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

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ABSTRACT: 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 harmful algal species, however, 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 (Δ) and toxin content using isolates of the dinoflagellate *Alexandrium catenella* in single-N and mixed-N experiments. Growth of *A. catenella* exclusively on nitrate (NO_3^-), ammonium (NH_4^+), or urea resulted in Δ 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 NH_4^+ and urea when exposed to mixed-N medium and only utilized NO_3^- after NH_4^+ decreased below 2 to 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 NO_3^-- and urea-rich environments, the $\delta^{15}N$ of *A. catenella* would resemble the source(s) of N utilized, supporting this tool's utility as a tracer of N source(s) facilitating bloom formation, but caution is advisable in NH_4^+-rich environments, where the large Δ value could lead to misinterpretation of the signal.

KEY WORDS: *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 yr, creating dire consequences for public health, aquatic ecosystems, fishery services, and local economies (Anderson et al. 2012). 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.

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Stable isotope ratios of N ($^{15}N/^{14}N$, expressed herein as $\delta^{15}N$ values relative to atmospheric N_2) in particulate organic matter (POM, $\delta^{15}N_{POM}$) 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, Žvab Rožič et al. 2015). For example, N derived from human or animal waste is often ^{15}N enriched relative to natural sources (Kendall 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 $\delta^{15}N_{POM}$. The utility of $\delta^{15}N_{POM}$ 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 N chemical forms. This deficit identifies a current limitation of $\delta^{15}N_{POM}$ 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, 2008, Dyhrman & Anderson 2003, Leong et al. 2004, 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 (µM) concentrations of NO_3^-, NH_4^+, urea, or a combination of the 3, following preconditioning to a specific form. The objectives of this study were to determine (1) N isotopic fractionation (i.e. Δ values) by *A. catenella* during growth on µM 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 $\delta^{15}N_{POM}$ 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 N-rich compounds that are unique to a dinoflagellate of concern, such as STXs or other biotoxins. We will compare fractionation on the species-specific compound to $\delta^{15}N_{POM}$ 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.

MATERIALS AND METHODS

Chemicals

Reagents for toxin quantification include Optima LC-MS grade formic acid (99–100%), Fluka ammonium hydroxide (NH_4OH, >25% in water, eluent additive for liquid chromatography-mass spectrometry [LC-MS]), and Honeywell Burdick & Jackson LC-MS grade acetonitrile (ACN) and methanol. During extraction and culturing efforts, high-performance liquid chromatography grade glacial acetic acid; American Chemical Society (ACS) certified ammonium chloride, urea, and sodium chloride (Fisher Scientific); and ACS reagent grade sodium nitrate (99%) (Alfa Aesar) were utilized. Certified reference toxin standards STX, NEO, GTX1/4, GTX2/3, GTX5, dcNEO, dcSTX, dcGTX2/3, and C1/2 (National Research Council [NRC], Halifax, NS, Canada) were used during toxin quantification.

Culture maintenance

The STX-producing dinoflagellate *Alexandrium catenella* clones GTCA-28 (western Gulf of Maine, USA, 1985, single cell from cyst germination) and ATSP7-D9 (Nauset Marsh System, Cape Cod, MA, USA, 2009, single vegetative cell) were chosen for their association with different 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 (WHOI). Cultures were grown in autoclaved 0.22 µm prefiltered seawater from Wachapreague, VA, USA, brought to a pH of 8.3 with HCl or sodium hydroxide, salinity of 35, and amended with sterile f/6 nutrients without silicon dioxide or NO_3^- (i.e. 36.2 µM monosodium phosphate, N source and concentration as described under 'Experimental conditions', trace metals, and vitamins; Guillard 1975). The cultures were illuminated at light intensities of around 60 µmol photons on a 14 h light:10 h dark cycle and kept at a set temperature of 15°C. Cells were grown for at least 3 generations, i.e. preconditioned, 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 nickel sulfate [$NiSO_4$]), prior to use in the experiment. Cultures were grown on a low concentration of N, to better represent natural concentrations and to avoid NH_4^+ toxicity at ca. 50 µM. Medium containing urea was amended with nickel because *Alexandrium* spp. utilize the nickel-dependent urease enzyme to hydrolyze urea into NH_4^+ (Dyhrman & Anderson 2003). When culture was used to inoculate experiments, less than 0.4 µmol of NO_3^-, NH_4^+, 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 NO_3^-, NH_4^+, or urea with 100 nM $NiSO_4$, was repeatedly replenished to avoid N limitation; and (2) a mixed-source study where batch cultures were supplied with a combination of NO_3^-, NH_4^+, and urea at inoculation and then allowed to deplete the mix of N. The initial $\delta^{15}N$ values of the NO_3^-, NH_4^+, and urea stock reagents were measured at 12.7 ± 0.8, 2.1 ± 0.4, and −0.3 ± 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, 2 strains of *A. catenella*, GTCA-28 and ATSP7-D9, were each inoculated into triplicate experimental flasks for the 3 treatments (i.e. NO_3^-, NH_4^+, 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 3 treatments. The NH_4^+ concentration was monitored in real time, using an ammonia (NH_3)/NH_4^+ test kit with a limit of detection of 0 to 8 ppm (0–443 µM) and a color chart with increments of 0.25 ppm (13.8 µM, Aquarium Pharmaceuticals), to avoid reaching toxic levels during N additions; the other 2 N chemical forms were added to their respective treatments assuming a similar uptake rate as NH_4^+. N was added to all treatments daily, to reach 30 µM N, based on the NH_3/NH_4^+ test kit results. On Day 5 and thereafter, the frequency of N amendment was increased to every 6 h to account for the increase in culture biomass, maintaining N at a concentration of over 10 µM N. Culture medium was collected, filtered, and frozen for the independent quantification of dissolved NO_3^-, NH_4^+, and urea at the start of the experiment and every 2 d 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 $\delta^{15}N$ of the dissolved NO_3^- ($\delta^{15}N_{NO_3^-}$) or NH_4^+ ($\delta^{15}N_{NH_4^+}$), allowing for source characterization most relevant to the end $\delta^{15}N_{POM}$ measurement. To end the experiment, i.e. when cell concentrations reached ~3000 cells ml^{-1} during exponential growth, endpoint samples were collected for measurement of the isotopic composition ($\delta^{15}N$) of particulate N ($\delta^{15}N_{POM}$) and media $\delta^{15}N_{NO_3^-}$

and $\delta^{15}N_{NH_4^+}$. 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 preconditioned to grow on NO_3^-, NH_4^+, or urea (25–50 µM N) for at least 3 generations (around 15 d). To mark the beginning of the experiment, nine 3 l Fernbach flasks per treatment were inoculated with a preconditioned 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 µM N each, or a total of 120 µM 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 3 flasks were enumerated daily with the use of Lugol's preservation, an S-R counting chamber, and light microscopy. Samples for the determination of N medium concentrations, $\delta^{15}N_{POM}$, and media $\delta^{15}N_{NO_3^-}$ and $\delta^{15}N_{NH_4^+}$ were taken from triplicate flasks every 12 h over a 6 to 7 d experimental period. Culture volume became depleted due to repeated sampling; at 60 and 96 h, 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 h, i.e. when subsequent measurements were then taken from another set of triplicate flasks inoculated at the same time. When comparing the 9 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 precombusted (4 h, 450°C) glass fiber filter (25 mm diameter, Whatman GF/F, catalog 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 4-channel segmented flow analyzer (Nutrient Analytical Facility, WHOI) was used to quantify NH_4^+, NO_3^-/nitrite, phosphate, and total dissolved N in the GF/F-filtered medium. Urea was quantified at Virginia Institute of Marine Science (VIMS) using the manual diacetyl monoxime thiosemicarbizide method adapted from Rahmatullah & Boyde (1980), modified by Price & Harrison (1987). Duplicate samples were diluted to fall within the standard curve (0–10 µM N, 6 points × 3 replicates) and analyzed on a Shimadzu UV-1601 UV-visible spectrophotometer equipped with a 10 cm cell (525 nm, Solorzano 1969).

Toxin extraction and analysis

The toxin samples were immediately pelleted by centrifugation at 3000 relative centrifugal force (RCF) for 15 min at 10°C, aspirated to remove overlying seawater, and the pellets were 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 3 freeze–thaw cycles, and probe sonicated 1 min at 40 W on ice (Branson Digital Sonifier 450). Subsequently, samples were centrifuged (3000 RCF, 10°C, 15 min) to remove cell debris. The supernatant was pushed through a 0.22 µm, 13 mm syringe filter (Millex-GV, Durapore), diluted 1:1 with ACN in an autosampler vial, and frozen (–20°C) for up to 2 wk before quantification.

Quantification was performed by hydrophilic interaction chromatography using an Acquity ultra-performance liquid chromatography coupled to a Xevo TQD triple quadrupole (Waters) tandem mass spectrometer (HILIC-MS/MS). Separation was achieved using a Waters BEH Amide 1.7 µm column (2.1 × 100 mm, product no. 186004801) with a Waters BEH Amide 1.7 µm guard column attached (product no. 186004799). The autosampler and columns were maintained at 10 and 60°C, respectively. Mobile phase A was water/formic acid/NH_4OH (500:0.075:0.3 v/v/v), mobile phase B was ACN/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 and negative 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; and GTX4, m/z 412.1 > 314.1 in positive mode and dcGTX2, m/z 351.1 > 164; GTX2, m/z 394.1 > 351.1; and 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, dcGTX3, C1, and C2 (NRC, Halifax, NS, 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 the 1:2000 dilution, the lowest calibration standard for each congener: 33.2 nM STX, 32.8 nM NEO, 30.2 nM GTX1, 27.9 nM GTX2, 17 nM C2, 56.7 nM C1, 27.9 nM GTX5, 21.7 nM GTX3, 9.9 nM GTX4, 14.7 nM dcNEO, 32.5 nM dcSTX, 50.1 nM dcGTX2, and 14.7 nM dcGTX3. The precision of toxin measurements, i.e. the percent relative standard deviation (RSD = SD/mean × 100) based on the peak area of 6 injections, was less than 6% for C2, GTX3, and GTX4 (the 3 most dominant toxins). The relative cellular toxicities (fmol STX equivalents [eq] cell^{-1}) of the congeners were calculated in terms of the parent toxin, STX, based on toxicity equivalency 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 precombusted for 4 h 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, catalog no. 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 no. 05-719-117) and frozen at −20°C. Prior to isotope analysis of POM ($\delta^{15}N_{POM}$), samples were dried at 60°C for 30 to 60 min until dry. Filtrate, 200 ml, for isotope analysis of dissolved NH_4^+ ($\delta^{15}N_{NH_4^+}$) was processed using a modified version of the NH_4^+ diffusion method of Holmes et al. (1998), whereby polypropylene membrane filters (25 mm, 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 µmol N, or modified at the elemental analyzer combustion furnace to reach a lower detection limit, 0.15 µmol N (Holtvoeth et al. 2006, York et al. 2007). The precision of $\delta^{15}N$ measurements on this instrument was 0.17‰. Filtrate, 20 ml for isotope analysis of dissolved NO_3^- ($\delta^{15}N_{NO_3^-}$), was transferred into duplicate 30 ml high-density polyethylene bottles and analyzed by bacteria denitrification assay using a ThermoFinnigan GasBench + PreCon trace gas concentration system interfaced to a ThermoScientific Delta V Plus isotope ratio mass spectrometer (Stable Isotope Facility, University of California, Davis).

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

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

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

$$\Delta = \delta^{15}N_{Medium} - \delta^{15}N_{POM} \tag{2}$$

For the experiments, Δ represents the difference between the $\delta^{15}N_{POM}$ at the end of the experiment and the isotope value of the medium ($\delta^{15}N_{medium}$) immediately after the final N addition, i.e. 6 h prior to the end of the experiment (Table 1). The only exception was for urea, where the Δ value was calculated based on the $\delta^{15}N$ value of the urea stock reagent prior to dissolving into seawater (Table 1).

Statistical analysis

A Shapiro-Wilks test was used to test for normality in the data and Bartlett's test to determine for equal variances among populations. Two-way 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, non-parametric 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 9 flasks was considered an independent treatment, as flask sets were changed at 60 and 96 h (n = 9). Statistics and graphs were generated using the R statistical program (version 0.99.879; Wickham 2009, R Core Team 2016, R Studio Team 2016), and α was set at 0.05.

Table 1. Growth rates (μ), nitrogen (N) isotope ratios of the medium ($\delta^{15}N_{medium}$) and *Alexandrium catenella* cells (i.e. particulate organic matter, $\delta^{15}N_{POM}$), and Δ value based on $\delta^{15}N$ of the medium in the last 6 h, $\delta^{15}N$ of the medium at the end of the experiment when grown on 1 of 3 N forms during the single-N source experiment. Samples performed in triplicate. Values are pre-sented as means ± SD. μ: ln(concentration$_2$/concentration$_1$)/(time$_2$ – time$_1$); Δ: $\delta^{15}N_{medium} - \delta^{15}N_{POM}$; na: not available

A. catenella strain	N source	μ (d^{-1})	$\delta^{15}N_{medium}$ (‰)		$\delta^{15}N_{POM}$ (‰)	Δ value based on $\delta^{15}N_{medium}$ (‰)	
			Last 6 h	End of exp.	End of exp.	Last 6 h	End of exp.
ATSP7-D9	Nitrate	0.32 ± 0.02	12.7 ± 0.1	13.3 ± 0.1	10.6 ± 0.2	2.09 ± 0.1	2.6 ± 0.1
	Ammonium	0.27 ± 0.01	24.8 ± 5.5	37 ± 4.8	–2.5 ± 1.6	27.3 ± 6.9	39.5 ± 3.6
	Urea	0.29 ± 0.01	–0.3 ± 0.4[a]	na	–0.75 ± 0.1	0.45 ± 0.1	na
GTCA-28	Nitrate	0.19 ± 0.01	12.6 ± 0.1	12.6 ± 0	9.2 ± 2.5	3.4 ± 2.5	3.4 ± 2.5
	Ammonium	0.28 ± 0.01	15.5 ± 1.1	30.1 ± 3.3	–2.3 ± 1.4	17.9 ± 3.1	32.4 ± 2
	Urea	0.29 ± 0.02	–0.3 ± 0.4[a]	na	–0.57 ± 0.2	0.27 ± 0.2	na

[a]$\delta^{15}N$ of urea in the medium was determined using stock reagent

RESULTS

Single-N source experiment

Growth rates

Over the 7 d experimental period, the cultures demonstrated a 2 d lag during which cell concentrations remained constant or declined and then transitioned into exponential growth. Overall, exponential growth rates were similar between the 2 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 N treatment and *Alexandrium catenella* strains (N form: $\chi^2_{2,2} = 0.72$, $p > 0.6$; strains: $\chi^2_{1,8} = 0.29$, $p > 0.5$), with ATSP7-D9 (Fig. 1a) demonstrating a growth rate (μ) of 0.34 to 0.37 d^{-1} and GTCA-28 (Fig. 1b) a growth rate (μ) of 0.26 to 0.29 d^{-1}. Treatments were terminated once cultures reached a minimum cell concentration of 3000 cells ml^{-1} but less than 6000 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.

N utilization

The initial seawater, preamendment, contained 0.6 μM NO_3^-, 1.9 μM NH_4^+, and 0.1 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 to 24 h. During the last 6 h of the experiment, the average N drawdown (hereafter inferred as uptake rates) for strain ATSP7-D9 was 0.02 ± 0.003, 0.01 ± 0.007, and 0.003 ± 0.003 N cell^{-1} d^{-1} for NO_3^-, NH_4^+, and urea, respectively. Strain GTCA-28 had a similar uptake rate for NO_3^- of 0.03 ± 0.02 N cell^{-1} d^{-1} and for NH_4^+ of 0.009 ± 0.001 N cell^{-1} d^{-1}. Urea uptake rates were not calculated for GTCA-28 because there was error with dilutions of the high urea concentrations.

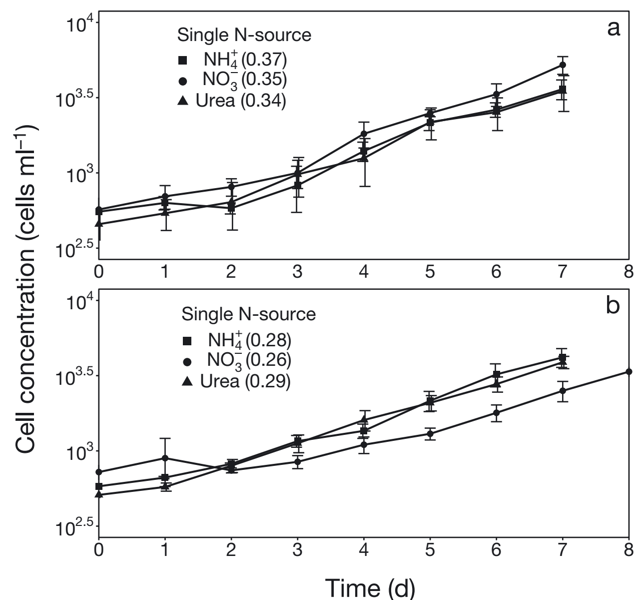

Fig. 1. Increase in cell concentration and (log scale) growth rates for *Alexandrium catenella* strains (a) ATSP7-D9 and (b) GTCA-28 over time when grown separately on nitrate (NO_3^-, ●), ammonium (NH_4^+, ■), or urea + nickel (▲) during the single-nitrogen (N) source experiment. Starting after growth lag, strain ATSP7-D9 cells grew at an average growth rate (μ) of 0.35 d^{-1} on NO_3^-, 0.37 d^{-1} on NH_4^+, and 0.34 d^{-1} on urea + nickel, and strain GTCA-28 grew at a growth rate (μ) of 0.26 d^{-1} on NO_3^-, 0.28 d^{-1} on NH_4^+, and 0.29 d^{-1} on urea + nickel. Statistical analyses performed using log-transformed cell concentrations indicated no significant difference between strains (p = 0.58) or N type (p = 0.69) (nonparametric Kruskal-Wallis test) (n = 3, mean ± SD)

N 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^+ (Δ = 23.5 ± 7.2‰), followed by NO_3^- (Δ = 2 ± 0.3‰) and urea (Δ = 0.3 ± 0.1‰) (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 $\delta^{15}N_{NH_4^+}$ value of the medium increased from 2.1 to 24.8 ± 5.5‰ in the ATSP7-D9 experiments over time and to 15.5 ± 1.1‰ in the GTCA-28 experiments (Table 1). The observed enrichment in ^{15}N in the NH_4^+ medium was likely not 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, total dissolved N (TDN), or dissolved organic N (DON) through the course of the experiment. The change in the $\delta^{15}N_{NO_3^-}$ value of the medium was minimal over the course of the experiment, increasing only from 12.7 to 13‰.

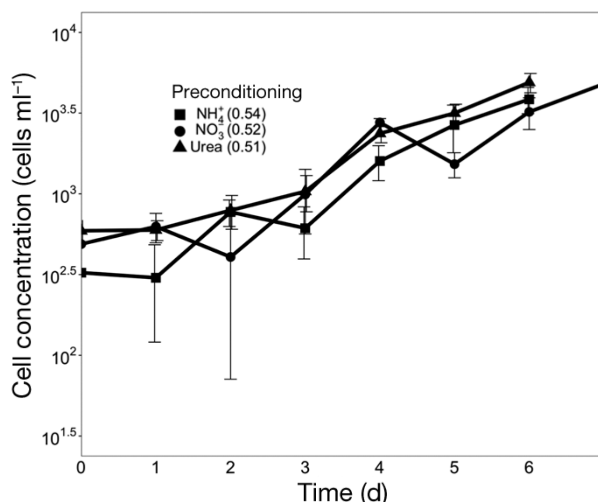

Fig. 2. Increase in cell concentration (log scale) for *Alexandrium catenella* strain ATSP7-D9 over time when preconditioned either on ammonium (NH_4^+, ■), nitrate (NO_3^-, ●), or urea + nickel (▲) and grown on all 3 nitrogen (N) chemical forms. Starting after growth lag, strain ATSP7-D9 cells grew at an average growth rate (μ) of 0.51 to 0.54 d^{-1}. Statistical analyses performed using log-transformed cell concentrations indicated no significant difference based on the N type the cells were preconditioned on (repeated measures ANOVA: $F_{2,2}$ =3.345, p = 0.172) (n = 3, mean ± SD)

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 to 0.54 d^{-1} over the course of the experiment (Fig. 2). No effect of N preconditioning was detected on growth rate or between growth curves of the 3 treatments ($F_{2,2}$ = 3.3, p > 0.13). All 3 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.

N utilization

During the mixed-N source experiment, there was no indication that preconditioning *A. catenella* cells to any of the 3 N forms affected the patterns of NH_4^+ utilization (repeated measures ANOVA: $F_{2,2}$ = 0.9, p > 0.4) or NO_3^- 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 treatments, with cells preconditioned on NO_3^- taking up urea at a faster rate than cells preconditioned on NH_4^+ and urea (Figs. 3a, 4a & 5a). A consistent pattern of N selectivity held across treatments, whereby *A. catenella* cells took up NH_4^+ and urea initially and only assimilated NO_3^- after NH_4^+ was drawn down below 2 to 4 μM (Figs. 3a, 4a & 5a). In comparison, urea concentrations ranged from 5 to 30 μM N at the onset of NO_3^- utilization and continued to be utilized at the same time as NO_3^- (Figs. 3a, 4a & 5a). The N uptake rate varied between the N chemical forms, with the highest uptake on NH_4^+ (0.036 N $cell^{-1}$ d^{-1}), followed by NO_3^- (0.031 N $cell^{-1}$ d^{-1}) and then urea (0.025 N $cell^{-1}$ d^{-1}).

N isotope fractionation

The $\delta^{15}N_{POM}$ was at its lowest at around 48 h (averaging −5‰) during co-utilization of NH_4^+ and urea and then increased by 84 h (averaging 0.9‰) during the uptake of NO_3^- and urea (Figs. 3b, 4b & 5b). This pattern was consistent between treatments, demon-

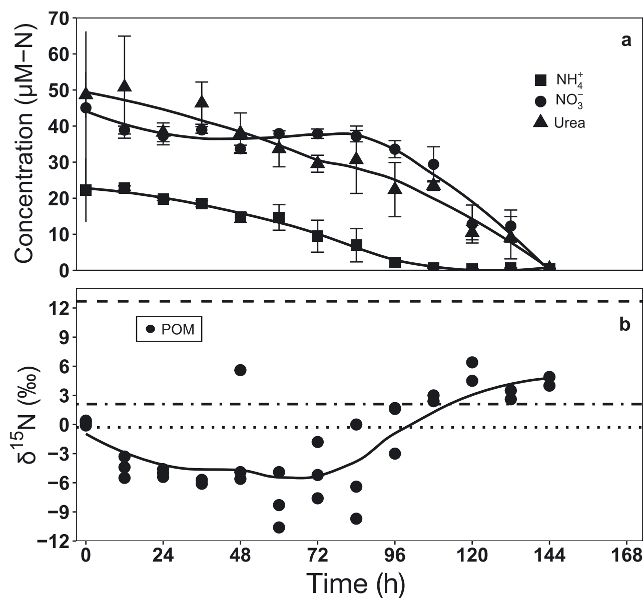

Fig. 3. (a) Nitrogen (N) depletion in medium and (b) associated stable N isotope ratio ($\delta^{15}N$) of particulate organic matter (POM, i.e. cells) over time for *Alexandrium catenella* culture preconditioned on ammonium and then grown in mixed medium (all 3 N chemical forms) during the mixed-N source experiment. Nitrate (NO_3^-, ●), ammonium (NH_4^+, ■), or urea + nickel (▲) concentrations are presented in µM N with a best fit line. The $\delta^{15}N$ values of whole cells are represented by black circles with a best fit line added; horizontal lines represent the initial isotope values of NO_3^- (dashed), NH_4^+ (dot-dashed), and urea (dotted) (n = 3, mean ± SD)

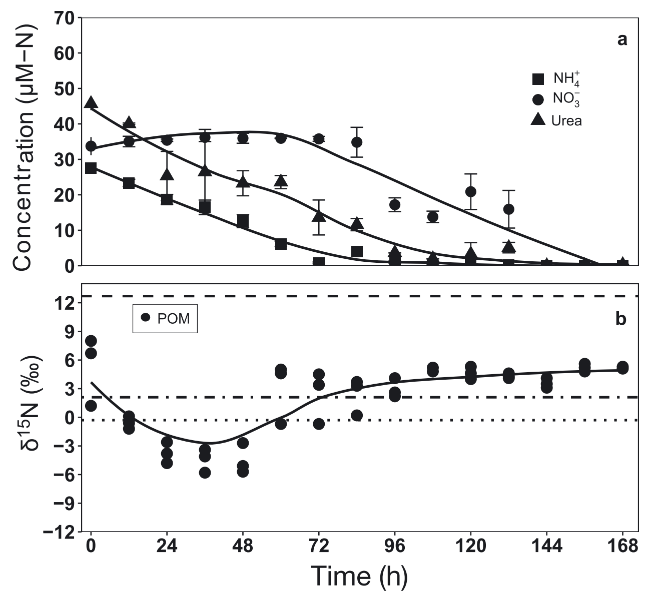

Fig. 4. Same as Fig. 3 but preconditioned on nitrate

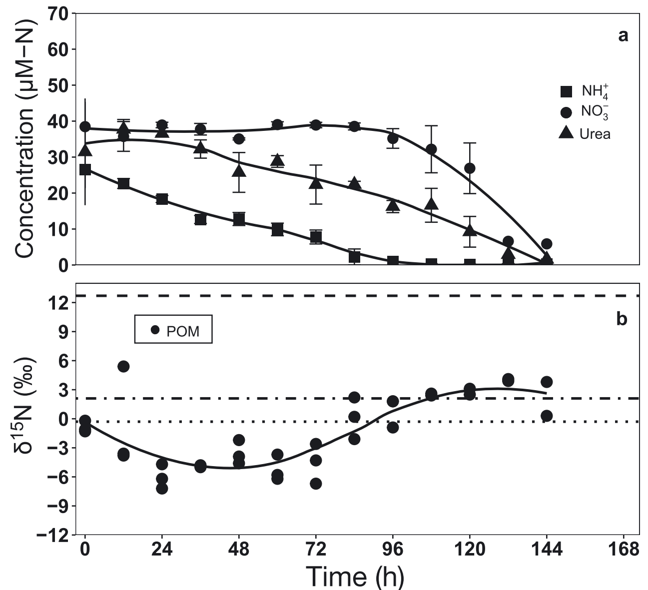

Fig. 5. Same as Fig. 3 but preconditioned on urea

strating that preconditioning cells to NO_3^-, NH_4^+, 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 $\delta^{15}N$ of the dissolved NH_4^+, and to a lesser extent NO_3^-, increased over time in the medium. The $\delta^{15}N$ of the dissolved NH_4^+ increased from 2.6 ± 0.7 to 19.5 ± 2.4‰ by 48 h, and NO_3^- increased from 12.6 ± 0.2 to 13.5 ± 0.2‰ by 132 h. 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 the concentration of dissolved NO_3^-, NH_4^+, urea, TDN, or DON through the course of the experiment.

Toxin 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 (Fig. 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 TEFs, reported as STX eq cell^{-1}, Fig. 7) (ANOVA: $F_{1,2}$ = 2.17, p = 0.16). When averaged over all N treatments, the total toxicities of ATSP7-D9 and GTCA-28 were 57.7 ± 13.6 and 46.3 ± 19.1 fmol STX eq cell^{-1}, respectively.

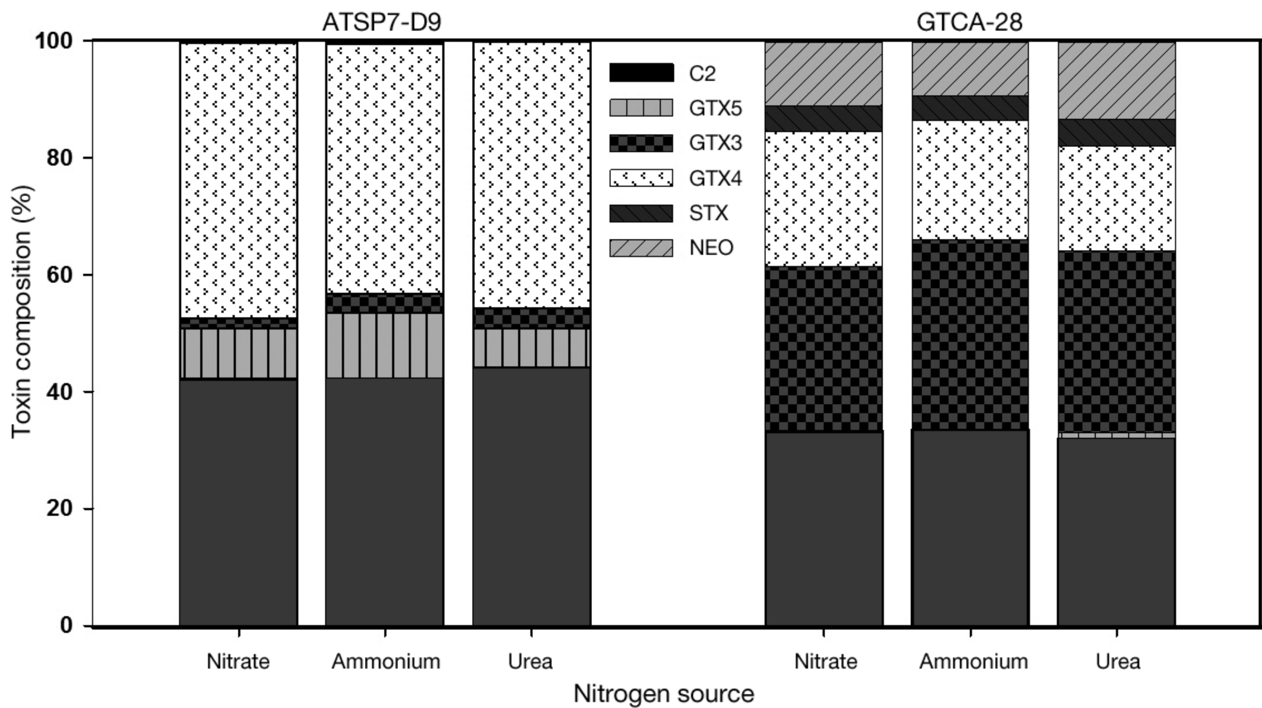

Fig. 6. Percent toxin composition, i.e. profile, of 2 strains of *Alexandrium catenella*, ATSP7-D9 and GTCA-28, when grown on ammonium, nitrate, or urea (n = 3) during the single-nitrogen source experiment. Variations in toxin composition within treatments were minimal with all standard deviations less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

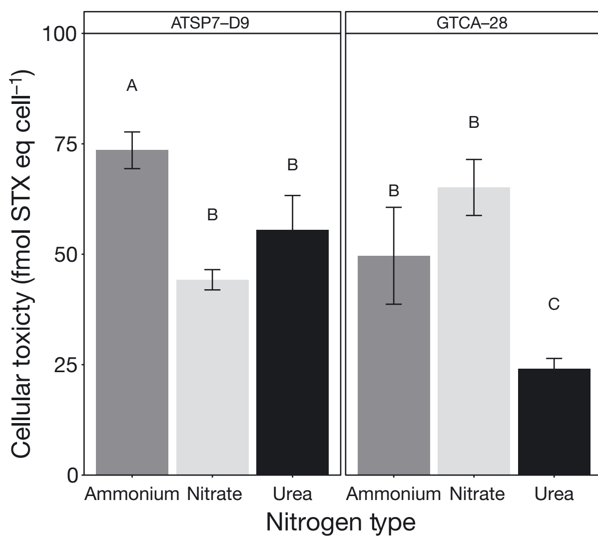

Fig. 7. Total toxicity of *Alexandrium catenella* strains ATSP7-D9 and GTCA-28 cells when exposed to ammonium, nitrate, or urea during the single-nitrogen source experiment. Total toxicity is calculated using the toxicity equivalency factors recommended by the European Food Safety Authority (2009). Data are plotted by mean ± SD. Letters indicate significant differences between means tested across strains (post hoc ANOVA: Tukey). STX eq: saxitoxin equivalents

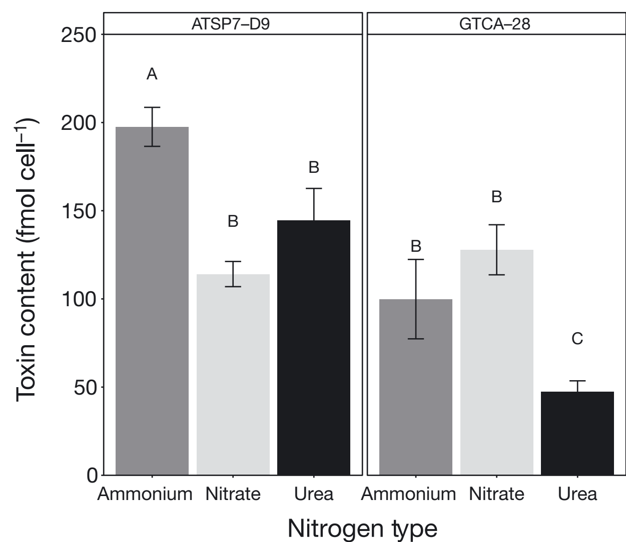

Fig. 8. Total toxin content of *Alexandrium catenella* strains ATSP7-D9 and GTCA-28 cells when exposed to ammonium, nitrate, or urea during the single-nitrogen source experiment. Data are plotted by mean ± SD. Letters indicate significant differences between means tested across strains (post hoc ANOVA: Tukey)

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; Fig. 7) and toxin content ($F_{2,2}$ = 13.61, p < 0.01; Fig. 8) across strains. For both strains, the NH_4^+-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 NO_3^--grown cultures, however, varied in toxin content; 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 NH_4^+-grown cultures. When comparing across both strains and all N treatments, cellular 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 utilizing urea (mean: 24 ± 2.4 fmol STX eq cell^{-1}; Fig. 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 dinoflagellates. The results presented here are consistent with the isotopic fractionation results found for other taxonomic groups, namely diatoms and coccolithophores, and add new insight into the ability of *Alexandrium catenella* to exploit many N forms available in the field as well as the dependency of toxicity upon the N chemical form being utilized. Here we also demonstrate that *A. catenella* can utilize NO_3^-, NH_4^+, or urea for growth and toxin production under N-sufficient conditions (Figs. 1, 7, & 8) and that while the chemical form of N available does not affect growth rate, it can lead to variations 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 NH_4^+ or urea assimilation by dinoflagellates. With the larger goal of determining the utility of stable N isotopes in linking N source to dinoflagellate blooms, we evaluated N isotope fractionation by *A. catenella* cells grown on NO_3^-, urea, or NH_4^+. Growth on urea and NO_3^- led to an average N isotope ratio in the cells, $\delta^{15}N_{POM}$, that was reliably lower than the source, with an average Δ value of 0.34 and 2.37‰ (average of 2 strains), respectively (Table 1). The fractionation observed during NO_3^- utilization was comparable to the results of Smith & Erdner (2011), who demonstrated that during stationary phase, *A. catenella* cells were 1.5‰ lower than the NO_3^- source. Similarly, Δ values of 1 to 3‰ are reported for other dinoflagellates grown on NO_3^- (Needoba et al. 2003). While no data have previously been reported for dinoflagellate fractionation when grown on urea, the average Δ value reported here (0.34‰) is similar to that reported for marine diatoms, 0.8‰ (Waser et al. 1998a). Overall, the results suggest that in NO_3^-- and urea-rich environments, the $\delta^{15}N$ of *A. 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 $\delta^{15}N_{POM}$ as a tracer in NH_4^+-rich environments. This large fractionation observed in dinoflagellates, 29‰, may, however, 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 exist 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, then the $\delta^{15}N_{POM}$ of dinoflagellate blooms will more effectively mimic the source in low concentrations of NH_4^+ and the utility of $\delta^{15}N$ 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 promising tool for tracking N use by dinoflagellates in aquatic systems. For example, if a dinoflagellate bloom is being driven by a ^{15}N-enriched source of N (such as sewage-derived NO_3^-), we can determine use of this source based on elevated $\delta^{15}N_{POM}$ relative to baseline or reference site values. With $\delta^{15}N_{POM}$ values that closely resemble the $\delta^{15}N$ of the NO_3^- source (i.e. a small Δ associated with NO_3^- use) and a consistent

response expected across subpopulations of *A. catenella*, the utility of this tool with respect to NO_3 is clear. It may also be possible to track use of urea if unique signals associated with specific sources can be identified, but little work has been done to define urea $\delta^{15}N$ source signals to date, and it is yet unclear if differences among urea sources are large enough and consistent enough to be used in this way. Establishing linkages with NH_4^+ sources using $\delta^{15}N$ values is less promising because of the larger and more variable Δ between $\delta^{15}N_{NH_4^+}$ values and $\delta^{15}N_{POM}$. It is possible in NH_4^+-dominated systems, however, that the Δ 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 $\delta^{15}N_{POM}$ values well below the $\delta^{15}N$ 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 or 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 $\delta^{15}N_{POM}$ 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 $\delta^{15}N_{POM}$ 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 (i.e. NH_4^+ or NO_3^-) forms simultaneously to support growth (Figs. 3a, 4a, & 5a). Co-utilization of organic and inorganic N forms also resulted in elevated growth rates when compared to cultures grown on a single-N source (Figs. 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 to 4 µM (Figs. 3a, 4a, & 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 5 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.

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 preconditioning the cultures to grow on different N forms, no difference was found in N preference, rates of uptake or utilization, and NH_4^+ and NO_3^- thresholds (Figs. 3a, 4a, & 5a). We also observed the same rapid switching between N sources in all preconditioned treatments once the same preferred chemical form, NH_4^+, had been depleted. In contrast, 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 (Figs. 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 are common within dinoflagellates, if these traits are independent of nutrient preconditioning, and if they manifest to a competitive advantage *in situ*.

In our mixed-N source experiments, the response rate of $\delta^{15}N_{POM}$ was rapid (within 24 h) to the new N source being utilized by the cells (Figs. 3–5). As expected based on the single-N source experiments, N isotope discrimination between source and POM was large during the first 2 d (Δ = 23‰), when cells were utilizing NH_4^+ and urea, and decreased to a smaller value (Δ = 3.3‰) upon exhaustion of NH_4^+ and switch to NO_3^- utilization (Figs. 3–5). Co-utilization of both inorganic and organic N forms was evident in the isotope ratios of POM for 2 reasons: (1) the Δ value was not as large in the first 48 h 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,

$\delta^{15}N_{POM}$ = 3.3‰, between the $\delta^{15}N$ value of urea (−0.7‰) and NO_3^- (12.7‰) in the medium. Together this suggests that the $\delta^{15}N_{POM}$ 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 *A. catenella* are utilizing more than 1 N source. When applied in systems where anthropogenic and natural N sources are isotopically distinct, $\delta^{15}N_{POM}$ could also discriminate between anthropogenic versus natural sources, and one could calculate the percent contributions of each N source utilized by the dinoflagellates.

More research is still needed, however, to confirm a consistent pattern of fractionation across diverse genera of dinoflagellates and to determine how broadly applicable these results are to dinoflagellates that rely on mixotrophy to meet their nutritional requirements. There is some evidence that species of *Alexandrium* are mixotrophic, but it is unknown how many species exhibit this feeding behavior and the contribution of different prey sources in their overall diet (Jauzein et al. 2015, Lee et al. 2016, Blossom et al. 2017). The isotope value of mixotrophic dinoflagellates will depend on the proportion of cellular N derived from heterotrophy versus direct N uptake. The internal N will likely follow the general rule of a +3‰ fractionation per trophic step for heterotrophy sources and follow the fractionation patterns found in this study during direct uptake of N sources, resulting in an overall fractionation representative of the mixed-N sources utilized.

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^+ (Figs. 7 & 8). Other studies have reported a similar reduction in toxin content under urea utilization, including STX-producing *A. 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; Fig. 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 (Fig. 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 varied between strains for the 2 *A. catenella* 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 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^+-supplied blooms may be relatively more toxic compared to urea-supplied blooms due to increased toxin per cell.

CONCLUSIONS

This study demonstrates the ability for $\delta^{15}N_{POM}$ to be used as a source tracer in systems dominated by either NO_3^- or urea (assuming distinct source signatures) 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 (Figs. 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 $\delta^{15}N$ 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, $\delta^{15}N_{POM}$ 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 catenella* blooms. Addi-

tionally, the N isotope fractionation information here is being used in the development of a compound-specific stable isotope analysis to describe the nutritional history of a specific HAB species instead of the constituents of bulk particulate organic matter samples. Nutrients are only 1 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 nevertheless necessary to understand and predict future decadal, annual, and compositional shifts in algal blooms and toxicity.

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