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Impact of nitrogen chemical form on the isotope signature and toxicity of a marine dinoflagellate

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Abstract

Despite a global interest in the relationship between harmful algal blooms (HABs) and eutrophication, the impact of natural versus anthropogenic nutrient sources on species composition or toxicity of HABs remains unclear. Stable isotopes are used to identify and track nitrogen (N) sources to water bodies, and thus can be used to ascertain the N source(s) used by the phytoplankton in those systems. To focus this tool for a particular species, the fundamental patterns of N isotope fractionation by that organism must first be understood. While literature is available describing N isotope fractionation by diatoms and coccolithophores, data are lacking regarding dinoflagellates. Here we investigated the effects of N chemical form on isotope fractionation () and toxin content using isolates of the autotrophic dinoflagellate, *Alexandrium catenella*, in single-N and mixed-N experiments. Growth of *A. catenella* exclusively on nitrate (NO₃⁻), ammonium (NH₄⁺), or urea, resulted in δ¹⁵N of 2.7±1.4‰, 29±9.3‰, or 0.3±0.1‰, respectively, with the lowest cellular toxicity reported during urea utilization. Cells initially utilized NH₄⁺ and urea when exposed to mixed-N medium, and only utilized NO₃⁻ after NH₄⁺ decreased below 2–4 μM. This pattern of N preference was similar across all N treatments, suggesting that there is no effect of preconditioning on N chemical preference by *A. catenella*. In NO₃⁻ and urea-rich environments, the δ¹⁵N of *Alexandrium catenella* would resemble the source(s) of N utilized, supporting this tool's utility as a tracer of N source(s) facilitating bloom formation, however, caution is advisable in NH₄⁺ rich environments where the large δ¹⁵N value could lead to misinterpretation of the signal.

Keywords

Alexandrium catenella; Dinoflagellates; Harmful Algal Blooms; Nitrogen; Saxitoxin; Stable Nitrogen Isotope

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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 contamination, sewage effluent, septic leaching, localized atmospheric deposition, and agricultural and aquaculture runoff (Anderson et al. 2002, Backer & McGillicuddy 2006). Nitrogen (N) is of particular interest because areas of high N, more specifically the reduced forms of N such as ammonium (NH_4^+) and urea, can be correlated with HABs comprised of toxic dinoflagellates (Glibert & Terlizzi 1999, Bronk et al. 2007). However, the link between anthropogenic N loading, chemical form, and HABs is site- and organism- specific.

Stable isotope ratios of nitrogen ($^{15}\text{N}/^{14}\text{N}$, expressed herein as $\delta^{15}\text{N}$ values relative to atmospheric N_2) in particulate organic matter (POM) are successfully used to characterize N sources in aquatic systems with the goal of understanding relative inputs and developing management strategies that minimize loads from the most destructive N contributors (Doi et al. 2004, Savage 2005, Dailer et al. 2010, Hattenrath et al. 2010, Zvab Rozic et al. 2015). For example, N derived from human or animal waste is often ^{15}N enriched relative to natural sources (Kendall & McDonnell 1998). During N utilization, phytoplankton incorporate ^{14}N at a faster rate than ^{15}N and thus alter the ratio of heavier and lighter isotopes in their cells as well as the residual, extracellular N pool, in a process called fractionation. Laboratory studies with diatoms and coccolithophores demonstrate that variability in the concentration or chemical form of N available can lead to changes in fractionation (Pennock et al. 1996, Waser et al. 1998a, b) and a subsequent misinterpretation of $\delta^{15}\text{N}_{\text{POM}}$. The utility of $\delta^{15}\text{N}_{\text{POM}}$ to identify N source can be preserved, however, if N fractionation by the dominant POM species is well-characterized. Although N isotope fractionation in diatoms and coccolithophores is well-documented (Pennock et al. 1996, Waser et al. 1998a, b), data are lacking regarding fractionation by dinoflagellates when grown on different chemical forms. This deficit identifies a current limitation of $\delta^{15}\text{N}_{\text{POM}}$ as a management tool as many marine HABs and associated human syndromes are caused by dinoflagellates (Van Dolah et al. 2001).

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 filter-feeding seafood products and subsequent intoxication of human consumers. Management of this syndrome is challenging as the growth and toxicity of a causative dinoflagellate, *Alexandrium* spp., can be affected by many environmental and biological factors, including the concentration and chemical form of N available (Anderson et al. 2002, Dyhrman & Anderson 2003, Leong et al. 2004, Anderson et al. 2008, Hattenrath et al. 2010). Both field and laboratory studies of *Alexandrium* species show that cells grown on NH_4^+ have higher toxin content than those grown on urea or nitrate (NO_3^- ; Dyhrman & Anderson 2003, Leong et al. 2004, Hattenrath et al. 2010, Li et al. 2011). Thus, while multiple nitrogenous compounds can be utilized for *Alexandrium* growth, NH_4^+ may be more effectively assimilated and incorporated into STXs.

Here we investigated how stable N isotope values and toxicity of *Alexandrium catenella* change when cells utilize micromolar concentrations of NO_3^- , NH_4^+ , urea, or a combination of the three, following preconditioning to a specific form. The objectives of this study were to determine: 1) N isotopic fractionation (i.e., $\delta^{15}\text{N}$ values) by *A. catenella* 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. catenella* when grown on either NO_3^- , NH_4^+ , or urea. We expect these results will aid in the interpretation of $\delta^{15}\text{N}_{\text{POM}}$ in a system experiencing a dinoflagellate bloom, with N contributions from multiple sources (i.e., mixed N sources) or where sources change rapidly. Knowledge of the nutrients and their source, e.g., anthropogenic or natural, that contribute to the intensification and expansion of *A. catenella*, as well as those that alter toxin quota, profile, or production rate, is necessary to mitigate, predict and/or prevent blooms and subsequent outbreaks. In ongoing studies, we will build on this information by focusing on an N-rich compound that is unique to a dinoflagellate of concern, such as saxitoxin or other biotoxins. We will compare fractionation on the species-specific compound to $\delta^{15}\text{N}_{\text{POM}}$ data found here to better interpret the nutritional history of a single species within the context of the community. Ultimately, we hope to create a species-specific N-tracer that identifies the N source and chemical form(s) facilitating blooms and toxin production.

Methods

Chemicals.

Reagents for toxin quantification include Optima LC-MS additive grade formic acid (99–100%) and Burdick and Jackson LC-MS additive grade acetonitrile, methanol, and ammonium hydroxide (>25% in H_2O ; PCode: 101612155). During extraction and culturing efforts, HPLC 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, GTX1/4, GTX2/3, GTX5, dcNEO, dcSTX, dcGTX2/3, and C1/2 (National Research Council, NRC, Halifax, Nova Scotia, Canada), were used during toxin quantification.

Culture Maintenance.

The saxitoxin producing dinoflagellate *Alexandrium catenella* 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 chosen for their varying locations with annual PSP closures (Anderson et al 2005, Crespo et al 2011) and diverse toxin profiles. Clones were obtained from the laboratory of D. Anderson at Woods Hole Oceanographic Institution. Cultures were grown in autoclaved, pre-filtered (pore size 0.22 μm) seawater from Wachapreague, VA brought to a pH of 8.3 with hydrochloric acid or sodium hydroxide, salinity of 35, and amended with sterile f/6 nutrients without silicon dioxide (SiO_2) or nitrate (NO_3^-) (36.2 μM monosodium phosphate (NaH_2PO_4), trace metals, and vitamins; Guillard 1975). The cultures were illuminated at light intensities of around 60 μmol photons on a 14:10h light:dark 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 NO_3^- , NH_4^+ , or urea (urea treatment amended with 100 nM

NiSO₄), prior to use in the experiment. Cultures were grown on a low concentration of N relative to f/2 (Guillard 1975), to better represent natural concentrations and to avoid NH₄⁺ toxicity at *ca.* 50 μ M. Medium containing urea was amended with nickel because *Alexandrium* spp. utilize the nickel-dependent urease enzyme to hydrolyze urea into NH₄⁺ (Dyhrman & Anderson 2003). When culture was used to inoculate experiments, less than 0.4 μ moles of NO₃⁻, NH₄⁺, or urea was carried over to the experimental flasks.

Experimental Conditions.

Two experiments were conducted: (1) A single-source study where N, in the forms of NO₃⁻, NH₄⁺, or urea with 100 nM nickel (NiSO₄), was repeatedly replenished to avoid N limitation, and (2) a mixed-source study where batch cultures were supplied with a combination of NO₃⁻, NH₄⁺, and urea at inoculation and then allowed to deplete the mix of N. The initial $\delta^{15}\text{N}$ value of the NO₃⁻, NH₄⁺, and urea stock reagents were measured at 12.7 $\pm$ 0.8‰, 2.1 $\pm$ 0.4‰, and -0.3 $\pm$ 0.4‰ (n = 3), respectively, providing sufficient disparity between N sources to allow us to track uptake and fractionation of the different sources. 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 (Boyer et al. 1987, Anderson et al. 1990, Persson et al. 2012). Light, temperature, and nutrients remained the same as the pre-experimental conditions. 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, but were gently swirled to homogenize twice a day.

Single-N Source Experiment.

In preparation for the single-source experiment, two strains of *A. catenella*, GTCA-28 and ATSP7-D9, were each inoculated into triplicate experimental flasks for the three treatments (i.e., NO₃⁻, NH₄⁺, or urea), at a concentration of 500 cells ml⁻¹. To avoid N limitation and minimize effects of recycled N, N was added throughout the experiment to all three treatments. The NH₄⁺ concentration was monitored in real-time, using a NH₃/NH₄⁺ test kit with a limit of detection of 0–8 ppm (0–443 μ M) and a color chart with increments of 0.25 ppm (13.8 μ M, Aquarium Pharmaceuticals), to avoid reaching toxic levels during N additions; the other two N chemical forms were added to their respective treatments assuming a similar uptake rate as NH₄⁺. Nitrogen was added to all treatments daily, to reach 30 μ M-N, based on the NH₃/NH₄⁺ test kit results. On day 5 and thereafter, however, 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 μ M-N. Culture medium was collected, filtered, and frozen for the independent quantification of dissolved NO₃⁻, NH₄⁺, 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.

Six hours before the end of the experimental period, i.e., after the last N addition, culture medium was collected to determine the $\delta^{15}\text{N}$ of the dissolved NO₃⁻ or NH₄⁺ ($\delta^{15}\text{N}_{\text{NO}_3^-}$ and $\delta^{15}\text{N}_{\text{NH}_4^+}$), 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 $\sim 3,000$ cells 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-source experiment, *A. catenella* strain ATSP7-D9 was pre-conditioned to grow on NO_3^- , NH_4^+ , or urea ($25\text{--}50\text{ }\mu\text{M-N}$) for at least three generations (around 15 days). To mark the beginning of the experiment, nine 3-L Fernbach flasks per treatment were inoculated with a pre-conditioned culture in exponential growth, at an initial concentration of ~ 100 cells ml^{-1} . Experimental flasks began with a mix of NO_3^- , NH_4^+ , and urea at concentrations of $40\text{ }\mu\text{M-N}$ each, or a total of $120\text{ }\mu\text{M-N}$ (plus 100 nM NiSO_4). In contrast 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. Samples for the determination of 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 became depleted due to repeated sampling; at 60 hours and 96 hours, sampling continued from the next set of triplicate flasks. 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: $F_{2,8} = 3.3$, $p = 0.172$).

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 Analytical 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. Urea was quantified at Virginia Institute of Marine Science using the manual diacetyl monoxime thiosemicarbazide method adapted from Rahmatullah and Boyde (1980), modified by Price and Harrison (1987). Duplicate samples were diluted to fall within the standard curve ($0\text{--}10\text{ }\mu\text{M-N}$, 6 points $\times$ 3 replicates) and analyzed 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 $\times 3$, 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-µm, 13-mm filter syringe (Millex-GV, Millipore, Durapore), diluted 1:1 with acetonitrile (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 µm column (2.1 mm × 100 mm; P/N: 186004801) with a Waters BEH Amide 1.7 µm guard column attached (P/N:186004799). The autosampler and columns were maintained at 10°C and 60°C, respectively. 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) and initial conditions were based on Boundy et al. (2015) and Turner 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 quantities were 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. Toxin quantity was reported as zero if extract concentrations were below 1:2000, the lowest calibration standard: 32.8 nM neoSTX, 33.2 nM STX, 17 nM C2, 56.7 nM C1, 27.9 nM GTX5, 21.7 nM GTX3, 9.9 nM GTX4, 14.7 nM dcNEO, 30.2 nM GTX1, and 27.9 nM GTX2. 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 three most dominant toxins). 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 N contamination. 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 (NO₃[−] and NH₄⁺ of medium). Filters were transferred into 20-ml glass vials (Fisher, catalog #05-719-117) and frozen at −20°C. Prior to isotope analysis of POM (δ¹⁵N_{POM}), samples were dried at 60°C for 30–60 minutes until dry. Filtrate, 200 ml, for isotope analysis of dissolved NH₄⁺ (δ¹⁵N_{NH4+}) was processed using a modified version of the NH₄⁺ diffusion method of Holmes (1998), whereby

polypropylene membrane filters (25 mm, lot #: 151579, Sterlitech) replaced Teflon filters (Holmes et al. 1998; Hannon & Bohlke 2008). 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 $\mu\text{moles N}$ (Holtvoeth et al. 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).

The N isotope ratios, $\delta^{15}\text{N}$, of the samples are reported here as a δ value (‰ = per mil) with respect to N_2 gas in air (~ 0.004) (equation 1).

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

The difference between N isotope values of the dissolved NH_4^+ or NO_3^- and the POM are defined by the discrimination factor () (equation 2) (Peterson & Fry 1987).

$$\Delta = \delta^{15}\text{N}_{\text{Medium}} - \delta^{15}\text{N}_{\text{POM}} \quad (\text{Equation 2})$$

For the experiments, Δ 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 1). The only exception was for urea, where the Δ value was calculated based on the $\delta^{15}\text{N}$ value of the urea stock reagent prior to dissolving into seawater (Table 1).

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 source experiment.

Growth Rates.—Over the seven-day experimental period, the cultures demonstrated a two-day lag during which cell concentrations remained constant or declined transitioning 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. No difference in growth rate was detected between nitrogen treatment and *Alexandrium catenella* strains (Nitrogen Form: $\chi^2_{2,2} = 0.72$, $p > 0.6$; Strains: $\chi^2_{1,8} = 0.29$, $p > 0.5$), with ATSP7-D9 (Figure 1A) demonstrating a growth rate (μ) of $0.34\text{--}0.37\text{ d}^{-1}$ and GTCA-28 (Figure 1B) a μ of $0.26\text{--}0.29\text{ d}^{-1}$. Treatments were terminated once cultures reached a minimum cell concentration of $3,000\text{ cells ml}^{-1}$, but less than $6,000\text{ cells ml}^{-1}$ to ensure cultures remained in exponential growth throughout the sampling period. As such, NO_3^- , NH_4^+ , and urea treatments concluded on day 7, with the exception of the NO_3^- treatment for strain GTCA-28, which required an additional day to reach appropriate densities.

Nitrogen Utilization.—The initial seawater, pre-amendment, contained $0.6\text{ }\mu\text{M NO}_3^-$, $1.9\text{ }\mu\text{M NH}_4^+$, and $0.1\text{ }\mu\text{M-N urea}$. These N chemical forms remained under $2.5\text{ }\mu\text{M}$ 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, the average N draw-down (hereafter inferred as uptake rates) for strain ATSP7-D9 was $0.02\pm0.003\text{ N cell}^{-1}\text{d}^{-1}$, $0.01\pm0.007\text{ N cell}^{-1}\text{d}^{-1}$, $0.003\pm0.003\text{ N cell}^{-1}\text{d}^{-1}$, for NO_3^- , NH_4^+ and urea, respectively. Strain GTCA-28 had a similar uptake rate for NO_3^- of $0.03\pm0.02\text{ N cell}^{-1}\text{d}^{-1}$, and NH_4^+ of $0.009\pm0.001\text{ N cell}^{-1}\text{d}^{-1}$. Urea uptake rates were not calculated for GTCA-28 because instrument error was excessive at such high concentrations.

Nitrogen Isotope Fractionation.—The degree of N fractionation by *A. catenella* varied significantly between N treatments ($\chi^2_{2,2} = 14.3$, $p < 0.01$), with the largest difference in isotopic ratios measured between cells and source N during the uptake and assimilation of NH_4^+ ($\delta = 23.5\pm7.2\text{‰}$), followed by NO_3^- ($\delta = 2\pm0.3\text{‰}$), and urea ($\delta = 0.3\pm0.1\text{‰}$) (Table 1). The degree of source-mediated isotopic fractionation, however, followed the same trend between strains, suggesting consistent uptake processes across the species (Table 1; Kruskal Wallis ANOVA: $\chi^2_{1,8} = 0.6$, $p > 0.4$).

The $\delta^{15}\text{N}_{\text{NH}_4^+}$ value of the medium increased from 2.1‰ to $24.8\pm5.5\text{‰}$ in the ATSP7-D9 experiments over time, and to $15.5\pm1.1\text{‰}$ in the GTCA-28 experiments (Table 1). 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 the concentration of dissolved NO_3^- , NH_4^+ , urea, TDN, or DON through the course of the experiment. 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‰ .

Mixed-N source experiment.

Growth Rates.—Similar to the single-N experiment, all cultures in the mixed-N experiment showed a lag followed by exponential growth, with μ ranging from 0.51–0.54 d⁻¹ over the course of the experiment (Figure 2). No effect of N pre-conditioning was detected on growth rate or between growth curves of the three treatments ($F_{2,2}=3.3$, $p>0.13$). 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). Samples were excluded from data analysis if insufficient N was captured during filtration.

Nitrogen Utilization.—During the mixed-N source experiment, there was no indication that preconditioning *A. catenella* cells to any of the three N forms affected the patterns of NH₄⁺ utilization (repeated measures ANOVA: $F_{2,2}=0.9$, $p>0.4$) or NO₃⁻ utilization (repeated measures ANOVA: $F_{2,2}=2.7$, $p>0.07$) over the course of the experiment. There was, however, a detectable difference in urea utilization (repeated measures ANOVA: $F_{2,2}=12.61$, $p<0.01$) between the preconditioned treatments, with cells preconditioned on nitrate taking up urea at a faster rate than cells preconditioned to NH₄⁺ and urea (Figure 3A, 4A, 5A). A consistent pattern of N selectivity held across treatments, whereby *A. catenella* cells took up NH₄⁺ and urea initially, and only assimilated 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 N uptake rate 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).

Isotope Fractionation.—The $\delta^{15}\text{N}_{\text{POM}}$ was at its lowest at around 48 hours (averaging -5‰) during co-utilization of NH₄⁺ and urea, and then increased by 84 hours (averaging 0.9‰) during the uptake of NO₃⁻ and urea (Figures 3B, 4B, 5B). This pattern was consistent between treatments, demonstrating that preconditioning cells to NO₃⁻, NH₄⁺, or urea did not affect the fractionation processes within cells (repeated measures ANOVA: $F_{2,8}=3.236$, $p=0.05$). As in the single-N source experiment, the $\delta^{15}\text{N}$ of the dissolved NH₄⁺, and to a lesser extent NO₃⁻, increased over time in the medium. The $\delta^{15}\text{N}$ of the dissolved NH₄⁺ increased from 2.6±0.7‰ to 19.5±2.4‰ by 48 hours and NO₃⁻ increased from 12.6±0.2‰ to 13.5±0.2‰ by 132 hours. As in the first set of experiments, we do not expect that the observed enrichment in ¹⁵N in the NH₄⁺ medium was due to recycling or remineralization of N by the dinoflagellate or microbial community as there was no measured increase in the concentration of dissolved NO₃⁻, NH₄⁺, urea, TDN, or DON through the course of the experiment.

Toxicity Content 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 (Figure 6). Toxin profiles, however, varied between strains, with ATSP-D9 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 7) (ANOVA: $F_{1,2} = 2.17$, $p = 0.16$). 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.

A comparison of N treatments (2-way ANOVA: $F_{2,2} = 18.33$, $p < 0.01$) showed similar trends in cellular toxicity ($F_{2,2} = 34.53$, $p < 0.01$; Figure 7) and toxin content ($F_{2,2} = 13.61$, $p < 0.01$; Figure 8) across strains. For both strains, the ammonium-grown culture (ATSP: 73.5 ± 4.2 fmol STX eq cell⁻¹, GTCA: 49.7 ± 11 fmol STX eq cell⁻¹) was significantly more toxic than the urea-grown culture (ATSP: 55.5 ± 7.75 fmol STX eq cell⁻¹, GTCA: 24 ± 2.4 fmol STX eq cell⁻¹). The nitrate-grown cultures, however varied in toxin content; when grown on nitrate, strain ATSP7-D9 had a relatively low toxin content (44.2 ± 2.3 fmol STX eq cell⁻¹), similar to levels contained in urea-grown cultures, while strain GTCA-28 had a higher toxin content (65.1 ± 6.3 fmol STX eq cell⁻¹), similar to levels contained in NH₄⁺-grown culture. When comparing across both strains and all N-treatments, cellular toxicity was greatest under NH₄⁺ utilization by strain ATSP7-D9 (mean: 73.5 ± 4.2 fmol STX eq cell⁻¹), and was the lowest when strain GTCA-28 was utilizing urea (mean: 24 ± 2.4 fmol STX eq cell⁻¹; Figure 7).

Discussion

This study is the first to show the impact of N chemical form and N preference on N isotope fractionation during uptake by autotrophic dinoflagellates. The results presented here for an autotrophic dinoflagellate are consistent with the isotopic fractionation results found for other taxonomic groups, namely diatoms and coccolithophores, and adds new insight into the ability of *Alexandrium catenella* to exploit any N available in the field as well as the dependency of toxicity upon the N chemical form being utilized. Here we also confirm that *Alexandrium catenella* can utilize NO₃⁻, NH₄⁺, or urea for growth and toxin production under N-sufficient conditions (Figures 1,7,8), and that while the chemical form of N available does not affect growth rate, it can lead to variation in cellular toxicity.

Dinoflagellate N fractionation

Although dinoflagellates are the most cosmopolitan and prolific of the HAB groups, there have been few isotopic fractionation studies on this taxonomic group, and no research to date has specifically investigated fractionation during NH₄⁺ or urea assimilation by dinoflagellates. 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. catenella* cells grown on NO₃⁻, urea, or NH₄⁺. Growth on urea and NO₃⁻ 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 value of 0.34‰ and 2.37‰ (average of two strains), respectively (Table 1). The fractionation observed during NO₃⁻ utilization was comparable to the results of Smith and Erdner (2011) who demonstrated that during stationary phase, *A. catenella* cells were 1.5‰ lower than the NO₃⁻ source (Smith & Erdner 2011). Similarly, values of 1–3‰ are reported for other dinoflagellates grown on NO₃⁻, (Needoba et al. 2003). While no data have previously been reported for dinoflagellate fractionation when grown on urea, the average values reported

here (0.34‰) is similar to those reported for marine diatoms, 0.8‰ (Waser et al. 1998a). Overall, the results suggest that in NO_3^- and urea-rich environments, the $\delta^{15}\text{N}$ of *Alexandrium catenella* would resemble the source(s) of N utilized, and therefore, may be an appropriate tool for describing N source contributions to a system and *A. catenella* bloom proliferation.

The magnitude of fractionation was much greater, however, when cells utilized NH_4^+ (Table 1), suggesting a possible limitation to the use of $\delta^{15}\text{N}_{\text{POM}}$ as a tracer in NH_4^+ rich environments. This large fractionation observed in dinoflagellates, 29‰, may only be a limitation in systems with elevated NH_4^+ concentrations, i.e., similar to those used in our study, 25 μM - NH_4^+ . While no other NH_4^+ -derived fractionation data exists for dinoflagellates, previous studies have reported a reduced fractionation in diatoms, 7.8‰, when NH_4^+ concentrations were lowered to 5–20 μM - NH_4^+ and an elevated level of isotopic fractionation, 27.2‰, when NH_4^+ concentrations were raised to 20–50 μM (Pennock et al. 1996, Vavilin et al. 2014). A concentration-dependent change in enzymatic pathways was proposed as the driver of this variability in fractionation during N transport in marine bacteria and diatoms (Hoch et al. 1992, Pennock et al. 1996). 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^+ . Additionally, if N is limiting in the environment, then the phytoplankton will utilize all available N and no fractionation will be observed.

Our results provide managers with a powerful tool for tracking N use by autotrophic dinoflagellates in aquatic systems. For example, if dinoflagellates are predominantly using N emanating from wastewater, with an isotope signature of ~20‰, we would be able to determine which N source the dinoflagellate utilized based on the expected $\delta^{15}\text{N}_{\text{POM}}$ value of 19.6‰, 17.6‰, or -5‰, when taking up urea, NO_3^- , or NH_4^+ , respectively. With $\delta^{15}\text{N}_{\text{POM}}$ values that will closely resemble the $\delta^{15}\text{N}$ of the source, and a consistent response expected across subpopulations of *A. catenella*, the utility of this tool in urea and NO_3^- dominated systems becomes clear. The large fractionation when utilizing NH_4^+ , however, could complicate the use of $\delta^{15}\text{N}_{\text{POM}}$ as a tracer in NH_4^+ rich environments, e.g., 25 μM , as the $\delta^{15}\text{N}_{\text{POM}}$ could misleadingly mimic an alternative source. It is possible in NH_4^+ -dominated systems, however, that the fractionation value resulting from *A. catenella* growth on NH_4^+ would be so large that the N source could be identifiable through its production of isotopically light cells, cells with $\delta^{15}\text{N}_{\text{POM}}$ values well below the $\delta^{15}\text{N}$ of any of the other identified N sources.

Co-utilization and N preference

While investigating N isotope fractionation on a single N source was a critical first step in our application of this tool to a dinoflagellate, the N isotopic composition of nutrients in estuarine and coastal ecosystems 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. To investigate the utility of $\delta^{15}\text{N}_{\text{POM}}$ in more complex systems, i.e., with multiple N inputs, we also incubated cells in mixed-N medium of NO_3^- , NH_4^+ and urea.

The growth of *A. catenella* on mixed N sources led to co-utilization, periods of inhibition or preference, and overall increased growth rates, demonstrating that, indeed, mixed N culturing experiments were necessary to apply and interpret $\delta^{15}\text{N}_{\text{POM}}$ in natural systems dominated by dinoflagellates. More specifically, when *A. catenella* was exposed to multiple N forms, cultures utilized both organic (i.e., urea) and inorganic forms (i.e., NH_4^+ or NO_3^-) simultaneously to support growth (Figures 3A, 4A, and 5A). Co-utilization of organic and inorganic N forms also resulted in elevated growth rates when compared to cultures grown on the chemicals in solidarity (Figures 1, 2). Nutrient preference was apparent during the mixed-N source experiments; the cultures initially selected NH_4^+ and urea, while NO_3^- was only utilized when NH_4^+ was below a threshold of 2–4 μM (Figures 3A, 4A, and 5A). This inhibition of NO_3^- uptake by NH_4^+ is already well-documented in diatoms and dinoflagellates, but can vary between species and concentrations of available N (Dortch 1990, Dortch et al. 1991, Waser et al. 1998b, Dugdale et al. 2007, Maguer et al. 2007, Shankar et al. 2014). Jauzein et al. (2008) compared uptake rates in five strains of *A. catenella* and found low ambient concentrations of NH_4^+ to inhibit the uptake of urea under urea-rich conditions in a subset of strains, suggesting strain variability exists.

Our results from the mixed-N study also suggest that past environmental N conditions likely do not affect future bloom responsiveness, and that *A. catenella* could rapidly proliferate when new sources of urea and NH_4^+ are introduced. Despite pre-conditioning the cultures to grow on different N forms, we found no difference in nitrogen preference, rates of uptake or utilization, and NH_4^+ and NO_3^- thresholds (Figures 3A, 4A and 5A). We also observed the same, rapid switching between N sources in all pre-conditioned treatments once the same preferred chemical form, NH_4^+ , had been depleted. Alternatively, 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 (Dortch et al. 1991). The observed ability for N switching and enhanced growth when co-utilizing multiple N forms (Figures 1, 2), may provide this dinoflagellate a competitive advantage over other algal species during variable N conditions (Glibert & Terlizzi 1999, Collos et al. 2007, Jauzein et al. 2008). However, this is a singular comparison, as few experiments have studied the impact of preconditioning cells on phytoplankton preference and growth rates. Further studies should be done with *A. catenella* and other dinoflagellates to elucidate if N-switching and enhanced growth during co-utilization is common within dinoflagellates, if these traits are independent of nutrient pre-conditioning, and if they manifest to a competitive advantage *in situ*.

In our mixed-N source experiments, the response rate of $\delta^{15}\text{N}_{\text{POM}}$ was rapid (within 24 hours) to the new N source being utilized by the cells (Figures 3, 4, and 5). As expected based on the single-N source experiments, N-isotope discrimination between source and POM was large during the first two days ($\delta = 23\text{‰}$) when cells were utilizing NH_4^+ and urea, and decreased to a smaller value ($\delta = 3.3\text{‰}$) upon exhaustion of NH_4^+ and switch to NO_3^- utilization (Figures 3, 4, and 5). Co-utilization of both inorganic and organic N forms was evident in the isotope ratios of POM as 1) the value was not as large in the first 48 hours as would be expected if the cells had only been utilizing NH_4^+ (Table 1), and 2) after the depletion of NH_4^+ , the isotopic signature of the cells rapidly rose to a value, $\delta^{15}\text{N}_{\text{POM}} = 3.3\text{‰}$, between the $\delta^{15}\text{N}$ value of urea (-0.7‰) and NO_3^- (12.7‰) in the medium. 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. As such, the results of this study suggest that a mixed isotope model (Waser et al. 1998b, Vavilin et al. 2014), which considers dinoflagellate fractionation, should ideally be adapted for systems where autotrophic *A. catenella* are utilizing more than one N source. When applied in systems where anthropogenic and natural N sources are isotopically distinct, $\delta^{15}\text{N}_{\text{POM}}$ could also discriminate between anthropogenic vs. natural sources, and one could calculate the percent contributions of each N source utilized by the autotrophic dinoflagellates. More research is still needed, however, to confirm a consistent pattern of fractionation across diverse genera of autotrophic dinoflagellates and to determine how broadly applicable these results are to dinoflagellates that rely on mixotrophy to meet their nutritional requirements. We speculate that once mixotrophy is introduced to this model, POM dominated by dinoflagellates will become isotopically heavier, following the general rule of a +3‰ fractionation per trophic-step, possibly making the isotopic tool more difficult to use.

Effect of N chemical form on cellular toxicity

While the utilization of different singular N forms did not alter *A. catenella* growth rates, the strains' toxin content (toxin per cell) and cellular toxicity (calculated from TEFs) varied significantly between N treatments of NO_3^- , NH_4^+ , and urea. For both strains of *A. catenella*, toxin content and cellular toxicity were significantly lower in cells grown on urea relative to cells grown on NH_4^+ (Figures 7 and 8). Other studies have reported a similar reduction in toxin content under urea utilization, including STX-producing *Alexandrium tamarense* and an axenic strain of *A. catenella*, and a pinnatoxin G-producing *Vulcanodinium rugosum* (Dyhrman & Anderson 2003, Leong et al. 2004, Abadie et al. 2015). Leong et al. (2004) provided an explanation for this anomaly, suggesting that the competition for N between toxin production and other metabolic pathways, e.g., growth, may be different among N chemical forms.

Interestingly, the observed changes in cellular toxicity in response to N chemical form were not due to changes in toxin profile (i.e., percent composition of congeners, Figure 6), as the relative percent of each congener remained essentially constant within strains across N chemical forms. Instead, differences in cellular toxicity were due to changes in the amount of toxin per cell (toxin quotas or content) between N treatments (Figure 7). This contrasts the results from a previous study (Dyhrman & Anderson 2003) showing a change in the toxin profile of an axenic strain of *A. catenella* and an increased toxicity when grown on urea. Additionally, the toxin content of the two *A. catenella* varied between strains when grown on NO_3^- , emphasizing the importance of using multiple isolates in such an experimental design and the possible differences in response between subpopulations. Further research is necessary to identify if this contrast in results is due to the presence of bacteria or variation between strains. Together these growth and toxicity studies suggest that the size of an *A. catenella* bloom is not restricted by the N form available, but that systems with mixed N forms may support larger blooms and NH_4^+ - compared to urea-supplied blooms may be relatively more toxic due to increased toxin per cell.

Conclusions

The study demonstrates the ability for $\delta^{15}\text{N}_{\text{POM}}$ to be used as a source tracer in systems dominated by either NO_3^- or urea, and the need for fractionation to be considered in single and mixed N models. Both this dinoflagellate's ability to assimilate urea along with multiple inorganic forms of N, and its increased growth rate when utilizing multiple N sources simultaneously (Figures 1, 2), should be considered during nutrient and bloom management, as urea-based fertilizers now comprise over half of the market (Anderson et al. 2002, Glibert et al. 2006). Further research must identify a method for analyzing the $\delta^{15}\text{N}$ of dissolved urea, within the range of natural ^{15}N abundances in seawater, as this chemical form can be substantial in the field. With an established 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 *A. catenella* blooms. Additionally, the N isotope fractionation information here is being used in the development of a compound specific stable isotope analysis (CSIA) to describe nutritional history of a specific HAB species instead of the constituents of bulk particulate organic matter samples. Nutrients are only one factor influencing bloom dynamics, but an understanding of the relative importance of natural and anthropogenic nutrients in the development of a specific toxic bloom is necessary to predict future decadal, annual, and compositional shifts in algal blooms and toxicity.

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Figure 1.

The increase in cell concentration and growth rates for *Alexandrium catenella* strains ATSP7-D9 (A) and GTCA-28 (B) over time when grown separately on nitrate ($\bullet$), ammonium ($\square$), 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 0.35 d^{-1} on nitrate, 0.37 d^{-1} on ammonium, and 0.34 d^{-1} on urea+Ni and GTCA-28 grew at growth rate (μ) of 0.26 d^{-1} on nitrate, 0.28 d^{-1} on ammonium, and 0.29 d^{-1} on urea+Ni. Statistical analyses performed using log-transformed cell concentrations indicated no significant difference between strains ($p = 0.58$) nor N type ($p = 0.69$) (nonparametric Kruskal-Wallis Test) ($n = 3$, mean $\pm$ SD).

Figure 2.

The increase in cell concentration for *Alexandrium catenella* strain ATSP7-D9 over time when preconditioned either on ammonium (□), nitrate (●), or urea+Ni (▲) and grown on all three N chemical forms. Starting after growth lag, strain ATSP7-D9 cells grew at an average growth rate (μ) of 0.51–0.54 cells•ml⁻¹. Statistical analyses performed using log-transformed cell concentrations indicated no significant difference based on the N type the cells were preconditioned (Repeated Measures ANOVA: $F_{2,2} = 3.345$, $p = 0.172$) ($n = 3$, mean $\pm$ SD).

Figure 3.

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

Figure 4.

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

Figure 5.

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

Figure 6. Percent toxin composition, i.e., profile, of two strains of *Alexandrium catenella*: ATSP7-D9 and GTCA-28, when grown on ammonium, nitrate, or urea ($n = 3$) during the single-N source experiment. Variation in toxin composition within treatments were minimal with all standard deviations less than 5%, and all but two congeners less than 2%.

Figure 7.

The total toxicity of *Alexandrium catenella* strains ATSP7-D9 and GTCA-28 cells when exposed to ammonium, nitrate, or urea during the single N source experiment. Total toxicity is calculated using the toxicity equivalent factors (TEFs) recommended by EFSA (2009). Data are plotted by mean $\pm$ SD. Letters indicate significant differences between means tested across strains (Post-Hoc ANOVA: Tukey).

Figure 8. The total toxin content of *Alexandrium catenella* strains ATSP7-D9 and GTCA-28 cells when exposed to ammonium, nitrate, or urea during the single N source experiment. Data are plotted by mean $\pm$ SD. Letters indicate significant differences between means tested across strains (Post-Hoc ANOVA: Tukey).

Table 1.

Growth rates (μ), nitrogen isotope ratios ($\delta^{15}\text{N}$) of the medium and *Alexandrium catenella* cells, i.e., particulate organic matter ($\delta^{15}\text{N}_{\text{POM}}$), and 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 value between the two strains ($\chi^2_{1,8} = 0.59$, $p = 0.44$) but there are changes in fractionation based on the nitrogen source ($\chi^2_{2,2} = 14.31$, $p\text{-value} < 0.01$) being taken up by the cells. Samples performed in triplicate.

<i>Alexandrium catenella</i> Strain	Nitrogen Source	μ (d^{-1})	$\delta^{15}\text{N}$ medium (‰)		$\delta^{15}\text{N}$ POM (‰)		value based on medium (‰)	
			Last 6 hours	End of exp.	Last 6 hours	End of exp.	Last 6 hours	End of exp.
ATSP7-D9	Nitrate	0.32 $\pm$ 0.02	12.7 $\pm$ 0.1	13.3 $\pm$ 0.1	10.6 $\pm$ 0.2		2.09 $\pm$ 0.1	2.6 $\pm$ 0.1
	Ammonium	0.27 $\pm$ 0.01	24.8 $\pm$ 5.5	37 $\pm$ 4.8	-2.5 $\pm$ 1.6		27.3 $\pm$ 6.9	39.5 $\pm$ 3.6
	Urea	0.29 $\pm$ 0.01	-0.3 $\pm$ 0.4*	na	-0.75 $\pm$ 0.1		0.45 $\pm$ 0.1	na
GTCA-28	Nitrate	0.19 $\pm$ 0.01	12.6 $\pm$ 0.1	12.6 $\pm$ 0	9.2 $\pm$ 2.5		3.4 $\pm$ 2.5	3.4 $\pm$ 2.5
	Ammonium	0.28 $\pm$ 0.01	15.5 $\pm$ 1.1	30.1 $\pm$ 3.3	-2.3 $\pm$ 1.4		17.9 $\pm$ 3.1	32.4 $\pm$ 2
	Urea	0.29 $\pm$ 0.02	-0.3 $\pm$ 0.4*	na	-0.57 $\pm$ 0.2		0.27 $\pm$ 0.2	na

* $\delta^{15}\text{N}$ of urea in the medium was determined using stock reagent

Mean $\pm$ SD

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

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

na = data not available