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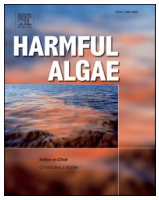

Original Article

Nitrogen liberated via allelopathy can promote harmful algal blooms

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ABSTRACT

Allelopathy is a biological mechanism that can promote harmful algal blooms (HAB) via the inhibition of sympatric phytoplankton. While nutrient loading can also promote HABs, the ability of allelopathy to stimulate HABs via the regeneration of nutrients has yet to be explored. To examine the impacts of allelopathically liberated N on HAB species, a series of experiments were performed using multiple allelopathic HAB species including the dinoflagellates *Alexandrium catenella* and *Margalefidinium polykrikoides*, and the pelagophyte, *Aureoumbra lagunensis*. These HAB species were paired with the cosmopolitan dinoflagellate, *Akashiwo sanguinea*, that was labeled with $^{15}NO_3^-$ or $^{15}NH_4^+$, allowing the release and transfer of N to be traced as a time course during allelopathic interactions. During all experiments, the allelopathic inhibition of *Akashiwo* was accompanied by increases in cell densities, growth rates, and the $\delta^{15}N$ content of the HAB species, evidencing the transfer of N liberated from *Akashiwo*. The cellular transfer of ^{15}N and release of dissolved N was higher when *Akashiwo* was grown with $^{15}NO_3^-$ compared to $^{15}NH_4^+$ suggesting a differential subcellular-compartmentalization of N sources. Regardless of the type of N, HAB species obtained 60 – 100% of their cellular N from lysed *Akashiwo* cells and there was an enrichment of the $\delta^{15}N$ content of the dissolved NH_4^+ pool post-lysis of *Akashiwo*. Collectively, the results demonstrate that beyond facilitating species succession, allelopathy can supply HABs with N and, therefore, is likely important for promoting and sustaining HABs. Given that allelopathy is known to be a dose-dependent process, allelopathy may induce a positive feedback loop, whereby competitors are lysed, N is liberated, HABs are intensified and, in turn, become more strongly allelopathic.

1. Introduction

The impacts of harmful algal blooms (HABs) on economies, ecosystems, and human health have increased in recent years (Anderson et al., 2012). While anthropogenic nutrient loading is known to promote HABs (Glibert et al., 2018a), the precise adaptive strategies of HABs in response to nutrient availability can vary regionally in a species-specific manner (Glibert et al., 2018b). Nitrogen (N) is a primary driver of HAB dynamics in estuarine and coastal ecosystems (Kudela et al., 2008; Paerl et al., 2014). During the formation of high biomass of HABs in coastal waters, levels of dissolved inorganic nitrogen (DIN) can be reduced to low levels and additional exogensous sources of N are often required to sustain blooms (Gobler et al., 2012; Love et al., 2005; Sunda et al., 2006).

N frequently limits the growth of phytoplankton in marine systems (Cloern et al., 2014). The regeneration of N is biologically facilitated as phytoplankton grow and/or interact within microbial food webs and such regeneration of N is crucial for supporting primary production (Fernández et al., 2009; Kang et al., 2021b; Morando and Capone, 2018). While NH_4^+ is traditionally considered the primary form of regenerated N, up to 40% of DIN incorporated by phytoplankton is released as DON (Bronk et al., 1994). Although the regeneration of N via zooplankton grazing and excretion (Møller et al., 2003; Valdés et al., 2017), viral infections (Shelford et al., 2012) or physiological stress (Bronk and Ward, 1999) has been widely characterized, little is known regarding N regeneration from allelopathy.

Allelopathy is the inhibition of one primary producer by another, typically via the release of chemical compounds that cause cellular impairment. Chemically-mediated competition among sympatric phytoplankton can influence phytoplankton dynamics in the ocean (Dias et al., 2017; Fistarol et al., 2003; Suikkanen et al., 2005) and microbial food webs (Weissbach et al., 2011). Regarding HABs, this mechanism has drawn increasing attention, as it has been hypothesized to be a driver of HAB formation via the suppression co-occurring phytoplankton (Graneli et al., 2008). A variety of plankton classes including cyanobacteria (Chia et al., 2018), dinoflagellates (Poulson

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et al., 2010), pelagophytes (Kang and Gobler, 2018), prymnesiophytes (Schmidt and Hansen, 2001), and raphidophytes (Fernández-Herrera et al., 2016) produce allelochemicals that can impair the physiology or other phenotypical characteristics (e.g. growth rates, morphology, membrane integrity) of competing phytoplankton. The allelopathic compounds of some algae (e.g., *Aureococcus anophagefferens* and *Aureoumbra lagunenesis*) can either suppress or intensify the growth of different target cells, thus demonstrating the potential role of allelopathy in influencing succession and the dynamics of coastal phytoplankton communities (Kang and Gobler, 2018). While some species are more allelopathically potent via direct contact (Rengefors et al., 2008), previous studies have demonstrated that filtrate of many HABs (e.g. *Alexandrium fundyense, Aureoumbra lagunensis,* and *Margalefidinium polykrikoides*) can strongly inhibit the growth of co-existing algae (Hattenrath-Lehmann and Gobler, 2011; Kang and Gobler, 2018; Tang and Gobler, 2010). Phytoplankton cell lysis can release nutrients that can relieve the nutrient limitation of other phytoplankton (Gobler et al., 1997), and in turn, the remineralization of released organic matter by heterotrophic bacteria can promote primary production (Kirchner et al., 1996). It can be hypothesized, therefore, that nutrients released via allelopathy may promote the occurrence and persistence of HABs in coastal ecosystems.

Here, we test this hypothesis in a series of experiments with three strongly allelopathic HAB species: *Alexandrium catenella, Aureoumbra lagunensis* and *Margalefidinium polykrikoides* (Hattenrath-Lehmann and Gobler, 2011; Tang and Gobler, 2010; Kang and Gobler, 2018). The cosmopolitan dinoflagellate, *Akashiwo sanguinea*, was used as a model, target alga and labeled with $^{15}NO_3^-$ or $^{15}NH_4^+$. The transfer of ^{15}N to each HAB species and to dissolved pools was traced over the course of multi-day experiments. Findings implicated allelopathy as an important mechanism for providing regenerated N that sustains and promotes HABs.

2. Materials and methods

2.1. Culture maintenance

Algal cultures utilized during this study included strains of *Alexandrium, Aureoumbra, Margalefidinium*, and *Akashiwo* that were established by isolating single cells from the Bay of Fundy, Canada (in 2003), Indian River Lagoon FL, USA (in 2013), Old Fort Pond on Long Island, NY, USA (in 2008) and Northport Bay on Long Island, NY, USA (in 2008), respectively (Table 1). All stock cultures were maintained in GSe medium (Doblin et al., 1999) prepared with autoclaved and filtered (0.22 μm) North Atlantic Ocean seawater (N 40° 48′ 0.13″, E –72° 28′ 26.61″) with a final salinity of 32.5‰. For *Aureoumbra*, GSe media was altered to contain 100 μM NH_4^+ and 6 μM PO_4^{-3}, as *Aureoumbra* grows poorly on nitrate (Muhlstein and Villareal, 2007). Since cultures were established, all cultures have been maintained with a final concentration of 1% antibiotic solution (primary stock was a mixture of 10,000 I.U. penicillin and 10,000 μg mL^{-1} streptomycin; Mediatech. Inc., USA) to minimize bacterial proliferation. Cultures were grown at 21 °C with a 12:12 h light:dark cycle, illuminated by fluorescent lights covered by one layer of neutral density screening, which reduced light levels to 80 μmol quanta m^{-2} s^{-1}.

2.2. Separation tests

The cellular diameters of the *Alexandrium, Aureoumbra, Margalefidinium*, and *Akashiwo* used in this study were ~ 20 μm, 5 μm, 30 μm and 50 μm. Separation tests to assess the isolation of HAB species from *Akashiwo* were performed prior to conducting the experiments. A 10 μm nylon mesh efficiently separated all *Aureoumbra* cells from *Akashiwo*, whereas the 30 μm nylon mesh was used to separate >90% of *Alexandrium* cells and a 40 μm mesh was used to separate *Margalefidinium* cells from *Akashiwo*. While *Margalefidinium* and *Akashiwo* cultures were not well separated due to their similar sizes, the severe allelopathic impacts of *Margalefidinium* on *Akashiwo* caused rapid and complete lysis of *Akashiwo* cells during all experiments.

2.3. Co-culturing experiments

Mixed culture experiments were conducted to examine the mechanisms through which liberated N from target species was transferred to the HAB species. For the experiments, *Akashiwo* was initially grown GSe media with N supplied exclusively as $^{15}NO_3^-$ (100 μM) or $^{15}NH_4^+$ (100 μM) and transferred to identical fresh media each week for three weeks. Prior to performing the experiments, harmful algae were isolated using 5 μm nylon mesh and transferred to GSe medium without N added. *Akashiwo* cells were isolated as described above and transferred to the freshly made HAB cultures, creating co-cultures. The isolation of HAB species and *Akashiwo* from stock cultures using 5 μm nylon mesh prior to transferring to freshly made GSe medium further reduced a potential of bacteria presence. In addition, the presence of bacteria in co-cultures would not have reduced the allelopathic potency because allelochemicals of HAB species are known to be resistant to the bacterial degradation (Ma et al., 2009). The initial cell densities of HAB species and co-existing *Akashiwo* were consistent with the cell densities commonly found in coastal zones during blooms (Table 1; Gobler et al., 2012; Hattenrath et al., 2010; Kang et al., 2015; White et al., 2014). Triplicate co-cultures were monitored for 120 h. Triplicate control cultures were also established to compare the growth of the individual algal species when grown as monocultures in enriched GSe media. Every 24 h, 5 mL aliquots were preserved with Lugol's solution to quantify the cell densities of each species via a light microscopy (Primo Star, ZEISS, GmbH, Germany). At each time point, 20 mL of sample was filtered through pre-combusted GFF to analyze dissolved inorganic nutrients and dissolved organic nutrients using a QuikChem 8500 Series 2 flow-injection autoanalyzer (LACHAT Instruments, Loveland, CO, USA; Parsons, 2013). Additionally, at each time point, 20 mL aliquot of cultures were obtained and the HAB and non-HAB species were separated using nylon mesh as described above. The filtrates (< 10 or 30 μm) were then filtered

Table 1
Information of species that are tested in this study. Parenthesis indicates strain names of testing species.

HABs	Initial cell density (cells mL^{-1})	Cell size (μm)	Target	Initial cell density (cells mL^{-1})	Cell size (μm)	^{15}N-target enriched
Margalefidinium polykrikoides (CP1)	575	30	*Akashiwo sanguinea* (AS2)	349	50	$^{15}NO_3^-$
Aureoumbra lagunensis (FL2)	801,000	5	*Akashiwo sanguinea* (AS2)	349	50	
Margalefidinium polykrikoides (CP1)	500	30	*Akashiwo sanguinea* (AS2)	250	50	$^{15}NH_4^+$
Aureoumbra lagunensis (FL2)	794,000	5	*Akashiwo sanguinea* (AS2)	410	50	
Alexandrium catenella (CCMP2304)	566	15–20	*Akashiwo sanguinea* (AS2)	410	50	

onto pre-combusted (2 h at 450 ℃) glass fiber filters (GFF; 0.45 μm pore size), and the cells captured on the nylon mesh (10, 30, or 40 μm) were rinsed with filtered seawater onto a second pre-combusted GFF. The filters were frozen immediately at −20 °C for later ^{15}N analysis. Analysis of $^{15}NH_4^+$ in the cultures followed the ammonia diffusion method as described by Holmes et al. (1998). Briefly, after each 120 h experiment, a filter pack made of 1 cm diameter GF/D filters (combusted at 400 °C for 4 h) sandwiched between two 2.5 cm diameter 10 μm pore size Teflon membranes with the addition of 25 μL 2 M $KHSO_4$ was added to 20 mL of 0.2 μm sterile filtered sample in high–density polyethylene (HDPE) bottles. Afterward, 3 g L^{-1} of reagent grade MgO was added to the bottles, and the samples were incubated in a shaking table for two weeks at 40 °C. The filter pack was removed from the diffusion bottle after the incubation period, placed in a desiccator with silica gel with an open container of sulfuric acid to remove trace ammonia, and dried for 48 h. All ^{15}N samples were analyzed at the University of California Davis Stable Isotope Facility.

2.4. Data analysis and statistics

The maximal potential $\delta PO^{15}N$ (particulate organic nitrogen; ‰) for each HAB during each time point was determined to identify the highest possible $\delta^{15}N$ value that the HAB cultures could obtain if all of the N assimilated by the HAB came from the ^{15}N-labeled *Akashiwo* cells. The maximal potential $\delta PO^{15}N$ was, therefore, calculated as:

($\delta^{15}N$ of HAB_T / $\delta^{15}N$ of non–$HAB_{initial}$),

where $\delta^{15}N$ of HAB_T is the $\delta^{15}N$ of the HAB species at a given time point, $\delta^{15}N$ of non-$HAB_{initial}$ is the initial $\delta^{15}N$ of the non-HAB species at the start of the experiment.

The maximal potential $PO^{15}N$ percentage attained by HAB (%) was calculated as follows:

[^{15}N of HAB_T / Maximal potential $PO^{15}N$] x 100,

where ^{15}N of HAB_T is ^{15}N of the HAB species at each time point.

Growth rates (d^{-1}) of HAB controls and HAB species in co-cultures were estimated to compare the responses of HAB species to allelopathically regenerated N. Growth rates were calculated as follows:

ln [HAB_{final} cell density / $HAB_{initial}$ cell density] / d,

where $HAB_{initial}$ cell density is the initial cell density of HAB species, HAB_{final} cell density is the final cell density of HAB species at 120 h, and d is the experimental period in day.

One-way ANOVAs were performed on $\delta^{15}N$ to assess the significant difference among samples between time points. Correlational analyses were performed to assess relatedness of parameters. Student's *t*-test was conducted to assess differences in the growth rate of HAB species between treatment and control. All statistics were performed using R version 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Changes in cell density and growth rate during allelopathic interactions

Results are presented for the three HAB species (e.g. *Alexandrium, Aureoumbra*, and *Margalefidinium*) co-cultured with two different types of ^{15}N-enriched *Akashiwo* (e.g. $^{15}NO_3^-$ and $^{15}NH_4^+$). In all cases, after 0–72 h of direct contact, *Akashiwo* cell densities were dramatically diminished or were below detection limits, whereas densities of the HAB species increased (Fig. 1). In the case of $^{15}NO_3^-$–enriched *Akashiwo* co-cultured with *Margalefidinium*, the *Akashiwo* cell densities sharply declined from 400 cells mL^{-1} to undetectable levels within 48 h, whereas *Margalefidinium* cell densities steadily increased from 550 cells mL^{-1} to 1040 cells mL^{-1} at 120 h (Fig. 1A). In the experiment with

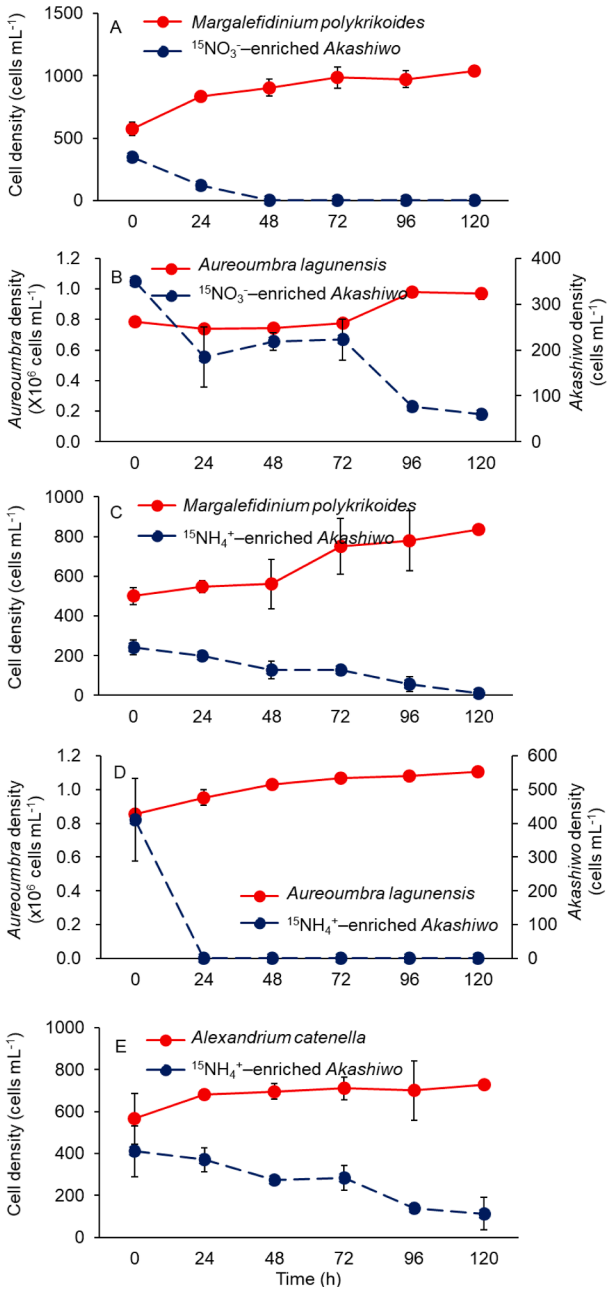

Fig.1. Cell density of HAB and non-HAB species in co-culture during the course of the experiments. (A) and (B) include *Akashiwo* enriched with $^{15}NO_3^-$ and (C) – (E) include *Akashiwo* enriched with $^{15}NH_4^+$.

Aureoumbra and $^{15}NO_3^-$–enriched *Akashiwo*, target cell densities decreased during the first 24 h and stabilized at 220 cells mL^{-1} until 72 h when levels further declined to 60 cells mL^{-1} at 120 h (Fig. 1B). Unlike *Margalefidinium*, the *Aureoumbra* cell density remained static until day three, after which it increased to 9.77 × 10^5 cells L^{-1} by 120 h (Fig. 1B). In the case of $^{15}NH_4^+$–enriched *Akashiwo* co-cultured with *Margalefidinium*, the cell density of *Akashiwo* gradually decreased from 220 cells mL^{-1} to undetectable levels by 120 h, whereas that of *Margalefidinium* steadily increased from 450 cells mL^{-1} to 837 cells mL^{-1} (Fig. 1C). In co-cultures with *Aureoumbra*, the cell densities of *Akashiwo* grown on $^{15}NH_4^+$ sharply decreased from 400 cells mL^{-1} to an undetectable level within 24 h whereas the *Aureoumbra* cell density gradually increased from 0.8 to >1.1 × 10^6 cells mL^{-1} at 120 h (Fig. 1D). Finally, in the experiment with *Alexandrium* and the $^{15}NH_4^+$–grown *Akashiwo*, cell densities of *Akashiwo* decreased from 400 to 113 cells mL^{-1} at 96 h,

whereas the *Alexandrium* cell density increased from 500 to 730 cells mL^{-1} (Fig. 1E).

The growth rates of HAB species were often significantly higher when co-cultured with *Akashiwo* without exogenously added N compared to control cultures grown in GSe with high levels of N ($p < 0.05$; *t*-test; Fig. 2). This effect was most striking during the first 24 h when the cell density of *Akashiwo* decreased most dramatically (Fig. 1). The growth rates of *Margalefidinium* for 120 h were 0.11 ± 0.03 d^{-1} when co-cultured with $^{15}NO_3^-$–enriched *Akashiwo* and 0.10 ± 0.01 d^{-1} when co-cultured with $^{15}NH_4^+$–enriched *Akashiwo*, whereas the control had a growth rate of 0.07 ± 0.03 d^{-1} ($p < 0.05$ for both; *t*-test; Fig. 2A). Moreover, the growth rates during the first 24 h were more than two-fold and significantly higher (0.38 ± 0.08 d^{-1}) in co-cultures with $^{15}NO_3^-$–enriched *Akashiwo* compared to the rates (0.14 ± 0.07 d^{-1}) in co-cultures with $^{15}NH_4^+$–enriched *Akashiwo* ($p < 0.05$; *t*-test; Fig. 2A). In the case of *Aureoumbra*, for the first 24 h incubation, the growth rates in control were 0.03 ± 0.04 d^{-1} whereas for $^{15}NO_3^-$–enriched it was 0.03 ± 0.02 ($p > 0.05$; *t*-test) and rates were 0.11 ± 0.05 d^{-1} in co-cultures with $^{15}NH_4^+$–enriched *Akashiwo* ($p < 0.05$; *t*-test; Fig. 2B). Over the full 120 h, growth rates were 0.01 ± 0.01 d^{-1} in the control, 0.04 ± 0.01 d^{-1} in co-cultures with $^{15}NO_3^-$–enriched *Akashiwo* and 0.05 ± 0.00 d^{-1} in co-cultures with $^{15}NH_4^+$–enriched *Akashiwo* ($p < 0.05$; *t*-test; Fig. 2B). In contrast to *Margalefidinium* and *Aureoumbra, Alexandrium* had higher growth rates for 120 h in control (0.11 ± 0.02 d^{-1}) compared to the co-cultures (0.07 ± 0.01 d^{-1}; Fig. 2A). However, the pattern was reversed when *Alexandrium* incubated for 24 h when growth rates of co-cultures (0.28 ± 0.02 d^{-1}) were significantly higher than the control (0.17 ± 0.08 d^{-1}; $p < 0.05$; *t*-test; Fig. 2A).

3.2. Variation in nutrients and $\delta^{15}N$ signatures during the growth of HAB species

During the co-cultures of HAB species with ^{15}N–enriched *Akashiwo*, DIN decreased during experiments, whereas DON either increased or remained relatively unchanged (Fig. 3). NO_3^- was rapidly reduced in co-cultures of *Margalefidnium* and $^{15}NO_3^-$–enriched *Akashiwo*, with levels decreasing from 1.47 ± 0.06 μM at the beginning of the experiment to an undetectable level at and after 48 h (Fig. 3A). Similarly, the levels continuously declined from 1.15 ± 0.01 μM to 0.38 ± 0.09 μM at 120 h in co-cultures of *Margalefidnium* and $^{15}NH_4^+$–enriched *Akashiwo* (Fig. 3B). NO_3^- levels in co-cultures of *Alexandrium* and $^{15}NH_4^+$–enriched *Akashiwo* also steadily decreased from 0.66 ± 0.14 μM to 0.44 ± 0.12

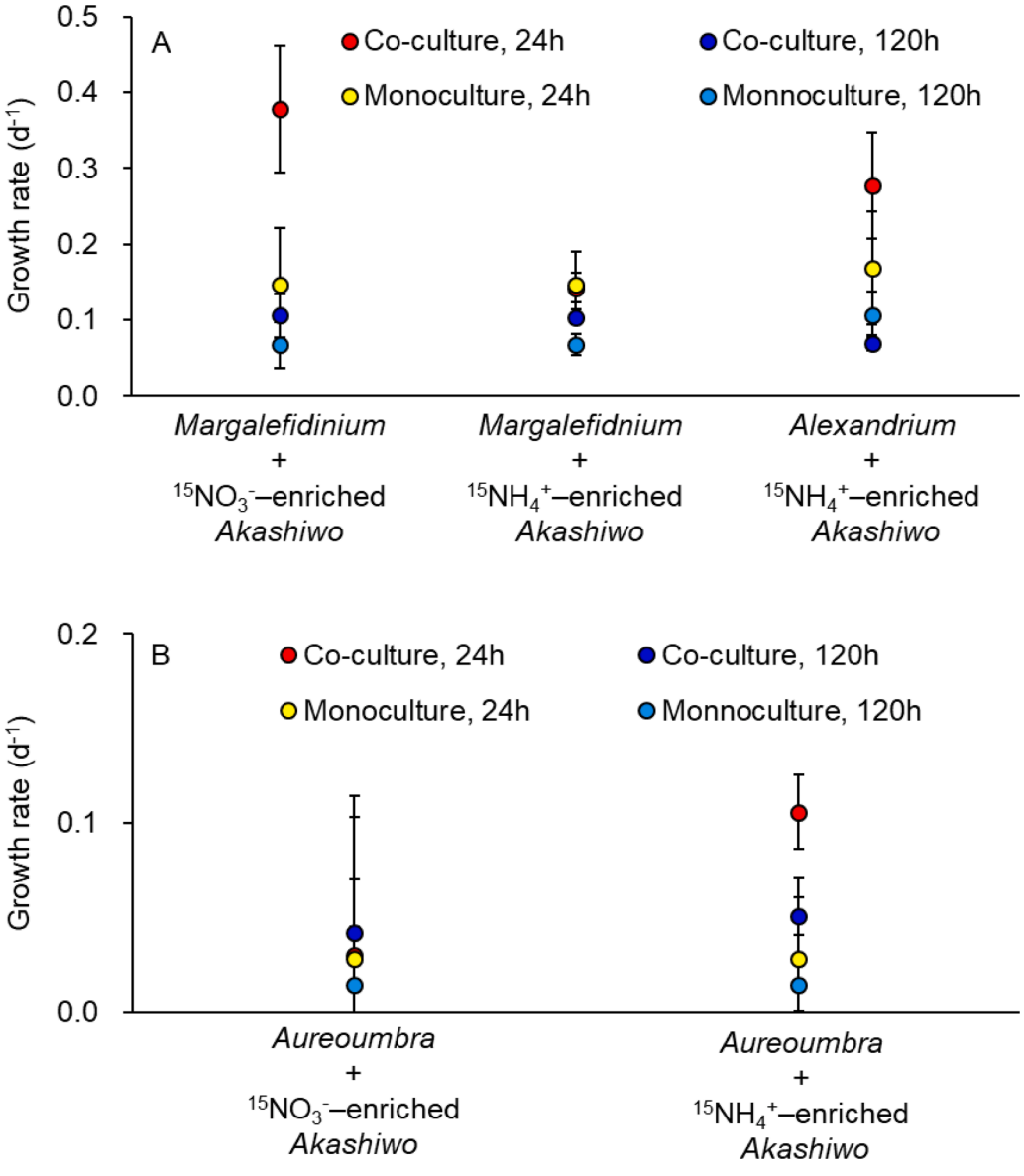

Fig. 2. Growth rates of HAB species (d^{-1}) during a course of experiments. (A) Growth rates of *Margalefidnium* in the presence of $^{15}NO_3^-$–enriched *Akashiwo* and $^{15}NH_4^+$–enriched *Akashiwo*, respectively and the growth rates of *Alexandrium* in the presence of $^{15}NH_4^+$–enriched *Akashiwo*. (B) growth rates of *Aureoumbra* in the presence of $^{15}NO_3^-$–enriched *Akashiwo* and $^{15}NH_4^+$–enriched *Akashiwo*, respectively. Red circles denote growth rate of HABs in co-cultures within 24 h, yellow circles denote growth rate of HABs in control within 24 h, blue circles denote growth rate of HABs for 120 h, and light blue circles denote growth rate of HABs in control for 120 h. T-test was performed to assess the significant difference in growth rate between treatment and control ($p < 0.05$; *t*-test).

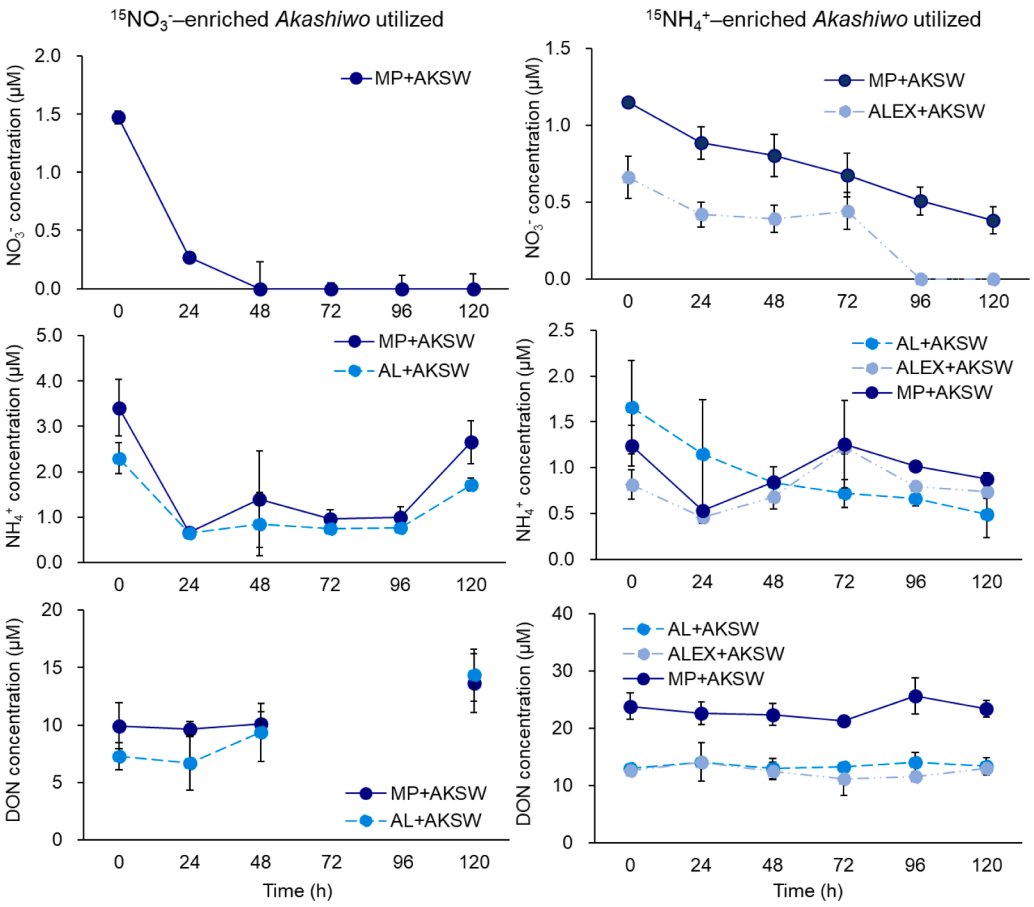

Fig. 3. Variation in nutrient concentration (μM) in co-cultures during the course of the experiments. (A) – (C) Results from experiments performed with *Akashiwo* enriched with $^{15}NO_3^-$. (D) – (F) Results from experiments performed with *Akashiwo* enriched with $^{15}NH_4^+$. (A) and (D) NO_3^- (μM), (B) and (E) NH_4^+ (μM), (C) and (F) dissolved organic nitrogen (DON; μM) Fig 4. Variation in nutrient concentration (μM) in co-cultures during the course of the experiments. (A) – (C) Results from experiments performed with *Akashiwo* enriched with $^{15}NO_3^-$. (D) – (F) Results from experiments performed with *Akashiwo* enriched with $^{15}NH_4^+$. (A) and (D) NO_3^- (μM), (B) and (E) NH_4^+ (μM), (C) and (F) dissolved organic nitrogen (DON; μM). Abbreviations are as follows: MP = *Margalefidinium polykrikoides*, AL = *Aureoumbra lagunensis*, Alex = *Alexandrium catenella*, and AKSW = *Akashiwo sanguinea*.

μM for three days, after which they were undetectable (Fig. 3B). During the experiments with *Margalefidinium* and *Aureoumbra* with $^{15}NO_3^-$–enriched *Akashiwo*, NH_4^+ levels decreased more than two-fold within 24 h and remained stable until 96 h when the levels increased to 2.66±0.47 μM and 1.72 ± 0.14 μM, respectively (Fig. 3C). In experiments with $^{15}NH_4^+$–enriched *Akashiwo*, NH_4^+ gradually declined for 120 h regardless of the type of co-cultured HAB (Fig. 3D). Unlike the DIN, DON levels increased from 9.92 ± 2.01 μM to 13.64 ± 2.58 μM in co-cultures of *Margalefidinium* and from 7.31 ± 1.17 μM to 14.35 ± 2.26 μM in co-cultures of *Aureoumbra* grown with $^{15}NO_3^-$–enriched *Akashiwo* (Fig. 3E). In the case of co-cultures with $^{15}NH_4^+$–enriched *Akashiwo*, however, DON levels remained relatively stable (Fig. 3F).

In all experiments, the $\delta^{15}N$ (‰) of HAB species gradually increased during the course of the experiments (Fig. 4). In the case of $^{15}NO_3^-$–enriched *Akashiwo*, the initial $\delta^{15}N$ of *Margalefidinium* was 0.7 ± 0.2 ‰ and increased more than 30-fold over five days (22.8 ± 7.8‰ at 120 h; Fig. 4A). *Margalefidinium* achieved 37% of its maximal potential of $\delta^{15}N$ at 24 h and 56% after four days (Fig. 4B). $\delta^{15}N$ of *Aureoumbra* cultured with $^{15}NO_3^-$–enriched *Akashiwo* gradually increased from 0.5 ± 0.0‰ at the initial stage of the experiment to 16.6 ± 5.0‰ at 120 h (Fig. 4C), and the percentage of maximal potential $\delta^{15}N$ reached by *Aureoumbra* increased from 14% to 62% by 120 h (Fig. 4D). In the case of $^{15}NH_4^+$–enriched *Akashiwo*, the $\delta^{15}N$ of *Margalefidinium* sharply increased from 0.6 ± 0.1‰ to 6.6 ± 2.2‰ after 24 h and remained stable during the rest of the experimental period (Fig. 4E). The maximal potential percentage attained by *Margalefidinium* ranged from 81 to 118% (Fig. 4F). When $^{15}NH_4^+$–enriched *Akashiwo* was exposed to *Aureoumbra,* the $\delta^{15}N$ of *Aureoumbra* increased more than four-fold from 1.4 ± 0.1‰ to 6.6 ± 0.1‰ over three days and slightly decreased to 4.8 ± 1.0‰ by 120 h (Fig. 4G). *Aureoumbra* attained 44–94% of its potential maximal $\delta^{15}N$ during the experiment (Fig. 4H). The $\delta^{15}N$ of *Alexandrium* increased more than three-fold during its experiment, reaching 3.4 ± 0.4‰ (Fig. 4I). The potential maximal $\delta^{15}N$ percentage attained by *Alexandrium* increased from 45 to 103% over the course of the experiment (Fig. 4J).

The $\delta^{15}NH_4^+$ in the cultures reflected the $^{15}NH_4^+$ liberated from *Akashiwo* but not assimilated into HAB biomass (Fig. 5). $^{15}NH_4^+$ levels in the three HAB controls were fairly stable at 0.4 ± 0.0‰, whereas the levels in $^{15}NO_3^-$–enriched *Akashiwo* control and $^{15}NH_4^+$–enriched *Akashiwo* control were 1.2 ± 0.3‰ and 0.5 ± 0.0‰, respectively (Fig. 5A). In the co-cultures with $^{15}NO_3^-$–enriched *Akashiwo*, the $\delta^{15}NH_4^+$reached 10.2 ± 4.5‰ and 1.7 ± 0.3‰ when cultured with *Margalefidinium* and *Aureoumbra*, respectively (Fig. 5B). However, the levels of $^{15}NH_4^+$in experiments with *Margalefidinium, Aureoumbra*, and *Alexandrium* co-cultured with $^{15}NH_4^+$–enriched *Akashiwo* were significantly lower at 0.6 ± 0.0‰, 0.7 ± 0.0‰, and 0.5 ± 0.1‰, respectively (Fig. 5B). $^{15}NH_4^+$ levels in treatments were significantly greater than the levels in control ($p < 0.05$; *t*-test) in all experiments except for the experiment of *Alexandrium* exposed to $^{15}NH_4^+$–enriched *Akashiwo* ($p > 0.05$; *t*-test; Fig. 5B).

3.3. Correlation analyses

Cells densities of HAB species were inversely correlated with *Akashiwo* cell densities during the course of all experiments ($p < 0.05$ for all; Table 2). *Akashiwo* densities were positively correlated with NO_3^- concentrations ($p < 0.05$ for all; Table 2) and negatively correlated with NH_4^+ in the experiment with $^{15}NH_4^+$-enriched cells and *Aureoumbra* (Table 2). *Margalefidinium* densities were inversely correlated with NO_3^- levels in both experiments with this HAB ($p < 0.05$; Table 2). *Aureoumbra* was significantly positively correlated with DON when co-cultured with $^{15}NO_3^-$–enriched *Akashiwo* ($p < 0.05$) but not when co-cultured with $^{15}NH_4^+$-enriched *Akashiwo* ($p > 0.05$; Table 2).

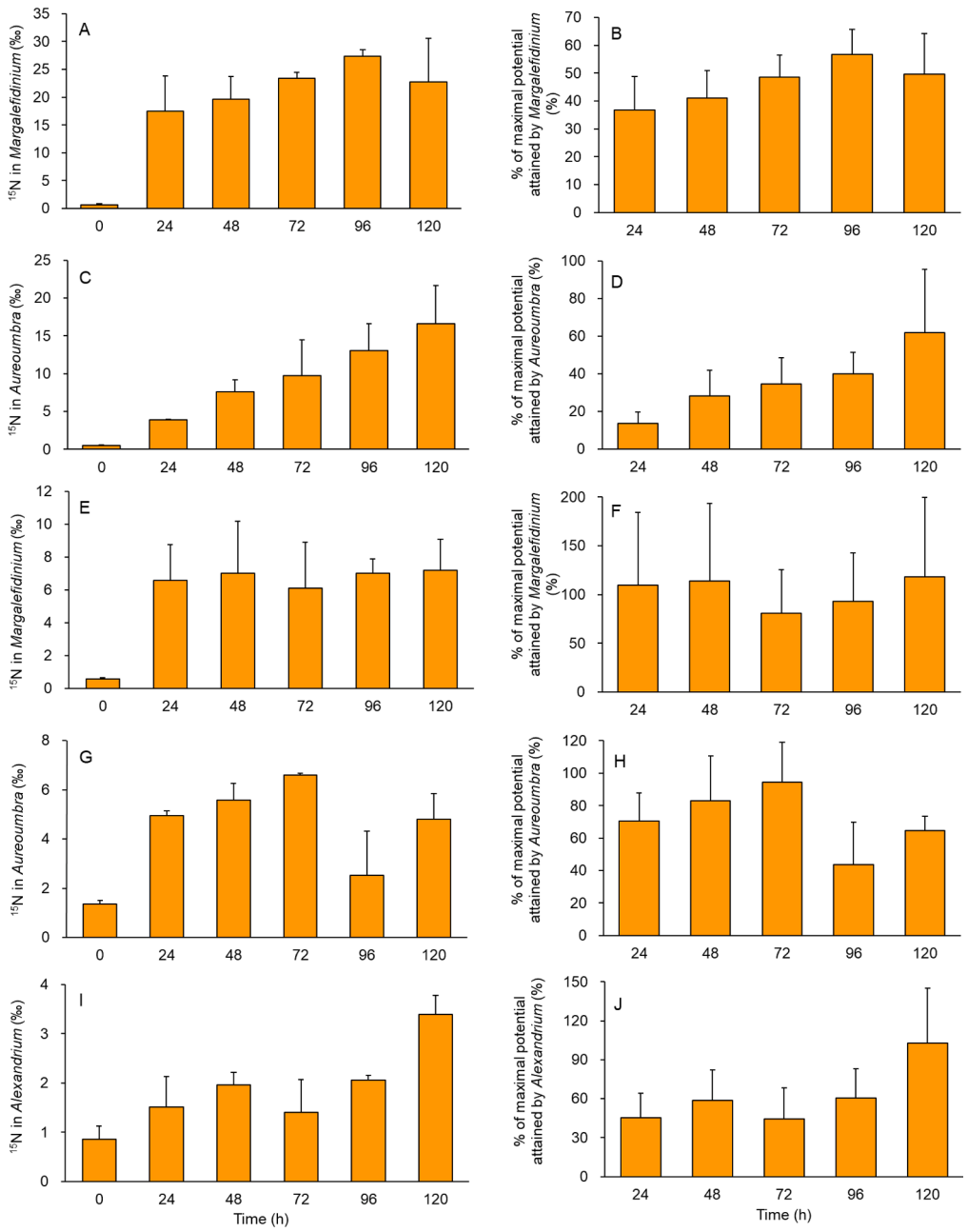

Fig. 4. The percentage of maximal potential attained by HAB (%). (A), (C), (E), (G) and (I) are results from co–culturing experiments conducted with *Akashiwo sanguinea*, that was enriched with $^{15}NO_3^-$ while (B), (D), (F), (H) and (J) are results from co–culturing experiments conducted with *Akashiwo sanguinea* that was enriched with $^{15}NH_4^+$.

4. Discussion

4.1. Impacts of released N on HAB growth

During all experiments, the HAB species studied displayed steady increases in cell densities that were accompanied by a sharp decline in *Akashiwo* densities, sometimes to undetectable levels, affirming the strong allelopathic nature of *Alexandrium* (Hattenrath-Lehmann and Gobler, 2011), *Aureoumbra* (Kang and Gobler, 2018), and *Margalefidinium* (Tang and Gobler, 2010). This growth occurred despite the facts that no exogenous N was added to these experiments and that ambient levels of DIN were low (<5 μM). Phytoplankton release both dissolved inorganic and organic N as they grow, lyse, and/or die (Lomas et al., 2000; Varela et al., 2005) and HABs may directly benefit from such released N, either via the direct use or the further degradation of DON into DIN (Gann et al., 2022; Kang et al., 2021a; Zhuang et al., 2015). All of the HAB species tested herein reached elevated bloom densities using solely N released from allelopathically targeted non-HAB alga. The increase in cell density and $\delta^{15}N$ of the HAB species during the rapid decline of the target species indicated that the N released by the target alga was utilized by the harmful, allelopathic algae. Supporting this notion, in four of five experiments, calculations of the maximal potential $\delta^{15}N$ content indicated that more than 60% of the N within the newly formed HAB cells was from the non-HAB species and in three experiments, this value was 100%. This supports the hypothesis that the allelopathically regenerated N promotes the proliferation of HABs.

During the first 24–48 h of most experiments, there was sharp decline in *Akashiwo* densities and the most rapid growth of the HAB species occurred. After 48 h, the rate of change in cell densities for both groups slowed. This trend suggests that the rapid lysis of target species released

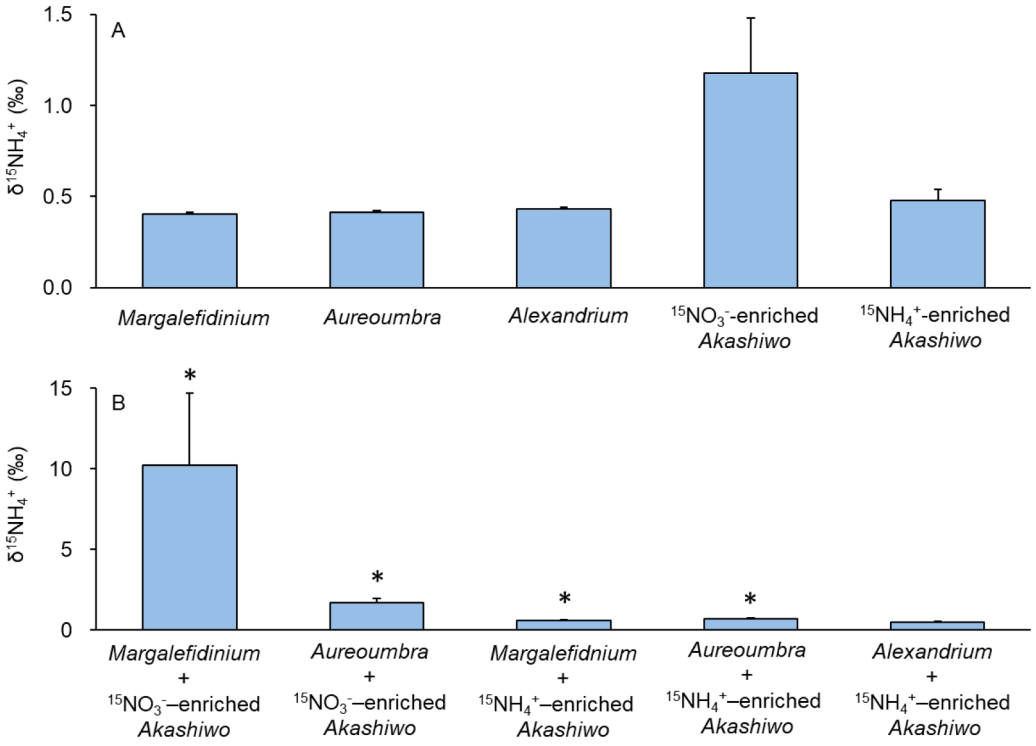

Fig. 5. $^{15}NH_4^+$ (‰) in cultures during a course of experiments. (A) $^{15}NH_4^+$ (‰) of controls, (B) $^{15}NH_4^+$ (‰) of HAB species in co-cultures after five-days of incubation. Asterisks indicate $^{15}NH_4^+$ values in treatment is significantly different from the values in control ($p < 0.05$; *t*-test).

Table 2
Correlation analysis between nutrients and each treatment during the course of the experiments. Blank indicates no correlation. Asterisk indicates significant correlation ($p < 0.05$).

Enrichment	Treatment	Factor	AKSW	NO_3^-	NH_4^+	DON
$^{15}NO_3^-$	*Margalefidinium* + *Akashiwo*	*Margalefidinium*	−0.97*	−0.95*	−0.52	0.68
		Akashiwo	–	0.99*	0.62	−0.53
	Aureoumbra + *Akashiwo*	*Aureoumbra*	−0.77		0.17	0.86*
		Akashiwo	–		0.37	−0.8
$^{15}NH_4^+$	*Margalefidinium* + Akashiwo	*Margalefidinium*	−0.90*	−0.94*	0.17	0.20
		Akashiwo	–	0.98*	0.06	−0.27
	Aureoumbra + *Akashiwo*	*Aureoumbra*	−0.83*		0.99*	0.27
		Akashiwo	–		−0.85*	−0.48
	Alexandrium + *Akashiwo*	*Alexandrium*	−0.74	−0.73	0.08	−0.12
		Akashiwo	–	0.96*	−0.1	0.29

more bioavailable N supporting more rapid growth of the HAB species compared to the slower growth when the loss rates of *Akashiwo* slowed and more rapid growth than control cultures grown on GSe media with nitrate. While phytoplankton can release DON via diffusion of low molecular weight compounds through cell membranes (Bjørrisen, 1988), phytoplankton also release more NH_4^+ under the stressful condition (Lomas et al., 2000). Phytoplankton preferentially utilize NH_4^+ (Cochlan and Bronk, 2003; Dortch, 1990), whereas additional enzymatic activity may be required to degrade DON to biologically available forms (Sipler and Bronk, 2015). Thus, the HAB species likely utilized the released NH_4^+ prior to the DON utilization. Additionally, previous studies have shown that the HAB species examined here exploit DON to increase abundance during a course of blooms (Collos et al., 2014; Gobler et al., 2012; Kang et al., 2015; Muhlstein and Villareal, 2007). During dense blooms, uptake rates of DON (e.g., urea) by HAB species can often exceed the ability of other co-occurring phytoplankton to utilize these compounds (Kang et al., 2017). Therefore, the rapid growth of HAB species in response to the rapid lysis of target species observed in our study might be attributed to rapid assimilation of NH_4^+ as well as the subsequent degradation and/or use of N from the DON pool.

Variations in the growth inhibition of target species coupled with prior studies suggest that different allelochemicals from HAB species have different effects on the co-occurring algae. Although the allelopathic elimination of *Akashiwo* cells exceeded 70% across all experiments, it reached 100% in both experiments with *Margalefidinium* and in the experiment with $^{15}NH_4^+$-grown *Akashiwo* cells exposed to *Aureoumbra* (Fig. 1). Previous studies have characterized the alleochemicals in these HAB species as non-PSP secondary metabolites for *Alexandrium* (Long et al., 2021; Tillmann and Hansen, 2009), hydrophobic secondary metabolites for *Aureoumbra* (Kang and Gobler, 2018), and reactive oxygen species (ROS) for *Margalefidinium* (Tang and Gobler, 2010). Each class of allelochemical is likely to cause inhibition in different ways and on different time courses and, in turn, may have contributed toward variations in the rates at which allelochemically impaired cells were lysed and released N.

Beyond allelopathy, phagotrophy is relatively common among HABs (Mitra et al., 2023). Some *Alexandrium* spp has been shown to be phagotrophic but not *A. catenella* (Jeong et al., 2004; Blossom et al., 2017) and while *M. polykrikoides* has been shown to be phagotrophic, it is not able to consume *A. akashiwo* (Jeong et al., 2005). Further, *Aureoumbra* is not known to be phagotrophic and is more than an order of magnitude smaller than *A. akashiwo*. Hence, N uptake by HAB species

shown in this study was likely the assimilation of allelopathically liberated N from *Akashiwo* and not a result of the phagotrophy. Still, allelopathically lysed cells likely liberate dissolved and particulate N and many HAB species are known to utilize combinations of allelopathy and phagotrophy for growth (Graneli et al., 2012; Place et al., 2012; Blossom et al., 2017). It is likely, therefore, that in an ecosystem setting, allelopathically liberated N is consumed by some HAB species using combinations of direct uptake, osmotrophy, and/or phagotrophy.

While bacterial densities were kept minimal during experiments via the use of aseptic techniques, antibiotics, and filtration/sieving, in an ecosystem setting, bacteria are ubiquitous, active consumers of labile organic matter (Wheeler and Kirchman, 1986). While many HABs including those studied here have been shown to assimilate organic nitrogen within ecosystem settings (Mulholland et al., 2002, 2009), competition with bacteria may reduce the direct transfer of allelopathy liberated N to HAB species. Still, HAB species may indirectly benefit from this allelopathically liberated N consumed by bacteria via bacterivory and/or phagotrophy of bacterivorous protists.

4.2. Nitrate vs ammonium grown target cells

During experiments with *Margalefidinium*, $^{15}NO_3^-$-enriched *Akashiwo* cultures dramatically decreased upon exposure to HAB species compared to $^{15}NH_4^+$-enriched cultures which displayed more gradual declines. In contrast, during experiments with *Aureoumbra* there was a more rapid and complete decrease in $^{15}NH_4^+$-enriched *Akashiwo* densities compared to a $^{15}NO_3^-$-enriched *Akashiwo* exposed to identical cell densities of the allelopathic brown tide alga. Taken together, these results might suggest that the type of N compounds utilized by *Akashiwo* affected its vulnerability to different HAB allelochemicals. Phytoplankton shift their allocation of photosynthetic energy and carbon between major metabolic pathways to optimize growth and persistence, sometimes as a function of N source they utilize (Geider et al., 2009), and HAB species can trade between cell growth and toxin production upon exposure to other organisms such as grazers (Park and Dam, 2021). Felpeto et al. (2019) demonstrated that allelopathy of *Synechocystis* sp. induces an increase in lipid production and a decrease in photosynthetic efficiency in target cells, evidencing a shift in energy allocation. In this study, we hypothesized that $^{15}NO_3^-$-enriched *Akashiwo* allocated energy to both cellular nitrate/nitrite reduction and defense against the allelopathic effect of *Margalefidinium*, whereas $^{15}NH_4^+$-enriched *Akashiwo* could allocate more energy solely to defense. Although *Akashiwo* could potentially allocate more energy for defense upon incorporation of NH_4^+, the instantaneous uptake of released NH_4^+ might have enabled *Aureoumbra* to more strongly inhibit *Akashiwo* given this pelagophyte grows efficiently on ammonium but not on nitrate (Muhlstein and Villareal, 2007). Conversely, the slower growth of *Aureoumbra* upon exposure to $^{15}NO_3^-$-enriched *Akashiwo* likely slowed the complete suppression on the *Akashiwo*.

DON levels during this study continuously increased for $^{15}NO_3^-$-enriched *Akashiwo*, whereas DON levels remained relatively consistent for $^{15}NH_4^+$-enriched *Akashiwo*. Consistent with these results, phytoplankton released more DON when grown with NO_3^- compared to NH_4^+ (Varela et al., 2005) as NH_4^+ utilization is highly correlated with biomass production (Bronk and Ward, 2005). Beyond the passive exudation from phytoplankton, zooplankton grazing is known to enhance the release of DON (Hasegawa et al., 2000; Jackson and Eldridge, 1992). Likewise, our results demonstrated that cellular damage due to allelochemicals induced a DON release from impaired cells that was enhanced in $^{15}NO_3^-$-enriched target cells. Consistent with the regeneration of DON, HAB species grown with $^{15}NO_3^-$-enriched *Akashiwo* obtained only 60% of their cellular N from *Akashiwo* whereas those grown with $^{15}NH_4^+$-enriched *Akashiwo* obtained close to 100% of their N from this non-HAB. Finally, $^{15}NO_3^-$-enriched *Akashiwo* yielded more $\delta^{15}NH_4^+$, and particularly when exposed to *Margalefidinium*. The $\delta^{15}NH_4^+$ signatures at the end of the experiments (at 120 h) were consistently higher in co-cultures with $^{15}NO_3^-$-enriched *Akashiwo* compared to co-cultures with $^{15}NH_4^+$-enriched *Akashiwo*. This suggests that a larger portion of released cellular N was regenerated to NH_4^+ in the mixed cultures that contained $^{15}NO_3^-$-enriched *Akashiwo*, further supporting the hypothesis that phytoplankton released more DON when grown with NO_3^- compared to NH_4^+ (Varela et al., 2005).

4.3. Ecological implications

N is generally considered the nutrient element that most commonly controls the proliferation and persistence of HABs in coastal ecosystems (Glibert et al., 2018a; Hattenrath-Lehmann et al., 2015; Hattenrath et al., 2010; Kang et al., 2015). The allelopathic effects of HAB species have been proposed as a mechanism important for sustaining and/or promoting HABs (Granéli and Hansen, 2006; Kang and Gobler, 2018; Tang and Gobler, 2010; Tillmann and Hansen, 2009), and the data in this study provides a mechanistic link for this process via the transfer and regeneration of N. Given that the allelopathic effects of HAB species on sympatric algae are often dose-dependent with the most potent effects likely occur at higher bloom densities (Jonsson et al., 2009; Kang and Gobler, 2018; Tang and Gobler, 2010), the N released via allelopathic interactions may be important when N levels in the ambient water are depleted to a limiting level due to high biomass levels present during bloom peaks. One of the major motivations of this study was to understand the extent to which allelopathically recycled N can promote HAB persistence. Our findings demonstrated that the growth of HAB species in response to the released N can be rapid and significant, and that the amount of N that is generated by allelopathy is sufficient to further increase the bloom intensity, despite an absence of any other exogenous N supply. Collectively, our findings evidenced an allelopathically-driven positive feedback loop for HABs whereby the elimination of competing plankton liberates N, that can be used by the HAB species and yield greater HAB biomass. Given allelopathy can be a dose-dependent process (Jonsson et al., 2009; Kang and Gobler, 2018; Tang and Gobler, 2010), increases in HAB biomass could strengthen the concentration of allelochemicals, further eliminate competitors, further regenerate N, and further intensify the HAB.

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Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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