammonium (\diamond), nitrate (\oplus), or urea+Ni (\blacktriangle) and given all three sources at the beginning of the experiment: time 0.

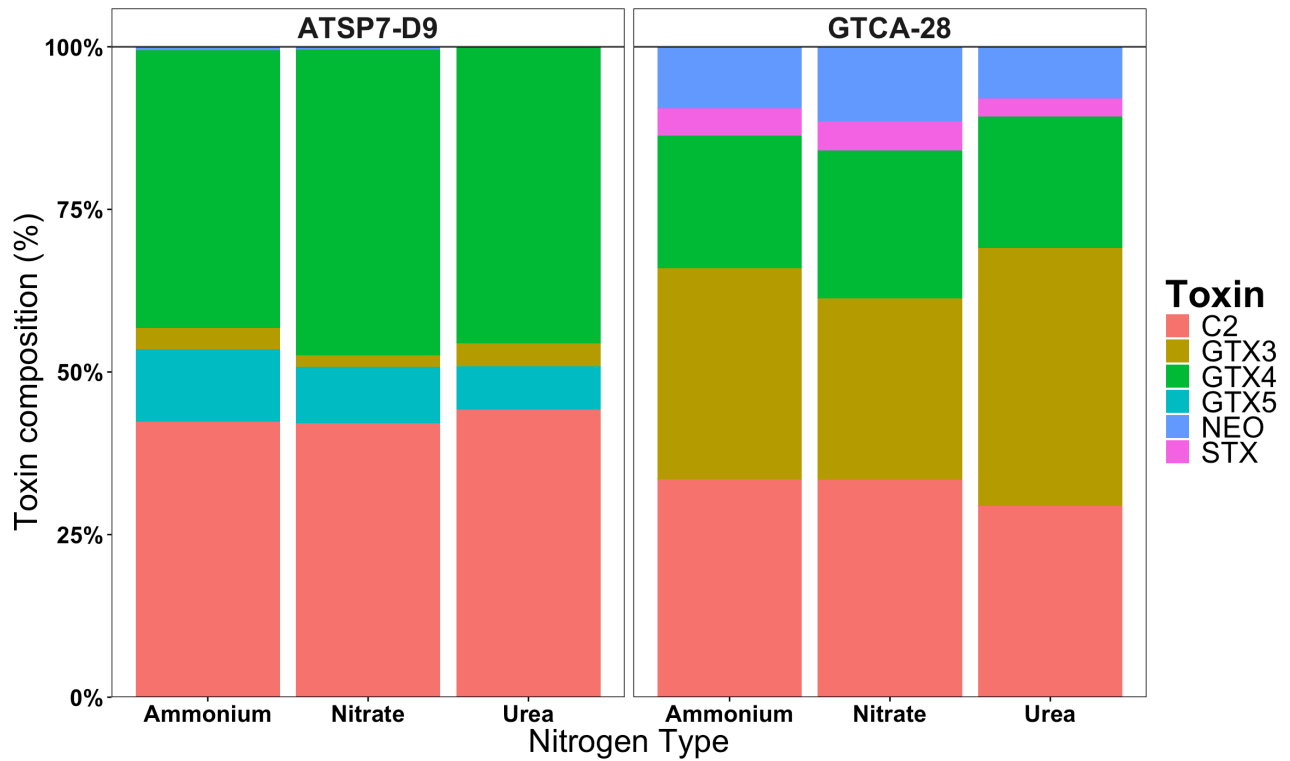


Figure 7. Percent toxin composition, i.e., profile, of two strains of *Alexandrium fundyense*: ATSP7-D9 and GTCA-28, when grown on three chemical forms of nitrogen: ammonium, nitrate, or urea (n=3) during the single-N source experiment. Variation in toxin composition within treatments were minimal with all standard deviation less than 5%, and all but two congeners less than 2%.

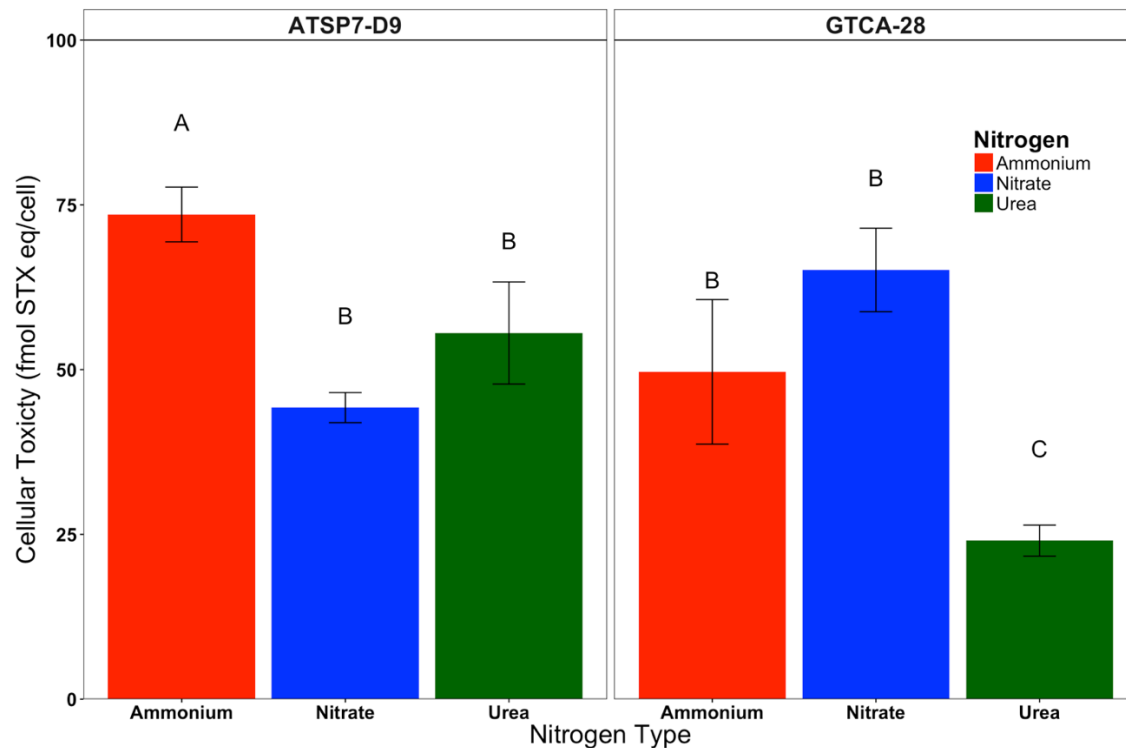


Figure 8. The total toxicity of *Alexandrium fundyense* strains ATSP7-D9 and GTCA-28 cells when exposed to ammonium, nitrate, urea during the single N source experiment. Total toxicity is calculated using the toxicity equivalent factors (TEFs) defined by Kasich, Taylor, Butler (2016). Data are plotted by mean \pm SD. Letters indicate significant differences between means (Post-Hoc ANOVA: Tukey).

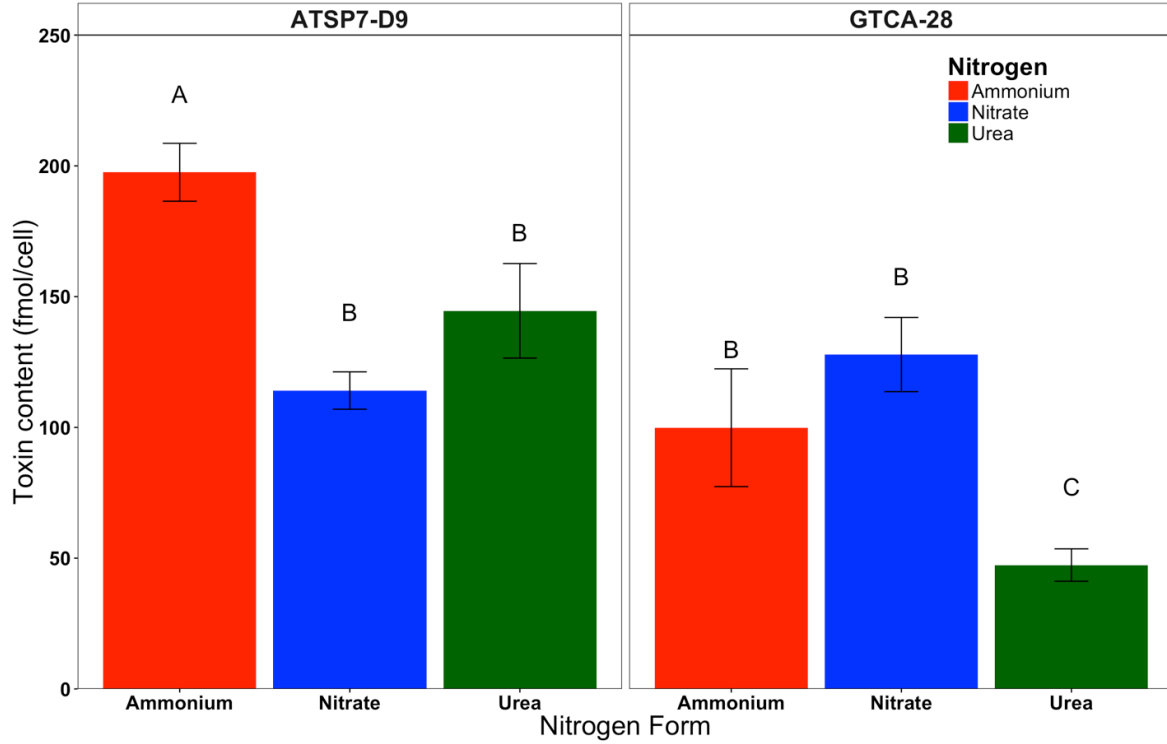


Figure 9. The total toxin content of *Alexandrium fundyense* strains ATSP7-D9 and GTCA-28 cells when exposed to ammonium, nitrate, urea during the single N source experiment. Data are plotted by mean \pm SD. Letters indicate significant differences between means (Post-Hoc ANOVA: Tukey).

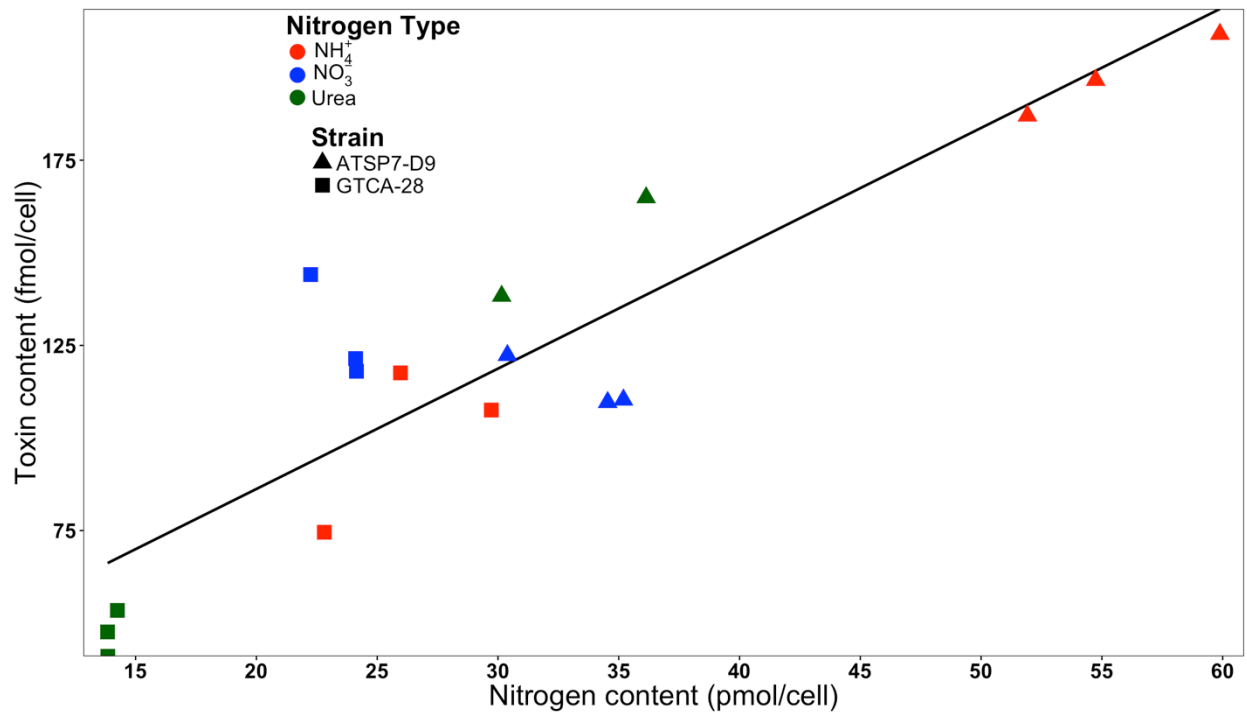


Figure 10. Relationship between nitrogen content per cell and total toxin content (or quota) per cell in two strains of *Alexandrium fundyense*: ATSP7-D9(▲) and GTCA-28(■), when grown on three chemical forms of nitrogen: ammonium (red), nitrate (blue), or urea+Ni (green) during the single N source experiment. $y = 3.25x + 21.24$; $r^2=0.81$.

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APPENDIX I-AMMONIUM DIFFUSION METHOD

Summary of Protocol

The ammonium (NH_4^+) diffusion method was modified from the procedure outlined by Holmes et al. (1998). This method was used to measure the isotopic signature of dissolved NH_4^+ in the medium, $\delta^{15}\text{N}_{\text{NH}_4^+}$. The method converts NH_4^+ into ammonia (NH_3) gas and traps the gas as $(\text{NH}_4)_2\text{SO}_4$ on an acidified glass fiber filter (1-cm diameter, Whatman GF/D, Cat #: 1823010). The NH_4^+ in the water is transformed into NH_3 by increasing the pH of water above 9 with MgO. The filter is then acidified with KHSO_4 and sealed between a polypropylene filter (25 mm diameter, Sterlitech, Cat #: PP0125100; Hannon & Bohlke 2008), allowing it to float on the surface of the water sample. A minimum of 0.5 μmoles nitrogen (N) is needed to exceed the lower detection limits of the elemental analyzer during isotope analysis of the $\delta^{15}\text{N}_{\text{NH}_4^+}$.

Abbreviations

MgO = Magnesium oxide

NaCl = Sodium chloride

KHSO_4 = Potassium bisulfate

$(\text{NH}_4)_2\text{SO}_4$ = Ammonium sulfate

NH_4^+ = Ammonium

NH_3 = Ammonia

K_2SO_4 = Potassium sulfate

Protocol

Preparation

1. Combust (450°C for 4hrs): 1 cm GF/D filters, MgO, NaCl (ACS grade)
2. Determine amount of sample needed based on the targeted 0.5-5 $\mu\text{moles-N}$ (7-70 μg or 2.5-25 μM) in 200-ml sample
3. Bring all samples to 200 ml with nanopure.

Filter Packets

1. Make 2.5M KHSO_4 as follows:
In a 50ml volumetric add
25ml nanopure water
3.5ml concentrated sulfuric acid
11 g K_2SO_4
add nanopure to 50ml mark
2. Clean sheet of aluminum foil, forceps, and a glass scintillation vial with 96% ethanol
3. Rinse polypropylene membranes in 10% HCl and then nanopure water
4. Layer paper towel sheets underneath aluminum foil and then spread out polypropylene membranes on sheet to dry
5. Place a 1 cm GF/D filter on a cleaned polypropylene membrane
6. Pipette 25ul of 2.5M KHSO_4 onto the GF/D filter
7. Fold the polypropylene membrane in half to wrap the GF/D filter and use the opened top of the scintillation vial to press the membrane sealed being careful not to press too hard and tear the filter but ensure it is sealed

8. Store filter packets in a clean vial capped tightly (parafilm the top under the cap) to avoid NH_3 from the atmosphere diffusing onto the acidified filter

¹⁵N_{NH4} Diffusion

1. Measure out sample volumes into acid-washed 250-ml polycarbonate bottles using graduated cylinder.
2. Add NaCl to bring the samples to 35ppt salinity as per refractometer
3. Add acidified filter packet to the surface of liquid in the 250-ml bottle
4. Add MgO 0.6g per 200 ml of sample
5. Seal bottle with parafilm and then cap
6. Place samples on a shaker table for 14 days in 35°C oven for diffusion to occur
7. Remove filter packet after incubation and place in pre-combusted scintillation vial and store uncapped in a desiccator containing a small beaker of concentrated H_2SO_4 for 1-2 days
8. Cap scintillation vials

Preparation of Blanks and Controls

1. Have at least one blank and three controls of known isotopic signature per run
2. Prepare blank with 200 ml nanopure water, 0.6 g MgO, 10 g NaCl, and filter packet
3. Prepare controls with 200 ml nanopure water, 2.5 μM NH_4^+ with known $\delta^{15}\text{N}$ value, 0.6 g MgO, 10 g NaCl, and filter packet

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APPENDIX II –SAXITOXIN PURIFICATION METHOD DEVELOPMENT

Saxitoxin as a Tracer

The effect of nutrient pollution on the development and growth of harmful algal blooms is site and organism specific. The use of nitrogen (N) stable isotopes in particulate organic matter (POM) is a way to identify whether the N in the environment is primarily from natural or anthropogenic sources and which of the sources are being utilized by the algae. If the $\delta^{15}\text{N}$ of POM is high, the N source is likely wastewater; if the $\delta^{15}\text{N}$ is low, then the N is due to natural sources or fertilizer runoff. However, the POM could come from multiple habitats/N sources and therefore, not be indicative of the N source fueling the specific toxic algae. This complication and the variation of isotope fractionation between POM species, makes deciphering the $\delta^{15}\text{N}$ of POM difficult and potentially misleading. Compound-specific stable-isotope analysis (CSIA) is often used in describing organic matter composition and for distinguishing between sources. Saxitoxins (STXs) have the potential to be used as species-specific tracers for CSIA, as most are only produced by one species of algae in an area at a given time and the toxins are relatively stable, nitrogen-rich compounds (6-7 N atoms per molecule). The isotopic signature of $\delta^{15}\text{N}_{\text{STX}}$ can be used to identify the N source fueling PSP producing blooms and causing increases in toxin production. Smith and Erdner (2011) exposed the dinoflagellate *Alexandrium fundyense* to 883 μM concentrations of nitrate and found the *Alexandrium* cells and saxitoxins were 1.5‰ and 3.0‰ lighter than the source, respectively, suggesting that STXs mimic the $\delta^{15}\text{N}$ of the N source. Saxitoxins also responded to the N source more rapidly than the bulk cells (Smith and Erdner 2011), indicating $\delta^{15}\text{N}_{\text{STX}}$ may be more appropriate tool than the $\delta^{15}\text{N}$ of POM when identifying N sources in more dynamic systems.

Before $\delta^{15}\text{N}_{\text{STX}}$ can be utilized for CSIA, an efficient and effective method for the concentration, extraction, and purification of STXs from *Alexandrium fundyense* cultures and

bulk POM field samples must be developed. The N in STX must be isolated and all other atoms of N removed, as isotopic analysis via EA-IRMS (elemental analyzer-isotope ratio mass spectrometry) quantitates all N atoms in the sample. Experiments have been performed to develop a method that removes any other N-containing cellular compounds from the extract and avoids the addition of other N atoms during extraction and processing. Once a successful method has been established, laboratory experiments can be performed to assess the changes in $\delta^{15}\text{N}_{\text{STX}}$ value due to physiochemical conditions and transportation pathways during metabolism. Once the changes in $\delta^{15}\text{N}_{\text{STX}}$ under different nutrient regimes are fully characterized, this compound-specific biomarker can be applied to the field. Beyond this project's application in nutrient management and bloom dynamics, the use of the $\delta^{15}\text{N}_{\text{STX}}$ value will provide additional information regarding STX synthesis within dinoflagellates.

Summary of Procedure

To assess the utility of saxitoxin as a compound-specific stable isotope tracer, we required extracts of extreme purity from large cell pellets, i.e., saxitoxins needed to be the only source of N in the final material. As such, we developed a saxitoxin purification method, modified from Boundy et al. (2015) and Laycock et al. (1994), that aimed to maximize cell lysing, maximize removal of endogenous N contaminants, and minimize toxin loss during purification using less expensive and time consuming steps than those presented in Smith and Erdner (2011). While the methods of Laycock et al. (1994) and Smith and Erdner (2011) yielded high purity material, our ability to routinely use the tool, $\delta^{15}\text{N}_{\text{STX}}$, in our laboratory relied upon the development of a more streamlined, faster protocol.

The method presented herein successfully extracted high quantities of all saxitoxin congeners with minimal steps or congener conversions, however, it requires additional

optimization to further minimize contamination by endogenous N. A minimum of 0.15 $\mu\text{moles-N}$ is needed to exceed the lower detection limits of the instrumentation, requiring a substantial pellet of ≥ 1.5 million cells of 60 fmol STX $\cdot\text{cell}^{-1}$. This method was developed for a maximum of 5 million cells, and there is potential that when purifying STX from a larger pellet, a larger cartridge would be necessary for additional solid phase extraction (SPE) binding. Separate experiments were performed to maximize cell breakage prior to toxin isolation, determine ideal SPE conditions, and determine toxin recovery over the method. Experiments were also conducted using bleach, pH, and temperature to degrade STXs in preparation for shipment and subsequent isotope analysis.

Abbreviations:

STX= Saxitoxin

NaOH=Sodium Hydroxide

SPE=Solid Phase Extraction

POM=Particulate Organic Matter

LC-MS/MS = Liquid Chromatography-Mass Spectrometer/Mass Spectrometer

N=Nitrogen

Protocol

Lyse Cells

- 1) 1.5-5 million cell pellet in 50 ml centrifuge tube: add 6 ml 1% acetic acid (1:99 v/v with water), vortex pellet
- 2) Freeze and thaw 3 times (vortex samples between each freeze/thaw cycle)
- 3) Store samples on ice between all steps
- 4) Sonicate on ice: 5x30s each, 10s break between, at 160W (40% amp) (Branson Digital Sonifier-450, Danbury, Connecticut)
- 5) Bead beat using vortex adapter (MO Bio, Carlsbad, CA, Part #: 13000-V1-50) with 1 g of 0.1 mm silica beads for 20 minutes in 15°C cold room
- 6) Centrifuge: 3000xg (rcf) 15 minutes at 10°C
- 7) Collect supernatant and transfer directly to 50-ml Millipore Amicon Ultra-15 centrifugal filter Ultracel-3 3K(Millipore, Darmstadt, Germany, Part #: UFC900324)
- 8) Add 6 ml 1% acetic acid (1:99 v/v with water) and repeat steps 4-7 to the remaining pellet (x1) for a final volume of 12 ml in the Millipore Amicon Ultra-15 centrifugal filter Ultracel-3 3K

Molecular Weight Size Exclusion

- 9) Millipore Amicon Ultra-15 centrifugal filter Ultracel-3 3K: centrifuge 3,000xg (rcf) for 40 minutes at 20°C

- 10) Add 2 ml 1% HOAc to top of Millipore Amicon Ultra-15 centrifugal filter and centrifuge 3,000xg (rcf) for 40 minutes at 20°C
- 11) Remove top filter and add 1 M NaOH to the solution to a pH of 6.9-7.1

Solid Phase Extraction (SPE)

- 12) Use a graphitised polymer carbon SPE cartridge, Supelclean ENVI-Carb 500 mg/6 ml cartridge with 15 ml open column adapter (Supelco, Bellefonte, Pennsylvania, Part #: 57094)
- 13) Condition cartridge with 6 ml methanol/water/acetic acid (20:79:1)
- 14) Condition cartridge with 6 ml water/1 M NaOH (999:1)
- 15) Load 5 million cells or less worth of sample from step 11 onto the cartridge at 24 ml/min
- 16) Rinse sample container with nanopure and pour into cartridge at 24 ml/min
- 17) Wash cartridge with 6 ml Nanopure
- 18) Elute sample with 4 ml methanol/water/acetic acid (40:55:5) into 15 ml centrifuge tubes

Sample Drying/Concentrating

- 19) Collect subsample (~20 µl) for STX quantification on LC-MS/MS
- 20) Store in -20°C until ready for N dry down
- 21) Take samples to near dryness, <500 µl, under a gentle stream of ultra-pure N₂
- 22) Spot 100 µl at a time onto a 21-mm GF/C filter placed on top of a 30-mm diameter tin disk (Elemental Microanalysis, Part #:D1066) place inside a drying oven set to 45°C and allow to dry, repeat with remaining concentrated sample, 100 µl at a time, until no more sample remains
- 23) Once filter is completely dried, pelletize sample in the 30-mm tin disc (and if needed place the pellet inside a 5x9 mm tin capsule; Costech, Part #: 041077) and place in a 20-ml scintillation vial and cap tightly
- 24) Store in a desiccator until ready for analysis on an IRMS

Cell Lysing Optimization Experiment

Experiments were performed to optimize cell breakage prior to saxitoxin purification.

This is novel work, as no study to our knowledge has extracted saxitoxin from pellets containing 1-10 million cells. To begin, an experiment was performed to assess the ability of probe sonication for cell lysing the large pellets (Branson Digital Sonifier-450, Danbury, Connecticut). Probe sonication has been successful in lysing 95% of pellets composing up to 500,000 cells (Harlow et al 2006). Two frozen samples of 10 million cells *Alexandrium fundyense*, strain NG-SP3, were freeze/thawed three times and then probe sonicated for 4x30s (with 10 sec pauses between pulses). A 10-µl subsample was preserved with 2 µl of Lugol's solution and analyzed under a microscope for cell lysis. Although there was signs of cell lysis, e.g., cellular debris, a significant number of cells remained intact. The pellets were then probe sonicated for an

additional 4x30s, resulting in additional lysis. Lysis, however, was deemed insufficient despite 8x30s (4 min total) and so another method, bead beating, was then utilized.

A set of bead-beater experiments was conducted using a FastPrep-24 Tissue and Cell Homogenizer (MP Biomedicals, Solon, OH, Model #: 6004-500) to determine the type of beads that maximized cell lysis and did not bind saxitoxins. The experiment contained the following treatments, where standards of STX, C1, and C2 congeners were mixed in 1% acetic acid and 500 μ l was added to;

- 1) an empty 4 ml bead beater vial and not beaten (n=3),
- 2) an empty 4 ml bead beater vial and beaten (n=3),
- 3) a 4 ml bead beater vial with 0.7 g garnet beads (n=3),
- 4) a 4 ml bead beater vial with 0.5 g silica beads (n=3), and
- 5) a 4 ml bead beater vial with 0.5 g matrix E (0.4 mm ceramic spheres, 0.1 mm silica spheres, and 4 mm glass beads; n=3).

Both an empty vial with and without beating was necessary to analyze any toxin conversion due to heating of the vial during beating. The samples were bead beat at 6 m/s for 1 minute.

Afterwards, supernatant was filtered through a 0.22 μ m GSWP nitrocellulose membrane filter (Millipore, Part #: GSWP04700) to remove any beads, and transferred to high-recovery vials of sample:acetonitrile (50:50) to be analyzed using hydrophilic interaction liquid chromatography (HILIC)-MS/MS as described in Chapter 1. Results indicated that there was toxin loss associated with all bead types but none during the actual beating of the vials (i.e., minimal potential for heat degradation) (Table 1). There was the least amount of toxin loss using silica beads with the exception of C1 congener.

Bead beating was then investigated using cell pellets of *Alexandrium fundyense*. Two ml of 1% acetic acid was added to two samples of 7 million cells of strain NG-SP3. Silica beads, 0.5 g, were added and the samples were beaten for 20 sec x 5 at 4 m/s. Subsamples of 20 µl x 5 were put into Lugol stain for microscopic analysis where minimal cell damage was observed. The same two vials were beaten again for 20 sec x 5 at 4 m/s and five subsamples were taken where significantly more cells were broken but some remained intact. An additional beating of 20 sec x 7 at 4 m/s showed some cells still intact. Ultimately, a mixture of probe sonication, 5 x 30 sec, and bead beating, 20 min x 2, led to sufficient cell damage (Table 3).

Table 1. Cell Lysing Optimization Experiment. Standards of STX, C1, and C2 congeners were mixed in 1% acetic acid and 500 µl was added to an empty 4 ml bead beater vial and not beaten, an empty 4 ml bead beater vial and beaten, a 4 ml bead beater vial with garnet beads, a 4 ml bead beater vial with silica beads, and a 4 ml bead beater vial with matrix E (0.4 mm ceramic spheres, 0.1 mm silica spheres, and 4 mm glass beads) (n=3 for each treatment). The samples were bead beat at 6 m/s for 1 minute and the resulting samples were then analyzed using HILIC-MS/MS for toxin content. The toxin content range and average in the three replicates for congeners STX, C1, and C2 are shown below.

Treatment	Toxin Range (µM)			Average (µM)		
	STX	C1	C2	STX	C1	C2
No Bead No Beater (Control)	131-186	190-243	75-94	160	214	82
No Bead	147-190	173-273	81-89	168	218	86
Garnet	105-115	62-114	72-86	110	88	79
Silica	111-191	0-168	68-93	144	56	80
Matrix E	143-167	0	65-77	152	0	73

SPE Optimization Experiment

An experiment was performed to optimize saxitoxin isolation when samples (with 1% acetic acid and NaOH brought to a pH of 7) were loaded onto a 250 g/3 ml ENVI-Carb Solid Phase

Extraction cartridge and washed with 1 ml of increasing percent methanol containing 1% acetic acid. Goals were to remove unwanted endogenous compounds and salts, while also isolating and concentrating the toxins. Triplicate samples consisted of a mixture of 663 nM STX, 2268 nM C1, and 678 nM C2 standards from NRC (Halifax, Nova Scotia, Canada). These congeners were chosen because they represent a range of polarity with STX as one of the most polar and C1/C2 the least polar. This range in polarity is necessary because the slight difference in composition is enough to change the physical properties of the congeners. Only by experimenting with the range of polarity can recovery of toxins be possible. All extracts were analyzed using HILIC-MS/MS methods as previously described in Chapter 1. Saxitoxin was found to elute off the cartridge with a wash of just 10% methanol. However, C1 and C2 congeners did not elute off the cartridge until 20-30% methanol solution was applied (Table 2). Around 25% of the C1 toxin was still eluting off the cartridge in the second ml of 40% methanol with 1% acetic acid. This experiment demonstrates the need for 2 ml of 40% methanol during elution from a 250 g/3 ml ENVI-Carb to recover the most toxin and that a 10% methanol wash would result in the loss of the congener STX. Thus, only water was used for washing the cartridge before elution in the final method. An additional experiment (not presented) demonstrated that the water wash without the 0.1% NaOH resulted in the same 0% toxin loss.

Table 2. SPE Optimization Experiment. Envi-Carb were washed with increasing percent methanol containing 1% acetic acid. The percent toxin recovered in each wash along with the total percent recovered from the original samples for congeners STX, C1, and C2 are shown. The range shown for the total percent recovery does not correspond to the sums of the maximum and minimum from the washes for an individual toxin but rather displays the range of total recovery for each replicate sample.

Elute	STX	C1	C2
Nanopure	0%	0%	0%
Nanopure + 0.1% 1 M NaOH	0%	0%	0%
10% MeOH + 1% Acetic Acid	54-58%	0%	0%
20% MeOH + 1% Acetic Acid	6-13%	0%	19-21%
30% MeOH + 1% Acetic Acid	0%	7-14%	49-61%
40% MeOH + 1% Acetic Acid (1st ml)	0%	25-36%	14-20%
40% MeOH + 1% Acetic Acid (2nd ml)	0%	12-20%	0%
Total Percent Recovery	63-68%	53-62%	86-96%

Recovery Efficiency Experiment for STX Purification Method

An experiment was performed to determine toxin recovery over the whole protocol, including cell lysis, molecular-weight filtration, and solid phase extraction. Subsamples were taken from supernatant after the cells were lysed (step 7, i.e. initial concentration), after samples went through the 3K centrifugal filter (step 10, i.e. loss during filtration), and after collection from the ENVI-Carb Solid Phase Extraction (SPE) cartridge (step 17, i.e. loss during NaOH/SPE binding). *Alexandrium fundyense*, strain NG-SP3, was used in this experiment, and each sample contained between 9.3-10.1 million cells. The samples of culture were pelleted, and stored in -20°C freezer prior to running the experiment. Each sample was taken through the initial steps of

the protocol, and then split into two equal portions before be loaded onto a 0.5 g or 1g ENVI-Carb SPE so that parallel samples could be compared. This allocation also assured that only 4.5-5 million cells went through each cartridge. The 1 g ENVI-Carb SPE received additional 4 ml of eluting solution to assure the recovery of all STXs on the larger stationary bed. All subsamples were analyzed using HILIC-MS/MS.

Overall the protocol demonstrated successful recovery, 78-93%, using a 0.5 g SPE cartridge (Table 3). Ultimately it was decided that the 8-ml required for elution of all toxins using 1 g SPE cartridge was too high a volume to dry down and spot onto a filter, and so the 0.5 g bed was selected. During the addition of 1 M NaOH to the samples, sample A was brought to a pH of 12 while the other three samples were taken to a pH of 7. The elevated pH led to transformation among congeners (Table 4) and overall toxin loss (Table 3). The second 4 ml 1 g SPE extract revealed that about 30 percent of the total toxins remained in the cartridge (Table 3), suggesting that maximum recovery would require additional elution volume. The recovery of 78-93% of the total toxins in the initial culture using the 0.5g SPE cartridge will result in a high enough concentration of STX for nitrogen isotope analysis. There did appear to be minimal congener transformation during the final purification step, leading to an increase of the congeners: STX, NEO, GTX1, and C1, and a decrease of the congeners: GTX3, GTX5, C2, and GTX4 (Table 4). However, this change in congeners will not have a large effect on the isotopic signature of STX as all the toxins were pooled together for analysis. Although this method achieved high recovery, N isotope analyses demonstrated the presence of more N in the final samples than can be contributed to STXs alone (Table 5). As such, this method requires further optimization to achieve enhanced purity.

Table 3. STX Retention Experiment. Initial concentrations are calculated in fmol per cell considering total of all congeners eluted from the 0.5g and 1g ENVI-Carb SPE cartridge (initial sample split and run through one of the two cartridges). The percent loss during the protocol was calculated as a percent of the initial concentration still present (divided by 2 for the SPE recovery samples where only half of the initial sample went through the cartridge). Culture A was brought to a pH of 12 prior to solid phase extraction, while all others were between 6.9-7.1 pH.

Culture (ID: # cells per pellet)	Initial Concentration (Total fmol•cell⁻¹)	Post-Filtration Recovery (%)	SPE Recovery (0.5g cartridge) (%)	SPE Recovery (1 g cartridge; 1st 4 ml extract) (%)	SPE Recovery (1 g cartridge; 2nd 4 ml extract) (%)
A: 10,176,000	23.91	105.1	59.92	92.69	2.65
B: 9,372,000	25.46	100.1	91.11	85.16	34.35
C: 9,936,000	23.89	88.69	78.15	70	30.59
D: 9,312,000	27.43	82.92	92.78	74.01	34.96

Table 4. STX Retention Experiment. Initial concentrations in fmol per cell of each STX congener and the percent of each congener eluted from the 0.5g ENVI-Carb SPE cartridge.

Culture	Initial Concentration(fmol•cell⁻¹)								Percent Recovery (0.5g cartridge) (%)							
	STX	NEO	GTX1	GTX3	GTX5	C1	C2	GTX4	STX	NEO	GTX1	GTX3	GTX5	C1	C2	GTX4
A	0.01	0.22	0	0.20	2.41	0	8.37	12.69	155.73	58.48	-	38.43	16.14	-	57.7	28.98
B	0.04	0.27	0	0.22	3.62	0	7.18	14.13	151.39	156.67	-	70.89	53.33	-	135.01	75.84
C	0.01	0.11	0	0.25	2.43	0	7.5	13.57	273.84	233.61	-	77.57	56.23	-	64.16	64.16
D	0.03	0.24	0	0.35	2.73	0	8.52	15.55	249.65	148.89	-	62.88	60.07	-	72.8	72.8

Table 5. STX Purification Experiment. The calculated and actual amount of nitrogen in each sample and the isotope signature are presented according to the strain of *Alexandrium fundyense* and nitrogen source utilized. The amount of N contributed by STX was calculated using STX analysis by HILIC-MS/MS.

Strain	N Source	Calculated STX N (μmol)	Actual N in Sample (μmol)	$\delta^{15}\text{N}$
ATSP7-D9	NO_3^-	1.22	16	13.21
ATSP7-D9	NO_3^-	1.47	18	12.90
ATSP7-D9	NO_3^-	1.01	12	13.22
ATSP7-D9	NH_4^+	1.22	31	5.4
ATSP7-D9	Urea	1.60	19.88	1.30
ATSP7-D9	Urea	1.23	11.61	0.86
ATSP7-D9	Urea	0	12.62	2.07
GTCA-28	NO_3^-	1.61	15.53	12.58
GTCA-28	NO_3^-	1.34	14.07	12.61
GTCA-28	NO_3^-	1.16	14.44	12.86
GTCA-28	NH_4^+	1.7	31	6.0
GTCA-28	NH_4^+	0.5	8	1.5
GTCA-28	Urea	0.8	8	0.6
GTCA-28	Urea	0.4	9	0.9

Future Experiments

The current protocol yielded high recovery of target analytes, however, isotope analysis determined that further cleanup is required to remove endogenous nitrogen contamination. On average, the actual nitrogen content was 16x greater than the nitrogen estimated from the cellular saxitoxin alone (Table 5). As such, the generated $\delta^{15}\text{N}$ values for saxitoxin (Table 5) cannot be used to assess N isotope fraction during STX biosynthesis on different N chemical forms.

Additional toxin purification is needed to separate STX from all remaining small molecular weight, dissolved N-rich molecules, e.g., amino acids. Prior to elution of the toxin off the ENVI-Carb SPE cartridge, further wash steps can be utilized to remove other sources of N. A wash experiment was performed during the development of the STX purification method, but loss of toxin occurred with every percent methanol wash attempted other than water. Further research

and method development, therefore, must try to identify a wash step in which toxin loss does not occur. Alternatively, purification steps from previously published methods (Smith & Erdner 2011) can be applied, including size-exclusion chromatography (Bio-Gel P2, Bio-Rad, Hercules, CA) and cation-exchange chromatography (Bio-Rex-70 resin, Bio-Rad, Hercules, CA).

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