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Ammonium and maintenance of bloom populations of *Alexandrium fundyense* in the Gulf of Maine and on Georges Bank: results of laboratory culture experiments

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ABSTRACT: Blooms of the toxic dinoflagellate *Alexandrium fundyense*, responsible for paralytic shellfish poisoning, occur annually in the Gulf of Maine–Georges Bank region of the northwest Atlantic Ocean and often reach highest cell densities in surface waters depleted of nitrate, suggesting a dependence on recycled ammonium. We report here the results of batch culture experiments with *A. fundyense* designed to: (1) describe the kinetics of ammonium uptake and cell growth rates over a range of ammonium concentrations encompassing those observed in the Gulf of Maine–Georges Bank region; (2) compare rates of nitrate and ammonium uptake in the presence of one another; and (3) determine whether growth rates of *A. fundyense* on ammonium at concentrations observed in the region are sufficient to maintain established bloom populations and the degree to which continued growth of the bloom populations may be sustained by ammonium. The resulting half-saturation constant for growth ($K_{s(g)}$) on ammonium was 0.93 µM, and the half-saturation constant for ammonium uptake ($K_{s(N)}$) was 1.74 µM. The maximum growth rate (μ_{max}) was 0.51 d^{-1}, and the maximum cell-specific uptake rate (V_{max}) was 7.0×10^{-5} µmol NH_4^+-N $cell^{-1}$ d^{-1}. Based on these results we show that observed ammonium concentrations in the Gulf of Maine–Georges Bank region during *A. fundyense* blooms are sufficient to sustain the blooms for extended periods (weeks) and will allow modest continued growth.

KEY WORDS: *Alexandrium* · Ammonium · Uptake · Growth · Half-saturation constant

INTRODUCTION

Dinoflagellates of the genus *Alexandrium* have long been implicated in paralytic shellfish poisoning (PSP) events as a result of their production of potent neurotoxins. Blooms of these toxic dinoflagellates are commonly called 'red tides', a name that stems from a discoloration of the water when the cells reach high enough densities (e.g. reviewed in McGillicuddy et al. 2014a). In the Gulf of Maine–Georges Bank region, growth of these dinoflagellates to cell densities high enough to cause discoloration of the waters (often greater than 100 000 cells l^{-1}) is rare, but such blooms have been known to occur (Martin et al. 2014, McGillicuddy et al. 2014a). However, exceptionally high cell densities are not necessary to produce PSP conditions in the filter-feeding shellfish, and as few as 100 cells l^{-1} may be sufficient (Anderson 1997, Bricelj & Shumway 1998).

Species of *Alexandrium* occur throughout the world ocean (Wyatt & Jenkinson 1997) and 3 'species' are known to occur in the Gulf of Maine region: *A. tamarense*, *A. fundyense*, and *A. ostenfeldii* (Anderson 1997, Gribble et al. 2005). One of these, *A. osten-*

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feldii, is a larger cell than either *A. tamarense* or *A. fundyense* (Tomas 1997), and *A. tamarense* and *A. fundyense* are considered varieties of the same species (Anderson et al. 1994, Scholin et al. 1995). For the purposes of this study, which concerns the Gulf of Maine region, we use *A. fundyense* to refer to both forms.

Blooms of *A. fundyense* in the Gulf of Maine–Georges Bank region generally occur between April and August (e.g. Stock et al. 2005, Anderson et al. 2014a), following the annual spring diatom bloom (e.g. Townsend et al. 2005), but in some years there may be significant bloom events as late as October (e.g. Anderson et al. 2014a McGillicuddy et al. 2014b). Initiation of annual blooms are dependent on a source of benthic resting cysts (Anderson 1997, Anderson et al. 2014b), and concentrations and flux rates of dissolved inorganic nitrogen (DIN), in the form of nitrate. Following the initiation of *A. fundyense* blooms, the highest *A. fundyense* cell densities are observed in surface waters with low and undetectable nitrate concentrations (Love et al. 2005, Poulton et al. 2005, McGillicuddy et al. 2014b, Townsend et al. 2014). Earlier work has shown that the rate of vertical diffusion of nitrate across the seasonal pycnocline is weak, and those nutrient fluxes are consumed by phytoplankton in the subsurface chlorophyll maximum, thus contributing little if any nitrate to surface water populations (Townsend 1991, 1998). Clauss (2000) examined the possibility that dissolved organic nitrogen (DON) provides *A. fundyense* with its nitrogen requirements, but no correlations between DON and *A. fundyense* cell densities were detected. However, bloom populations are observed to persist for weeks following nitrate depletion by *A. fundyense* and the associated phytoplankton community (e.g. McGillicuddy et al. 2014b, Townsend et al. 2014), suggesting that recycled ammonium (NH_4^+) sustains the blooms.

The utilization of ammonium by other species of *Alexandrium* has been reported previously. Maguer et al. (2004) showed that *A. minutum,* which blooms in the Penzé estuary in France, requires ammonium for growth at certain phases of the bloom even in the presence of high concentrations of nitrate. Collos et al. (2007) studied the relative contribution of 4 different nitrogen sources (nitrate, nitrite, ammonium and urea) during a bloom of *A. catenella* in the Thau lagoon in Southern France and found that ammonium was the most important. In subarctic Pacific, waters even at low ammonium concentrations (<1% of total DIN), ammonium-based nitrogen uptake along with urea accounted for 44 to 89% of the total N uptake, and with increasing ammonium concentration the dependence on nitrate became less (Wheeler & Kokkinakis 1990). Ammonium uptake by *A. fundyense* was reported some time ago by MacIsaac et al. (1979; then known as *Gonyaulax excavata*). Based on preliminary experiments, they reported a half-saturation constant for ammonium uptake of 1.95 µg-at · l^{-1}, but they did not report a half-saturation constant for growth.

In order to shed light on the possibility that maintenance and even continued growth of *A. fundyense* bloom populations (such that growth rates of the bloom populations equal or exceed cell mortality rates) depends on ammonium fluxes in waters of otherwise very low or undetectable nitrate concentrations, we first need a better understanding of the ammonium uptake kinetics and concomitant growth rates of *A. fundyense*. We report here the results of batch culture experiments with *A. fundyense* that were designed to: (1) describe the kinetics of ammonium uptake and growth rates over a range of ammonium concentrations encompassing those observed in the Gulf of Maine; (2) compare rates of nitrate and ammonium uptake in the presence of one another; and (3) determine whether growth rates of *A. fundyense* on ammonium, at concentrations observed in the Gulf of Maine region, are sufficient to maintain established bloom populations, and the degree to which continued growth of the bloom populations may be sustained by ammonium.

MATERIALS AND METHODS

Strain and culture conditions

All experiments were performed with non-axenic *Alexandrium fundyense* cultures isolated from surface waters of Georges Bank in August 2010 by Dr. Lee Karp-Boss at the University of Maine. We assume that the effect of bacterial metabolism (e.g. uptake and regeneration of ammonium) in our non-axenic cultures will be negligible. As we used the same initial volume of media, and approximately the same initial low number of *A. fundyense* cells for all our experiments (ca. 20 to 50 cells ml^{-1}), the effects of bacterial metabolism on our calculations are cancelled out. All experiments were conducted with batch cultures growing in an incubator maintained at ~15.5 to 16°C, with a 14 h light: 10 h dark cycle and light at 150 µmol quanta m^{-2} s^{-1} provided by cool fluorescent lights.

Culture media

All *A. fundyense* cultures were maintained in sterile media prepared by adding macronutrients, trace metals and vitamins to nutrient-depleted surface water collected from the offshore waters of the Gulf of Maine. Nutrient concentrations approximated those of subsurface Gulf of Maine waters prior to the onset of the winter–spring phytoplankton bloom (16 µM NO_3^-, 16 µM $Si(OH)_4$ and 3 µM PO_4^{3-}). Trace metals and vitamins were added at ¼ concentration of L1, described by Guillard & Hargraves (1993). Nutrient stocks were obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota, East Boothbay, ME, USA. Nutrient-depleted Gulf of Maine water (salinity ~33 ppt) was Whatmann™ GF/F filtered and autoclaved following the addition of nutrients.

Experimental design

A total of 41 different experiments were performed grouped by the specific objectives of each: 29 longer-term experiments focused on growth and uptake kinetics over a range of ammonium concentrations; 8 short-term experiments were designed to investigate uptake rates only, and 4 experiments were done to compare the uptake kinetics of both nitrate and ammonium in the presence of one another. Attempts to use replicates were unsuccessful due to slight differences in initial ammonium concentrations, and the difficulties in measuring low ammonium concentrations in the laboratory, and as a result, each intended replicate is presented here as an experiment.

A. fundyense ammonium uptake and growth experiments

All experiments were performed with the above-mentioned incubation conditions, and culture media for all experiments were prepared as described above, except that we varied the DIN concentrations. We substituted NH_4Cl for the 16 µM NO_3^- used to maintain the cultures, varying the experimental ammonium concentrations from about 0.1 to 8.3 µM NH_4^+. The *A. fundyense* inoculum for each experiment was from a stock culture that had just entered the stationary growth phase; cell concentrations ranged from 1000 to 1500 cells ml^{-1}. The required volume of inoculum was transferred directly into 500 ml of media made in 1 l experiment flasks in a sterile environment (in SterilGARD® III Advance°, a class II biological safety cabinet). This study was designed keeping in mind that *A. fundyense* cells in post-bloom waters are exposed to low and often undetectable concentrations of DIN. The responses of starved cells (e.g. stationary phase) in our experiments are intended to mimic such populations of *A. fundyense* in the surface waters of the Gulf of Maine region. In order to determine the relationship between initial experimental ammonium concentrations and the *A. fundyense* ammonium uptake rates, we recorded changes in the ammonium concentrations in the experimental flasks throughout the experiments; the withdrawn samples did not significantly alter cell densities. After gentle mixing, a 10 ml sample was collected from each flask every 24 h for 4 to 10 d (except for 3 of the short-term experiments, where sampling was done every 6 h during the first 24 h). The sample was filtered using 0.45 µm Millex HA filters and frozen immediately at −18°C for later nutrient analyses using a Bran-Luebbe autoanalyzer and standard techniques. The sensitivities (standard deviations) of our autoanalyzer for measurements of ammonium and nitrate + nitrite (measured together, and hereafter referred to as nitrate) are ±0.12 µM NH_4^+ at concentrations less than 1.0 µM and ±0.03 µM NH_4^+ at concentrations in the order of 4.0 µM; the sensitivity for nitrate is ±0.05 µM NO_3^- at the concentrations used in our experiments (3 to 5 µM). Concentrations of silicate and phosphate were also measured, but because there were no significant changes in these nutrients or any experimental artifacts revealed (e.g. phosphate did not become limiting), those results are not presented.

To evaluate the dependence of *A. fundyense* growth rates on initial nutrient concentration, another 10 ml sample was collected every 24 h during the course of the experiments (4 to 10 d; also after gentle mixing) and immediately fixed in a final solution of 0.2% formalin and stored in the dark. Triplicate cell counts were done on each sample using a gridded Sedgwick-Rafter counting chamber with 1 ml subsamples. The entire chamber was counted using a Nikon compound microscope and 100× magnification.

Nitrate vs. ammonium uptake

These experiments were performed similar to those described above, except that in this case, the Gulf of Maine media was prepared with nearly equal proportions of ammonium and nitrate, with total DIN concentrations (NH_4^+ plus NO_3^- + NO_2^-) ranging

from 6.5 to 11.2 µM. After gentle mixing, 10 ml samples were collected every 24 h from each culture flask during the course of the 9 d experiments for nutrient analysis and *A. fundyense* cell counts, with each processed as described in the above section. The uptake kinetics of each nitrogenous nutrient, ammonium and nitrate, in the presence of one another, were compared, and cell counts made to determine *A. fundyense* growth rates in the presence of both forms of nitrogen, but at different total DIN concentrations.

Calculations

Cell-specific ammonium-uptake rate and specific growth rates

The specific growth rate (µ) for each experiment was calculated using the formula: $\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 is the number of cells at time period 1, or t_1, and N_2 is the number of cells at a subsequent time period, or t_2. The time period t_1 to t_2 is selected to represent the period of exponential growth, identified as the period with the steepest slope in changes of *A. fundyense* cell density with time.

The cell specific ammonium uptake rate (V) was calculated using the formula $V = (X_0 - X_1)/n$, where X_0 is the initial ammonium concentration, X_1 is the ammonium concentration on Day 1 (after 24 h) of the experiment and n is the initial number of cells for that experiment. In most experiments, we observed no significant changes in cell densities over the first day. In experiments where the cell densities did change, an average of the number of cells between Day 0 and Day 1 was used for n.

Half-saturation constants for growth and uptake

As mentioned in 'Strain and culture conditions', all these experiments were performed with batch cultures, in which ammonium concentrations change over time as the cells grow, in contrast to a continuous culture where ammonium concentrations are held constant. We therefore used initial ammonium concentrations for all our calculations as described in the next paragraph. Had we used ammonium concentrations that coincided in time with log-phase growth (e.g. lower than initial concentrations), our calculations of half-saturation constants would be lower than those we report here; that is, we chose the more conservative approach.

The cell-specific ammonium-uptake rates (µmol NH_4^+-N cell^{-1} d^{-1}) and specific growth rates (d^{-1}) calculated for each experiment were used to calculate half-saturation constants for ammonium uptake and cell growth ($K_{s(N)}$ and $K_{s(g)}$, respectively). Half-saturation constants were calculated using the hyperbolic Monod equation, also referred to in the case of nutrient uptake as the Michaelis-Menten equation. For ammonium uptake, the equation is written as $V = V_{max} \times S/(K_{s(N)} + S)$ and for growth rate the same equation is re-written as $\mu = \mu_{max} \times S/(K_{s(g)} + S)$, where S is the initial ammonium concentration (µM), V is the cell-specific uptake rate (µmol NH_4^+-N cell^{-1} d^{-1}), V_{max} is the maximum cell-specific uptake rate (µmol NH_4^+-N cell^{-1} d^{-1}), µ is the specific growth rate (d^{-1}), and μ_{max} is the maximum specific growth rate (d^{-1}). The half-saturation constants for ammonium uptake ($K_{s(N)}$) and growth ($K_{s(g)}$) are computed as the substrate (ammonium) concentration corresponding to 50% of V_{max} and 50% of μ_{max}, as determined by the fitted hyperbolic equation. Values of V_{max}, $K_{s(N)}$, μ_{max} and $K_{s(g)}$ were estimated using R-studio software (version 2.13.1) and a non-parametric bootstrapping method as described by Baty & Delignette-Muller (2013), with α set at a value of 0.05.

RESULTS

Uptake and growth experiments

The results of our 41 *Alexandrium fundyense* culture experiments are given in Figs. 1–6. The 29 experiments in which ammonium uptake rates and cell growth rates were examined are divided here into 4 groups for presentation (Figs. 1–4) by the initial concentrations of ammonium: <1.0; between 1.0 and 3.0; between 3.0 and 6.0; and >7 µM NH_4^+. Fig. 1 gives results of changes in NH_4^+ concentrations and cell densities with time for the 7 experiments in which the initial NH_4^+ concentration was <1.0 µM. These results show rapid initial uptake of ammonium as revealed by rapidly decreasing ammonium concentrations over the first day of the experiment for all but 1 of the experiments and showed evidence of continued uptake after that, as the ammonium concentrations continued to decrease to concentrations less than about 0.05 µM by Day 3 (Fig. 1A). The experiment with an initial NH_4^+ concentration of 0.5 µM exhibited a decrease in ammonium over the first day that was less than that for the other experiments, and after the first day, the ammonium concentrations were variable and indicated little or no over-

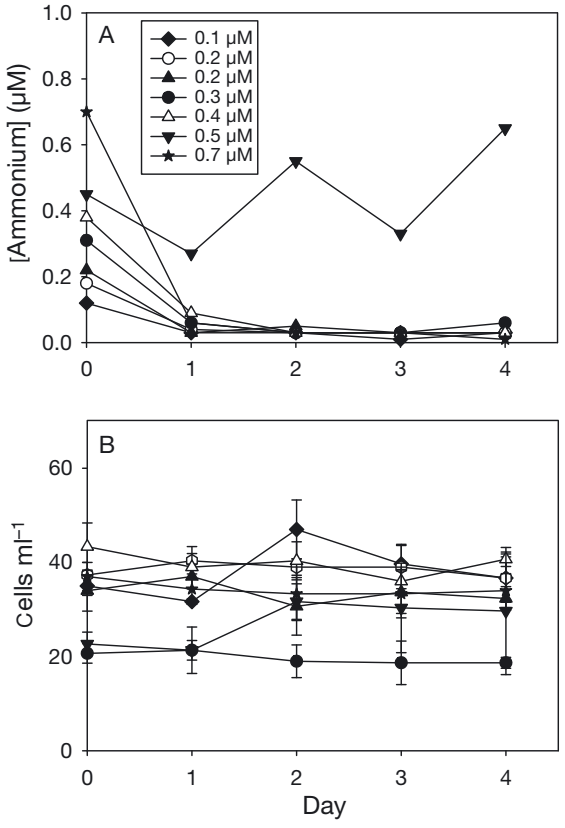

Fig. 1. Results of *Alexandrium fundyense* culture experiments with <1 µM initial ammonium concentrations ranging from 0.1 to 0.7 µM. Changes in (A) ammonium concentration and (B) cell densities during the course of experiments. The means of triplicate cell counts for each day are plotted and standard deviations are shown by error bars

all change in concentration over the 4 d experiment. One factor that can contribute to such an observation is higher natural variability in ammonium uptake at low concentrations (less than 1.0 µM). As shown in Fig. 1B, the growth rates were negative for 1 experiment, zero for another, and very low for the other 5 experiments, even though there was an initial ammonium uptake in these experiments with ammonium <1 µM. It would appear that despite the initial uptake of ammonium on the first day, the cell quota for nitrogen was not met, and cell division was limited by the low ammonium concentrations, which after the first day were depleted to <0.1 µM.

Fig. 2 presents the results of our 7 experiments with initial ammonium concentrations between 1.0 and 3.0 µM. There was a rapid decrease in ammonium concentration over the first day in all the experiments (Fig. 2A), but the cell densities did not exhibit any significant concomitant increases (Fig. 2B). Only after Day 1 was there evidence of growth; the exception was the experiment with an initial ammonium concentration of 1.0 µM, in which there was no growth after the second day. Some of the highest growth rates among our experiments were observed in this group, with rates from 0.48 to 0.59 d^{-1}.

Results of the 10 experiments in which the initial ammonium concentrations ranged from 3.3 to 5.9 µM are given in Fig. 3. Again, in all 10 experiments, there was an initial, rapid uptake of ammonium over the first day of the experiments, as revealed by rapidly decreasing ammonium concentrations, followed by continued but less rapid uptake (Fig. 3A). Also in this group, there were no significant changes in cell densities observed before Day 2, with the most rapid growth rates observed after Day 3 (µ ranged from 0.39 to 0.80 d^{-1}; Fig. 3B). Some of the experiments showed a delay in cell divisions (remaining in lag phase of growth) until Day 3. We discontinued our cell counts for some of the experiments after Day 6, when no evidence of growth was apparent after

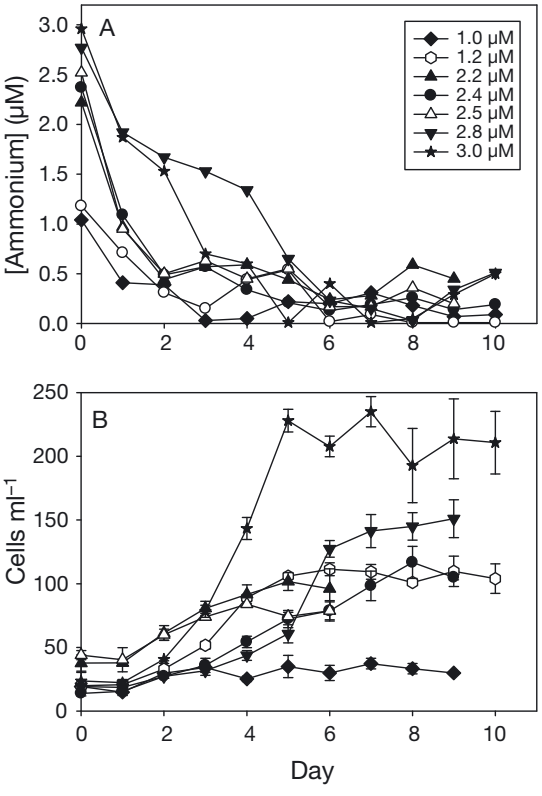

Fig. 2. Results of *Alexandrium fundyense* culture experiments with initial ammonium concentrations ranging from 1.0 to 3.0 µM. Changes in (A) ammonium concentration and (B) cell densities during the course of experiments. The means of triplicate cell counts for each day are plotted and standard deviations are shown by error bars

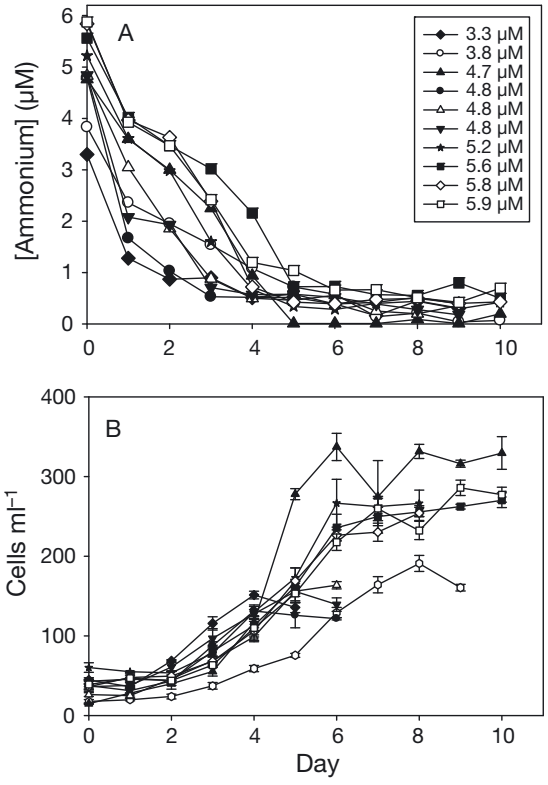

Fig. 3. Results of *Alexandrium fundyense* culture experiments with initial ammonium concentrations ranging from 3.3 to 5.9 µM. Changes in (A) ammonium concentration and (B) cell densities during the course of experiments. The means of triplicate cell counts for each day are plotted and standard deviations are shown by error bars

Day 4. Some of the highest cell-specific ammonium-uptake rates (7.0 to 8.0 $\times 10^{-5}$ µmol NH_4^+-N cell^{-1} d^{-1}) were observed in this group.

Fig. 4 shows the results of changes in ammonium concentrations and cell densities for 5 experiments with ammonium concentrations ranging from 7.1 to 8.3 µM. Once again there was a rapid initial decrease in ammonium concentrations over the first day (Fig. 4A). Increases in cell densities were not obvious in this group of experiments with the highest initial ammonium concentrations until Day 3 (Fig. 4B). Also, growth rates were slightly lower overall in these experiments than in those experiments shown in Fig. 3 (ranging from 0.32 to 0.41 d^{-1}), although some of the highest instantaneous cell-specific uptake rates (6.0 to 8.0 $\times$ 10^{-5} µmol NH_4^+-N cell^{-1} d^{-1}) were observed in these experiments.

The results of our short-term ammonium-uptake experiments are given in Fig. 5. As explained in the 'Materials and methods', the purpose of these experiments was to examine again, and more closely, the ammonium uptake over the first 24 h. Fig. 5A shows the results for 5 experiments with initial ammonium concentrations that ranged from 1.6 to 2.4 µM. In these 4 d incubations, the ammonium concentrations were drawn down to about 0.5 µM, with most of the uptake occurring in the first day. Fig. 5B shows results for the experiments in which initial ammonium concentrations were higher (6.9 to 7.3 µM) and which were sampled every 6 h for the first 24 h, and then again each day until Day 4. The results of these short-term ammonium uptake experiments confirm that uptake of ammonium is fastest in the first 6 to 12 h, and continues at a progressively slower rate for the next 36 h. For this experiment, cell counts were also made for each sample, but without replicate counts, which revealed that the first division appears to be synchronous and occurred after the first 24 h. The changes in cell densities for these short term uptake experiments are not shown here as they were not used in our calculations for $K_{s(g)}$.

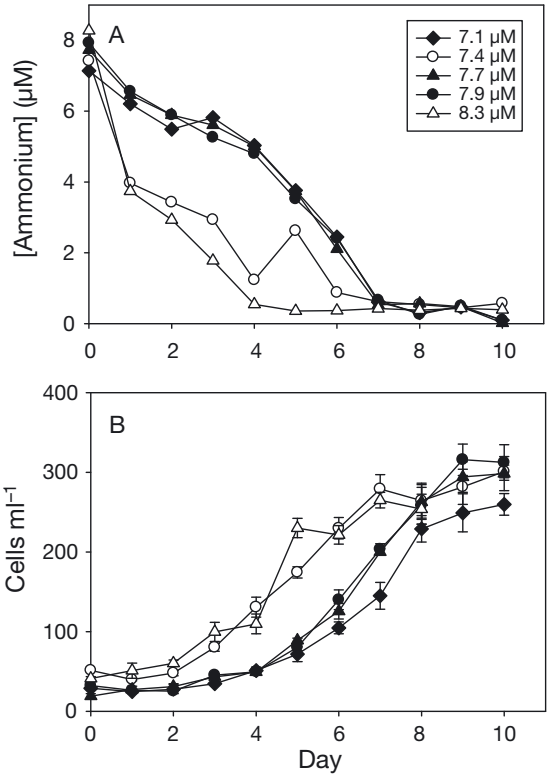

Fig. 4. Results of *Alexandrium fundyense* culture experiments with initial ammonium concentrations ranging from 7.1 to 8.3 µM. Changes in (A) ammonium concentration and (B) cell densities during the course of experiments. The means of triplicate cell counts for each day are plotted and standard deviations are shown by error bars

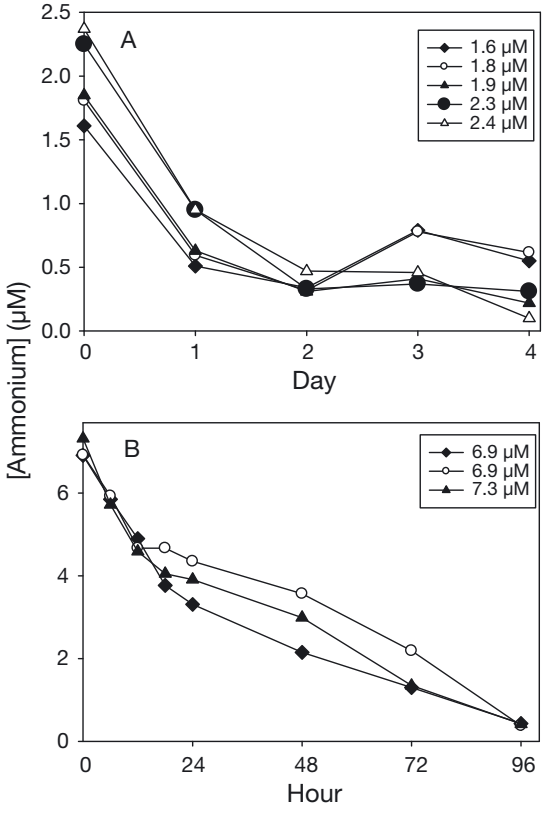

Fig. 5. Results of *Alexandrium fundyense* culture short-term uptake experiments. Changes in ammonium concentrations with time, with initial ammonium concentration ranging from (A) 1.6 to 2.4 µM and (B) 6.9 to 7.3 µM

Ammonium vs. nitrate uptake

Fig. 6 presents results for our experiments in which *A. fundyense* cells were cultured in media with varying concentrations of both ammonium and nitrate. As can be seen in Fig. 6A, in general, when presented with both forms of DIN at nearly equal concentrations, *A. fundyense* appears to prefer ammonium, except at the highest concentrations (>5 µM). At those highest concentrations of ammonium and nitrate, preference for ammonium is only evident over the first day of the experiment. At the lower initial concentrations, preference for ammonium over nitrate is more obvious throughout the course of the experiment. At the lower concentrations, a rapid decline in nitrate concentrations is observed only after ammonium concentrations have been nearly depleted. In 1 of the experiments, where the initial concentrations were 3.5 µM NH_4^+ and 3.0 µM NO_3^-, the cell-specific uptake rate for ammonium was some 24 times greater than that for nitrate (2.2×10^{-5} µmol NH_4^+-N cell^{-1} d^{-1} and 9.0×10^{-7} µmol NO_3^--N cell^{-1} d^{-1}). Fig. 6B shows the accompanying changes in cell densities with time. Except for the experiment with the highest DIN concentrations, the cells remained in lag phase until Day 3. The specific growth rates in these experiments were in the range of 0.34 to 0.54 d^{-1}.

$K_{s(g)}$ and $K_{s(N)}$, and μ_{max} and V_{max} rates

Specific growth rates and cell-specific ammonium-uptake rates determined from the above experiments, with values based on results from each of the 29 experiments presented in Figs. 1–4, were used to fit the hyperbolic Monod (Michaelis-Menten) equations, thus giving values of half-saturation constants for growth and uptake. Values based on results from each of the 8 short-term uptake experiments presented in Fig. 5 were used only in our calculation of the half-saturation constant for uptake. The fitted hyperbolic equations, as plotted in Fig. 7, gave a $K_{s(g)}$

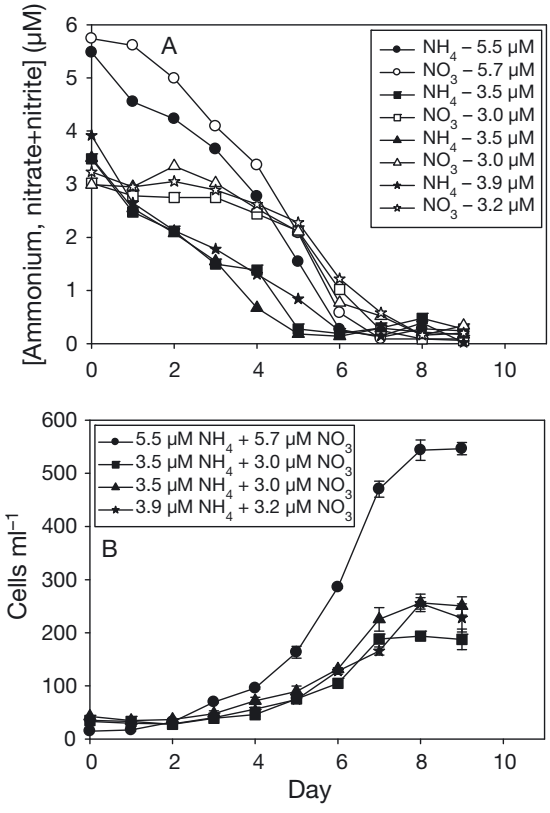

Fig. 6. Results of *Alexandrium fundyense* culture experiments in the presence of ammonium and nitrate. Changes in (A) each nutrient concentration and (B) cell densities during the course of the experiments

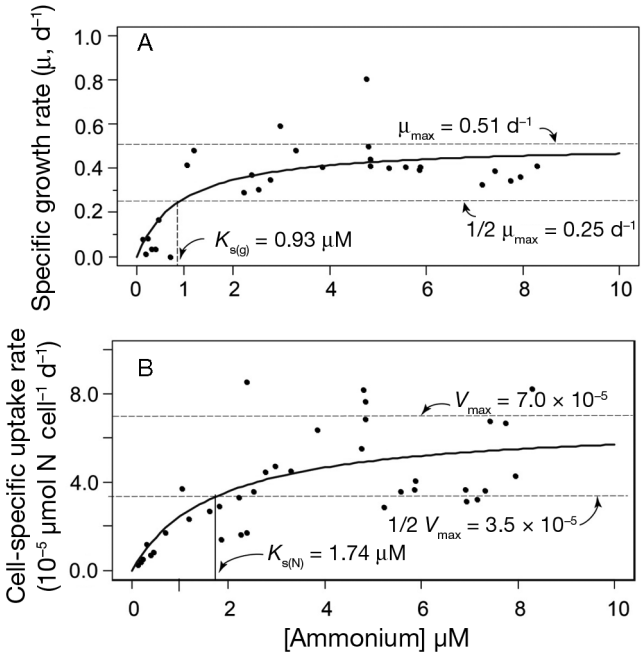

Fig. 7. Growth rate of *Alexandrium fundyense* strain (isolated from Georges Bank) as a function of initial ammonium concentration. (A) Values of the maximum growth rate (μ_{max}) and the half-saturation constant for growth ($K_{s(g)}$). Maximum cell-specific ammonium-uptake rate (V_{max}) of *A. fundyense* as a function of initial ammonium concentration. (B) Values of V_{max} and the half-saturation constant for ammonium uptake ($K_{s(N)}$). Both curves were fitted using a non-parametric bootstrapping method as described by Baty & Delignette-Muller (2013), with α set at a value of 0.05

of 0.93 µM for *A. fundyense* cultured with ammonium (Fig. 7A) and a $K_{s(N)}$ for ammonium of 1.74 µM (Fig. 7B). The value for the μ_{max} was 0.51 d^{-1} (Fig. 7A), and the V_{max} for ammonium was 7.0×10^{-5} µmol NH_4^+-N $cell^{-1}$ d^{-1} (Fig. 7B).

DISCUSSION

One of the most interesting aspects of our results is that *Alexandrium fundyense* is capable of taking up ammonium at concentrations below 0.5 µM. However, while our experiments clearly showed ammonium uptake by *A. fundyense* at initial concentrations that were less than 1 µM, there was little growth (Fig. 1). We assume that those cells were taking up ammonium initially for use in building up amino acid stores prior to undergoing cell division, but that after the first day, the population of cells had already depleted ammonium concentrations in our batch cultures such that remaining concentrations were insufficient, for the most part (<0.1 µM), to supply sufficient nitrogen to satisfy the cell quota needed for significant growth (cell division). Experiments by Van de Waal et al. (2013) with *A. tamarense* showed that starved cells in culture tend to have a very low amino acid content, and upon transfer to nutrient replete media, they remain in lag phase growth, as time and energy are expended in synthesizing a reserve of amino acids necessary for subsequent growth. We might expect, however, that had we maintained ammonium concentrations at or above about 0.5 µM in a continuous or semi-continuous culture with continuous N supply, rather than batch culture, we would have observed cell division after the first day. Our experiments with higher concentrations of ammonium would support this contention, since they did exhibit significant growth, but only following the first day or two, during which the cultures showed significant uptake and depletion of the initial stock of ammonium. In all of our experiments that exhibited cell growth, the cells remained in lag phase at least a day while, we assume, they built up a minimum required cell nitrogen quota and the required amino acids before dividing.

Our experiments reinforce earlier reports in the literature that phytoplankton in general prefer the reduced form of DIN, ammonium, over nitrate (e.g. McCarthy et al. 1977, Paasche & Kristiansen 1982, Herndon & Cochlan 2007). When presented with both forms of DIN in our experiments, *A. fundyense* took up ammonium preferentially to nitrate, exhibiting a higher affinity for ammonium in all of our experiments, with the possible exception of the single experiment with the highest concentrations of each (e.g. 5.5 µM NH_4^+ and 5.7 µM NO_3^-; Fig. 6A). This result is in general agreement with Dortch (1990), who, in her review of the literature, concluded that preference for ammonium over nitrate varies greatly. One ecological interpretation of such variability might be that when in the sustained presence of higher nutrient concentrations, such as in spring or in areas of new nutrient injections into surface waters, cells are better adapted to assimilate nitrate, which is almost always present in significantly higher concentrations than ammonium. Later in the season, however, situations in which concentrations of nitrate are relatively high (e.g. >5 µM) become less common, and cells present at those times are more likely to be adapted to preferentially take up recycled ammonium. Indeed, as discussed in Townsend et al. (2014), these blooms are most often found downstream of areas of new nitrogen injections and enhanced primary production; therefore,

particulate nitrogen so produced is the likely source of the recycled ammonium that maintains the populations. Certainly, in the Gulf of Maine and on Georges Bank, nitrate concentrations in near surface waters are usually depleted to near detection limits (below 0.2 µM) during the height of *A. fundyense* blooms, while ammonium, though patchy in distribution, is present at concentrations that average about 0.3 to 0.6 µM in the top 40 m (e.g. Love et al. 2005, Townsend et al. 2014), being highest in localized surface water patches in the range of 1 to 2 µM (D.W.T. unpubl. data). It has been argued (McCarthy & Goldman 1979) that such patches, and micropatches, of higher ammonium concentrations, formed by various metabolic and heterotrophic processes can be exploited by phytoplankton. Persistence of, and even continued growth of, *A. fundyense* blooms for weeks into the summer when nitrate is exhausted in surface waters (e.g. McGillicuddy et al. 2014b, Townsend et al. 2014) would therefore be expected to be significantly dependent on recycled ammonium. This raises the question: Are the low spatially averaged ammonium concentrations generally observed in the Gulf of Maine and on Georges Bank sufficient to support *A. fundyense* growth rates that exceed their natural mortality rates? We suggest that this is indeed possible, even probable, based on our analyses of growth and ammonium uptake kinetics.

The results of our uptake and growth experiments, with values of $K_{s(N)}$ and V_{max} for ammonium uptake, and $K_{s(g)}$ and μ_{max} for growth, are presented in Fig. 7. While, generally speaking, there is a relatively large body of literature with reports of half-saturation constants for nutrient uptake, $K_{s(N)}$ (e.g. Eppley et al. 1969, MacIsaac et al. 1979, Watanabe et al. 1982, Nakamura 1985, Yamamoto et al. 2004), that parameter has little meaning in ecology (Eppley et al. 1969). The half-saturation constant for uptake is the nutrient concentration at which the cell-specific nutrient uptake rate is half its maximum rate, which can be used to compare a species' affinities for nitrate versus ammonium, for example, as well as to draw inferences about interspecific competition for nutrients. Here, we report a $K_{s(N)}$ of 1.74 µM for *A. fundyense*, a fairly large value, but not unusual for such a large phytoplankton species. Literature values of $K_{s(N)}$ for ammonium uptake by species of *Alexandrium* are given in Table 1 along with literature values of $K_{s(g)}$. Larger phytoplankton cells in general have higher values of $K_{s(N)}$ (Eppley et al. 1969) and can therefore be viewed as being at a disadvantage during times of interspecific competition for nutrients, such as during the spring phytoplankton bloom in the Gulf of Maine.

Table 1. Published values of half saturation constants for ammonium uptake $K_{s(N)}$ and growth $K_{s(g)}$ for *Alexandrium* species. –: not given

Species	$K_{s(N)}$ (µM)	$K_{s(g)}$ (µM)	Reference
Gonyaulax excavata (*A. fundyense*)	1.95	–	MacIsaac et al. (1979)
Alexandrium catenella	–	3.3	Matsuda et al. (1999)
Alexandrium fundyense	1.74	0.93	This study

$K_{s(g)}$ is the concentration at which a nutrient is assumed to become limiting to growth, for which there are few reports in the literature specifically for ammonium (e.g. Matsuda et al. 1999). It too has limited ecological significance in that it merely indicates the sustained nutrient concentration at which the cell growth rates are half the maximum rate, but even rates that are less than half of a fast growth rate can still produce a bloom population, as long as natural mortality is less than that value. Our value for the $K_{s(g)}$ is 0.93 µM, and μ_{max} is 0.51 d^{-1}, which corresponds to a doubling time of 1.4 d. Therefore, at ammonium concentrations in the order of 1 µM, corresponding to a doubling time of 1.4 d, *A. fundyense* would be expected not only to maintain already established cell densities corresponding to bloom populations at times of nitrate exhaustion, but with relatively low ammonium fluxes, cell densities can continue to grow at a relatively fast rate. This does not happen in nature, however. Growth rates of *A. fundyense* in nature reflect a difference between the specific growth rate, µ, and the natural mortality rate, M, where net growth rate = ($\mu - M$). For the population to be maintained, or to grow, µ must exceed M. The natural mortality rate is poorly known, and is almost always estimated, not measured (e.g. McGillicuddy et al. 2005, Stock et al. 2005). Turner & Borkman (2005), however, in their study of zooplankton grazing on *A. fundyense*, reported a maximum mortality rate from grazing of about 0.06 d^{-1}, which is the only measured value that we are aware of. He et al. (2008), in their model of *A. fundyense* in the Gulf of Maine, estimated natural mortality based on a temperature-dependent formulation, with values that range from near zero at 1°C, to 0.03 d^{-1} at 8°C, 0.20 d^{-1} at 14°C, and rise quickly to 0.5 d^{-1} at 17°C. In the next paragraph, we explore how different rates of growth and mortality relate to observed *A. fundyense* cell densities during annual blooms.

Table 2. Approximate ammonium concentrations required to produce a specific gross growth rate (μ) that exceeds a range of specific mortality rates (M) such that the net growth rate ($\mu - M$) is equal to 0.05 d^{-1}, as given by the hyperbolic equation plotted in Fig. 7A

M (d^{-1})	Required μ (d^{-1})	Required [NH_4^+] (μM)
0.1	0.15	0.39
0.15	0.2	0.6
0.2	0.25	0.9
0.3	0.35	2.03

Earlier observations of *A. fundyense* cell densities in the Gulf of Maine–Georges Bank regions (McGillicuddy et al. 2014b, Townsend et al. 2014) would indicate that it can grow in the apparent absence of nitrate in the mixed layer. For example, as reported by Townsend et al. (2014) for waters along the Northern Flank of Georges Bank in 2010, there were roughly 10s of cells l^{-1} in the first week of May. About 1 mo later, cell densities on the Northeast Peak and Southern Flank of Georges Bank, downstream from the Northern Flank, were on the order of 200 cells l^{-1}. Still later that year, in the first week of July, there were on the order of 1000 cells l^{-1} across the central and southern portions of the Bank, suggesting a net growth rate of approximately 2.5 doublings every 4 wk, for a specific net growth rate of approximately 0.05 d^{-1}. That is, the difference between the specific growth rate and natural mortality rate was about 0.05 d^{-1}. Table 2 gives examples of ambient surface-water ammonium concentrations that would be required to produce a net growth rate of 0.05 d^{-1}, given a range of specific natural mortality rates between 0.1 and 0.3 d^{-1} (Stock et al. 2005). As can be seen, observed ammonium concentrations in the Gulf of Maine–Georges Bank (0.3 to 0.6 μM) could indeed result in a net growth rate that equals the natural mortality rate, thus sustaining the population of cells, and could result in a net growth rate of 0.05 d^{-1}, consistent with 2 to 3 doublings of cells over an 8 wk period. Based on the results of these experiments, and the resulting estimates of cell growth rates, we concur with the suggestion made by McGillicuddy et al. (2014b) and Townsend et al. (2014) that ammonium is an important nitrogenous nutrient for *A. fundyense* blooms in the Gulf of Maine–Georges Bank region, and we further speculate that even the low ammonium concentrations commonly observed can nonetheless sustain bloom populations, and even support continued growth in waters depleted of nitrate.

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