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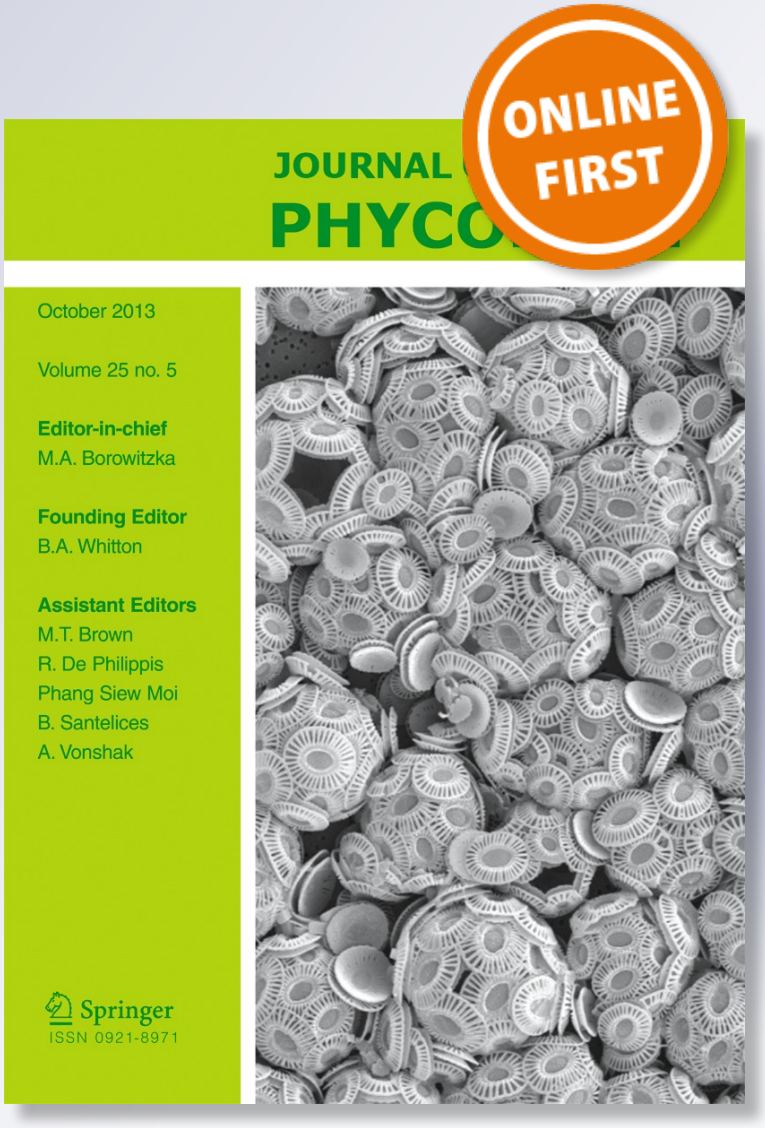

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Organic and inorganic nitrogen utilization by nitrogen-stressed cyanobacteria during bloom conditions

Justin D. Chaffin · Thomas B. Bridgeman

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Abstract Cyanobacterial blooms often occur in lakes that have high phosphorus (P) and low nitrogen (N) concentrations, and the growth rate of the blooms is often constrained by N. For these reasons, many researchers have suggested that regulation of both P and N is required to control eutrophication. However, because N occurs in many bioavailable forms, regulation of a particular form may be beneficial rather than regulation of all N forms. To address how N-stressed cyanobacteria respond to various N inputs, N enrichment experiments (nitrate, ammonium, urea, and alanine) were performed during N-limited cyanobacterial blooms in Maumee and Sandusky Bays of Lake Erie and in Grand Lake St. Marys (GLSM). Bioavailable N (nitrate, urea, and ammonium) concentrations were also determined. *Microcystis aeruginosa* dominated the Maumee Bay bloom, where the highest growth rates were in response to ammonium additions, and lowest growth rates were in response to nitrate. Urea and the amino acid alanine resulted in intermediate growth rates. *Planktothrix agardhii* dominated the Sandusky Bay and GLSM blooms, where nitrate, ammonium, and urea addition resulted in similar growth rates. Additions of alanine did not stimulate growth of the *Planktothrix* blooms. Incubations using stable isotope ^{15}N showed the cyanobacteria had a preference for ammonium, but the other forms were also assimilated in the presence of ammonium. These results show that cyanobacterial blooms will assimilate multiple forms of N to support growth. Thus, if lake managers do decide that N abatement is necessary, then all forms of bioavailable N need to be constrained.

Keywords Cyanobacteria · Eutrophication · *Microcystis* · Nitrogen · *Planktothrix*

Introduction

Eutrophication of surface waters has become a global problem due to excessive anthropogenic nutrient loading (Smith 2003). Symptoms of eutrophication include high biomasses of cyanobacteria, often called harmful algal blooms. Management of phosphorus (P) concentrations is paramount for minimizing cyanobacterial bloom (Schindler 1977), but the importance of nitrogen (N) in freshwater eutrophication is emerging (Lewis and Wurtsbaugh 2008). In many eutrophic lakes, N frequently limits growth and abundance of cyanobacteria during bloom conditions (Chaffin et al. 2013; Jeppesen et al. 2005; Xu et al. 2010). Furthermore, some limnologists support the idea of decreasing inputs of N into lakes in order to control eutrophication and cyanobacterial blooms (Conley et al. 2009; Paerl et al. 2011), although the idea of regulating both P and N is not agreed upon (Schindler et al. 2008). N abatement can be more complex than P abatement because N occurs in many bioavailable and refractory forms (Wetzel 2001).

Unlike P, which occurs mainly as phosphate, N occurs in many inorganic or organic forms and in many oxidation states. N is available to phytoplankton as either new allochthonous N or as recycled autochthonous N (Dugdale and Goering 1967). New N to the system occurs as nitrate (NO_3^-) and atmospheric dinitrogen gas (N_2); however, only a few cyanobacteria are able to utilize N_2. Recycled N occurs as ammonium (NH_4^+) or dissolved organic N (DON); however, many DON compounds are the refractory products of biological breakdown

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and not available to phytoplankton. Bioavailable DON includes urea and some amino acids. The assimilation of and preference for the bioavailable N forms differs among phytoplankton taxa and which form of N is present can shape phytoplankton communities (Blomqvist et al. 1994; Dai et al. 2012; Paerl et al. 2011). Therefore, for N abatement to be the most effective, we should consider placing constraints on the form of N that has the largest effect on enhancing cyanobacteria growth. However, if N deficient cyanobacteria will utilize all forms equally, then we should consider constrains on all form of N.

Nitrogen assimilation by cyanobacteria is regulated by the *ntcA* gene, which encodes for regulatory proteins that are required for all N uptake systems (Frías et al. 1993; Vega-Palas et al. 1992). Ammonium represses the *ntcA* at concentrations greater than 1 μmol L^{-1} (Lindell and Post 2001). In the absence of NH_4^+, *ntcA* gene products promote nitrate-reductase, nitrite-reductase, and urease gene expression (Frías et al. 1997; Herrero et al. 2001). Therefore, cyanobacteria, and phytoplankton in general, typically favor NH_4^+ uptake over any other N form, but uptake of other N forms may continue even when NH_4^+ concentration is greater than 1 μmol L^{-1} (Dortch 1990).

Cyanobacteria can obtain N from many forms of N-containing molecules. After N molecule enters a cyanobacterial cell, that N is reduced to NH_4^+ and incorporated into carbon skeletons (Herrero et al. 2001). Cyanobacterial cell membranes have NH_4^+ transporters when the external concentration of NH_4^+ is less than 1 μmol L^{-1}, but NH_4^+ can permeate cell membranes (Herrero et al. 2001). Nitrate enters the cyanobacteria cell via active transport and is reduced to nitrite then to NH_4^+ by nitrate-reductase and nitrite-reductase, respectively. The reduction of NO_3^- is dependent on iron (Fe) and molybdenum (Mo) ion cofactors (Flores and Herrero 2005). Cyanobacterial blooms can be constrained by trace metals, including Mo, (Downs et al. 2008), and NO_3^- utilization can be impaired by low Fe (Havens et al. 2012; Ivanikova et al. 2007; North et al. 2007). Urea reduction is carried out by urease that is dependent on nickel (Ni) (Flores and Herrero 2005). Urea is broken down into two NH_4^+ and one CO_2 molecule, and that CO_2 molecule can be incorporated by carbon-fixation (Finlay et al. 2010). Urea is associated with eutrophication in coastal oceans (Kudela et al. 2008), and urea in freshwater can increase the toxicity of non-N-fixing cyanobacteria (Donald et al. 2011; Finlay et al. 2010). Some amino acids can be assimilated by cyanobacteria and support growth (Dai et al. 2009; Flores and Herrero 2005). Assimilation of N from amino acid includes deamination (Dai et al. 2009) or conversion into urea (Quintero et al. 2000).

In this study, N utilization by N-stressed cyanobacteria occurring during blooms was addressed using growth and uptake experiments. Three aquatic ecosystems (Lake Erie's Maumee and Sandusky Bays and Grand Lake St. Marys (GLSM) in Ohio, USA) that typically have high biomasses of cyanobacteria (>1,000,000 cells mL^{-1}) and low levels of bioavailable N (<5 μmol L^{-1}) during bloom conditions were studied to address how cyanobacteria that become N-stressed respond to N enrichment. Two experiments were conducted in the laboratory using water collected from each lake during an N-limited cyanobacterial bloom. The first experiment included adding four different forms of N (NO_3^-, NH_4^+, urea, and the amino acid alanine) to lake water at concentrations ranging from 0.1 to 100 μmol L^{-1} N to generate Monod-style growth curves. The second experiment addressed which form of N is preferred in the presence of the other N forms. The stable N isotope ^{15}N was used in these experiments.

Materials and methods

Site descriptions

Maumee Bay is in the western corner of Lake Erie (Fig. 1) and receives a heavy nutrient load from the Maumee River (Richards et al. 2010). Maumee Bay has an average depth of 2 m. *Microcystis aeruginosa* dominates the phytoplankton community during summers (Millie et al. 2009), but dominance can shift to N-fixing *Anabaena spiroides* due to very low NO_3^- concentrations (Chaffin et al. 2013).

Sandusky Bay of Lake Erie (Fig. 1) is a drowned river mouth that has an average depth of 2.6 m (Conroy et al. 2007). Sandusky Bay is heavily impacted by nutrient loading from the Sandusky River (Richards et al. 2010). Sandusky Bay has been divided into west and east halves due to the construction of a highway bridge. The sample site for this study is in the eastern half and has a depth of 3 m. The filamentous cyanobacterium *Planktothrix agardhii* dominates the phytoplankton community throughout the summer (Conroy et al. 2007). Total N decreases throughout the summer (Conroy et al. 2007), and NO_3^- is below 1 μmol L^{-1} during the summer (Chaffin et al. 2013).

GLSM is a man-made inland lake in western Ohio (Fig. 1). The watersheds of GLSM have an average of 240 livestock animals per square kilometer, and the nutrient-laden runoff from spreading of the manure heavily impacts GLSM (Hoorman et al. 2008). During the summer of 2010, recreational use of GLSM was restricted due to an intense bloom of *Aphanizomenon gracile* (Davenport and Drake 2011). *Planktothrix* blooms have also been observed in GLSM (Filbrun et al. 2013). In order to combat eutrophication in GLSM, short-term solutions, such as sediment dredging, rough fish removal, and alum (aluminum sulfate) additions, have been attempted with little success (Filbrun et al. 2013). Many inland lakes in this area of Ohio have either N-fixing or non-N-fixing cyanobacteria, but heterocysts are very low in

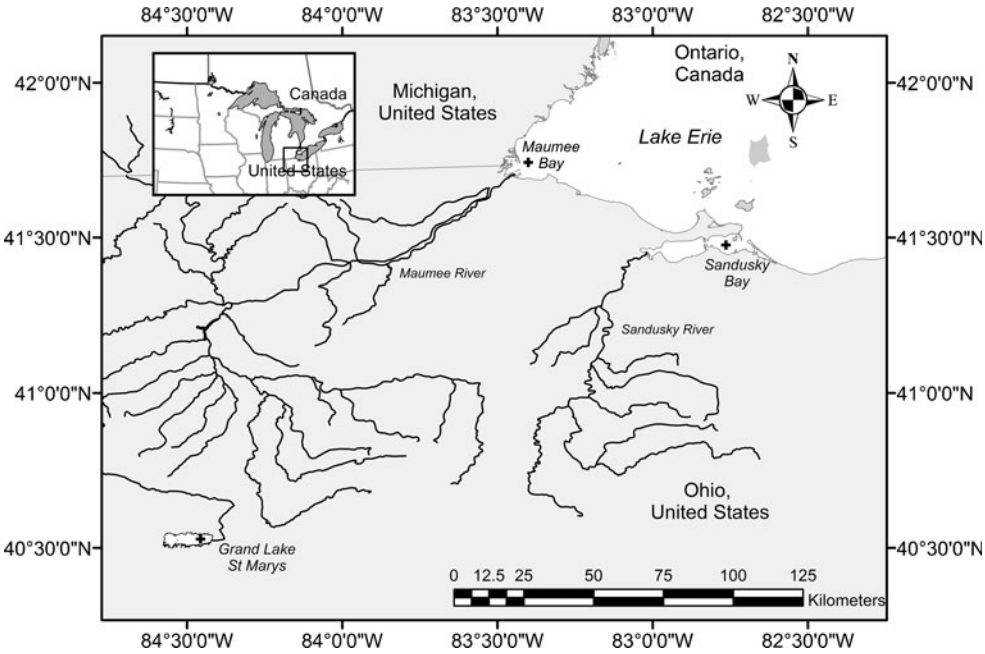

Fig. 1 Sample locations for the N uptake and growth experiment

density (Beaver et al. 2012). NO_3^- concentrations are very low during summer and below detectable limits during the fall (Hoorman et al. 2008).

Field methods

Lake water was collected from the surface to 1-m depth using an integrated tube sampler from Maumee Bay on July 14, Sandusky Bay on July 24, GLSM on August 6, and again from Maumee Bay September 1 during 2012. Water was poured into clean buckets then into acid-washed polyethylene jugs. A 500-mL glass jar was filled with lake water, and then Lugol's solution was added for identification of phytoplankton. Water temperature was recorded with a YSI sonde (Yellow Springs Instruments #6600) from surface to lake bottom at 0.5-m intervals. Secchi disk depth was recorded. Jugs and phytoplankton samples were kept in a dark cooler during transportation back to the laboratory, which was 0.5 h for Maumee Bay, 1.25 h for Sandusky Bay, and 2.5 h for GLSM.

Nutrient and chlorophyll analysis

Dissolved nutrients (NH_4^+, nitrite (NO_2^-), NO_3^-, dissolved Kjeldhal N, urea, and dissolved reactive P) were determined on lake water that passed through a 0.45 μm membrane filter, while total nutrients (total Kjeldhal N (TKN) and total P (TP)) were determined on unfiltered water. The dissolved and total nutrient samples were stored at −20 °C until analysis. All nutrient analysis except for urea were determined by the National Center for Water Quality Research at Heidelberg University (Tiffin, Ohio, USA) using USEPA approved methods (Richards et al. 2010). Urea was determined spectrophotometrically (Goeyens et al. 1998; Mulvenna and Savidge 1992). Briefly, 5 mL of filtered sample (allowed to thaw to room temperature) were added to 15-mL tubes and then 0.357 mL of diacetyl monoxime (96.15 % v/v of 34 mg mL^{-1}) and thiosemicarbazide (3.85 % v/v of 9.5 mg mL^{-1}) solution were added. The tube was capped and vortexed briefly to mix the reactants. Then, 1.143 mL of 56 % sulfuric acid and ferric chloride (0.093 % v/v of 15 mg mL^{-1}) were added (Mulvenna and Savidge 1992). The tube was capped and vortexed to mix. The samples were then kept in the dark for 72 h at room temperature (Goeyens et al. 1998). Absorbance at 520 nm was read using a UV-1650 PC Shimadzu spectrophotometer. Urea was quantified using a standard curve with urea concentrations from 0 to 25 μmol L^{-1} (0 urea-N to 50 urea-N). The curve was linear throughout the known range. Urea standards and reactants were prepared fresh for each analysis.

Chl *a* was determined by collecting phytoplankton on GF/F filters. Filters were stored on silica gel at −80 °C until analysis via DMF extraction and quantification by acid-corrected fluorometry (Speziale et al. 1984). Briefly, the filter with phytoplankton was placed in a 15-mL centrifuge tube and 10 mL of DMF was added. Then the samples were incubated over night at −20 °C and heated to 70 °C for 15 min. After heating, the samples were centrifuged at 2,000 rpm. Fluorescence was recorded using a Turner Designs 10 AU fluorometer. Fluorescence was recorded again after two drops of 6 N HCl were added. Chl *a* concentration was quantified from a standard curve.

Monod growth experiments

N-stressed cyanobacteria growth rate responses to additions of four N forms were determined at ten concentrations (0.0, 0.1, 0.5, 1.0, 3.0, 5.0, 10.0, 25.0, 50.0, and 100.0 μmol N L^{-1}). The four N forms were NO_3^- ($NaNO_3$), NH_4^+ ($(NH_4)_2SO_4$), urea, and the amino acid L-alanine. These concentrations represent the molar concentration of the N atom. Urea has two N atoms per molecule, and, therefore, the urea concentrations would be half that listed above. The 3.0 μmol L^{-1} addition was not used during the Maumee Bay July 14 experiment. Stock solutions of urea and alanine were prepared the day of the experiment.

Incubations used 250-mL acid-washed polycarbonate flasks with 200 mL of lake water. Flasks were incubated for 24 h in a growth chamber (Percival model: E-36HO, Geneva Scientific, USA) at lake temperature at time of collection under a light intensity of 300–350 μmol photon per square meter per second on a 12:12 h light/dark cycle. The light intensity was selected because it is greater than the light saturation point for phytoplankton (Kirk 1994). Incubations began in the afternoon and were terminated the following afternoon. Cyanobacteria abundance was determined as chl *a* at hour 0 and after 24 h. Growth rate was calculated as follows: $[\ln(\text{chl } a_{h24}) - \ln(\text{chl } a_{h0})]/1$ day. Growth rate curves were produced by plotting growth rate versus N concentration. Monod growth parameters were calculated as follows: growth rate = $\mu_{max} * [S/(K_s+S)]$, where μ_{max} is the maximum growth rate (per day), S is the N concentration, and K_s is the N concentration that half saturates growth rate. If the growth rate versus N concentration plot resulted in a linear relationship, linear regression was used rather than the Monod equation.

^{15}N assimilation

To determine if cyanobacteria could directly assimilate each form of N, incubations were conducted using stable ^{15}N (98 % atom ^{15}N, Sigma-Aldrich), and $\delta^{15}N$ was determined. In the 250 mL polycarbonate flasks, 200 mL lake water were spiked with 30 μmol L^{-1} $^{15}NO_3^-$, 5 μmol L^{-1} $^{15}NH_4^+$, 10 μmol L^{-1} urea-^{15}N, or 10 μmol L^{-1} L-alanine-^{15}N. An additional treatment included spiking lake water with all four ^{15}N forms to determine if multiple N forms are used (if cyanobacteria are using more than one N compound, $\delta^{15}N$ here should be greater than any one ^{15}N compound). To determine if abiotic ^{15}N uptake occurred, a formalin-killed (2 %) treatment was used and included adding all four ^{15}N forms at above concentrations. Background $\delta^{15}N$ was determined on initial samples before incubation, and a zero N control was also incubated. All incubations were 1 h in the growth chamber. All treatments were replicated with three separate flasks. After incubation, cyanobacteria were collected on precombusted (550 °C for 30 min) GF/F filters with a vacuum pressure less than 200 mmHg. Filters were immediately dried at 60 °C for 72 h. Excess filter was trimmed, then filters were placed in tin capsules. Isotope analysis was conducted at the Stable Isotope Facility at University of California Davis and followed their methods (http://stableisotopefacility.ucdavis.edu/13cand15n.html). Data were log normalized then analyzed with ANOVA with post hoc Tukey test using SPSS software version 20.

To determine if N-stressed cyanobacteria have a preference for a certain N form in the presence of other forms of N, experiments were conducted that involved adding one ^{15}N form but also enriching with ^{14}N of the other N forms. For example, preference of nitrate was determined by adding $^{15}NO_3^-$ with $^{14}NH_4^+$, ^{14}N urea, and ^{14}N L-alanine, using the above concentrations. All N enrichments followed that as above and were replicated with three separate flasks. To determine N preference index (ρ) for each N form, the $\delta^{15}N$ values were normalized for the ^{15}N and ^{14}N added to the experiment and the ambient N concentration, using the following equation: $\rho_x = \delta^{15}N/[^{15}N_X/(^{14}N_{add}+TDN_{amb})]$, where ρ_x is the preference index for ^{15}N compound X, $^{15}N_X$ is the concentration of ^{15}N added of N form X, $^{14}N_{add}$ is the concentration of ^{14}N added to the experiment, and TDN_{amb} is the ambient concentration of nitrate, ammonium, and dissolved organic N. The ρ_x data were log normalized then analyzed with ANOVA with post hoc Tukey test using SPSS software version 20.

To determine if assimilation of each form of N was inhibited by the presence of other N forms, we compared $\delta^{15}N$ values for the single ^{15}N enrichment to the enrichment that included the same ^{15}N form with ^{14}N of the other form. The data log was analyzed with a Student's *t* test using SPSS software version 20.

Most research studying N uptake by phytoplankton using stable isotope ^{15}N as a tracer generally enrich ^{15}N at 10 % of the ambient N concentration (Dugdale and Goering 1967), which allows for calculation of uptake rate. In our experiments, we want to show how N-stressed cyanobacteria may respond to a pulse of high-concentration N loading. Therefore, we enriched lake water to N concentrations that are typical of early summer, which are much greater than late summer. Because no equation can correct for high tracer concentration relative to ambient concentration (Legendre and Gosselin 1996), we do not attempt to calculate uptake rates but provide $\delta^{15}N$ as a means to determine which form of N cyanobacteria assimilated.

Co-limitation by metal cofactors

Nitrate and urea assimilation can be constrained by metal cofactors Fe, Mo, and Ni, respectively. In order to address

Table 1 Temperature, Secchi disk depth, chl *a*, and the dominant cyanobacterium of the four experiments

Date	Lake	Temp. (°C)	Secchi depth (cm)	Chl *a* (μg L^{-1})	Dominant Cyanobacterium
July 14	Maumee Bay	25.8	85	11.4	*Microcystis aeruginosa*
July 24	Sandusky Bay	26.2	25	158.9	*Planktothrix agardhii*
Aug. 6	Grand Lake	24.9	16	565.3	*Planktothrix agardhii*
Sept. 1	Maumee Bay	24.0	95	82.8	*Microcystis aeruginosa*

the possibility of co-limitation by metal ions, an additional flask spiked with 100 μmol L^{-1} NO_3^- was also spiked with 100 nmol L^{-1} Fe and 250 pmol L^{-1} Mo (Guillard and Lorenzen 1972), and an additional flask spiked with 100 μmol L^{-1} urea-N was also spiked with 40 pmol L^{-1} Ni (Ji and Sherrell 2008). Chl *a* levels in these additional metal-spiked flasks were compared to the chl *a* levels in the 100 μmol L^{-1} flasks that did not receive the metals and analyzed using a two-tailed paired sample *t* test.

Results

Lake properties

Microcystis aeruginosa was the dominant cyanobacterium in Maumee Bay on July 14 and September 1, and the total chl *a* level was 11.4 and 82.8 μg L^{-1} (Table 1). A few eukaryotic algae were observed, but their density relative to *Microcystis* was low and likely due to sampling surface water during calm conditions. *P. agardhii* was dominant in Sandusky Bay and chl *a* was 158.9 μg L^{-1}. *P. agardhii* was dominant in GLSM and chl *a* was 565.3 μg L^{-1}. No other phytoplankton species were observed from the two *Planktothrix* bloom samples. Maumee Bay was visited on August 22 during an *A. spiroides* bloom, but only surface to 0.5 m water was collected for the experiments and chl *a* level was 1,249 μg L^{-1}. The results of this *Anabaena* experiment are not presented because it was likely that too much N was trapped on the filter for isotope analysis.

Ambient NO_3^- concentration was less than 7 μmol L^{-1} in all experiments and below detection limits (<0.7 μmol L^{-1}) in Maumee Bay on July 14, Sandusky Bay, and GLSM (Table 2). Ambient NH_4^+ concentration was less than 4 μmol L^{-1} in three of the four experiments. Sandusky Bay had the highest urea concentration of 30.6 μmol N L^{-1}, and GLSM had 18.5 μmol N/L urea. Urea in the two Maumee Bay experiments was 6.7 and 8.7 μmol N L^{-1}. Total N (sum of TKN and NO_3^-) ranged from 31.6 to 340.2 μmol L^{-1}.

N Enrichment growth

The final chl *a* concentration was very similar between the 100 μmol L^{-1} NO_3^- enrichment with and without Fe and Mo (p =0.461, df=3, T=−0.842). Likewise, 100 μmol L^{-1} urea enrichment resulted in similar final chl *a* levels with and without Ni (p =0.288, df=3, T=−1.87). These results indicate that metal ions were not constraining the uptake of and growth on NO_3^- or urea in these experiments.

The addition of NO_3^-, NH_4^+, and urea stimulated growth in all experiments (Fig. 2). Alanine stimulated growth only in the two Maumee Bay experiments. Monod growth was followed for the two Maumee Bay experiments (Fig. 2a, d), while growth rate increased linearly with increasing N for the Sandusky Bay and GLSM experiments (Fig. 2b, c). In the two Maumee Bay experiments, greatest μ_{max} was achieved on NH_4^+, while NO_3^- resulted in the lowest μ_{max} (Table 3). In the Sandusky Bay and GLSM experiment, μ_{max} could not be determined due to linear growth, but growth rates were very similar among NO_3^-, NH_4^+, and urea at the 100 μmol N L^{-1} addition (Fig. 2b, c). When zero N was added, three of the four experiments had negative growth; only the Maumee Bay September 1 experiment maintained positive growth without excess N.

Table 2 Ambient ammonium (NH_4^+), nitrate (NO_3^-), urea-N, total dissolved organic N (DON, * includes urea), total N (TN), dissolved reactive P (DRP), and total P (TP) concentrations of the four experiments. All values are μmol L^{-1}

Date	Lake	NH_4^+	NO_3^-	Urea-N	DON*	TN	DRP	TP
Jul. 14	Maumee B.	2.00	BDL	8.68	20.37	31.61	0.0048	1.5061
Jul. 24	Sandusky B.	3.96	BDL	30.60	34.98	136.70	0.9540	6.4942
Aug. 6	GLSM	2.32	BDL	18.54	65.91	340.20	0.1082	9.9713
Sep. 1	Maumee B.	16.63	6.43	6.69	30.73	96.89	0.3487	4.5328

BDL below detectable levels

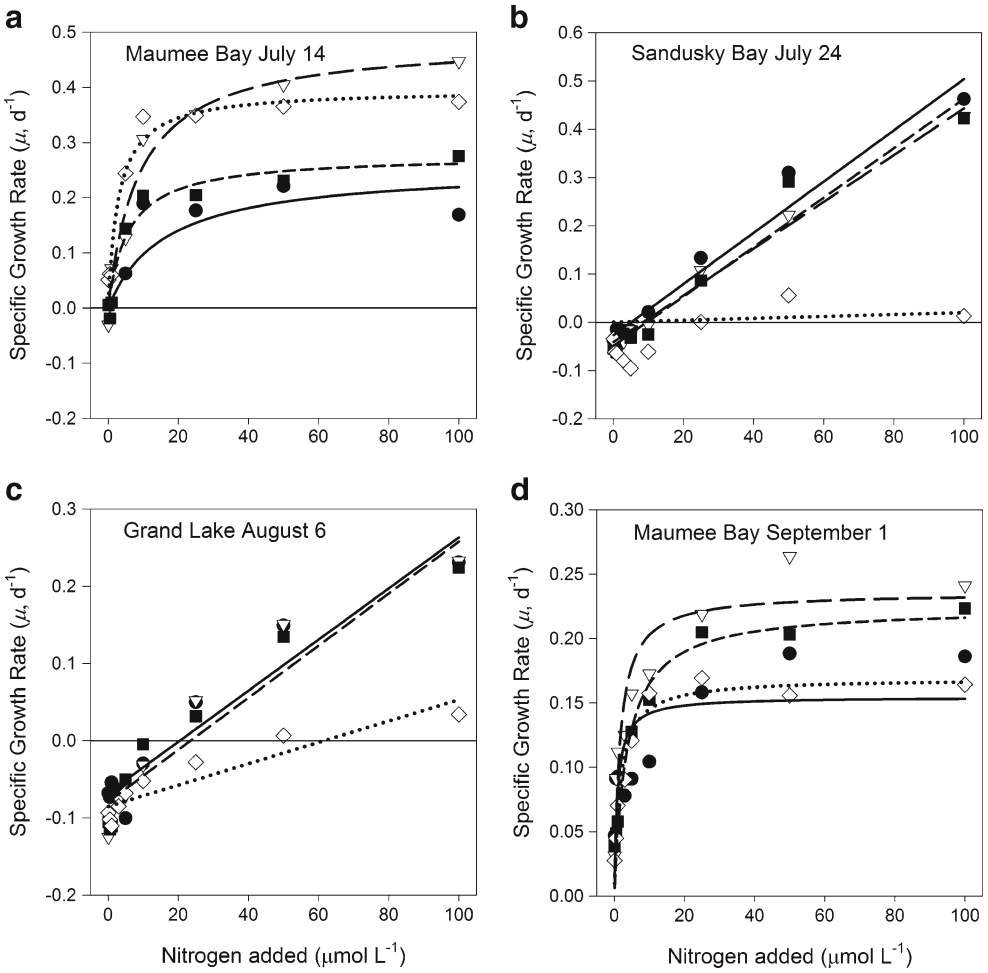

Fig. 2 Growth response of N-stressed cyanobacteria to addition of nitrate, ammonium, urea, and L-alanine. Note the difference in the *y*-axis scale

Table 3 Monod growth parameters to the N addition growth experiment. Monod model was fitted to the growth curves of Maumee Bay (MB) July 14 and September 1

Experiment	Nitrogen	K_s (μmol L^{-1})	μ_{max} (d^{-1})	alpha	r^2	*p* value
MB July 14	Nitrate	18.443	0.260	0.014	0.261	0.1604
MB July 14	Ammonium	9.476	0.491	0.052	0.972	<0.0001
MB July 14	Urea	5.692	0.276	0.049	0.952	<0.0001
MB July 14	Alanine	3.478	0.401	0.115	0.961	<0.0001
SB July 24	Nitrate	NA	NA	0.005	0.967	<0.0001
SB July 24	Ammonium	NA	NA	0.005	0.991	<0.0001
SB July 24	Urea	NA	NA	0.005	0.959	<0.0001
SB July 24	Alanine	NA	NA	0.001	0.535	0.0046
GL Aug 6	Nitrate	NA	NA	0.003	0.928	<0.0001
GL Aug 6	Ammonium	NA	NA	0.004	0.923	<0.0001
GL Aug 6	Urea	NA	NA	0.003	0.919	<0.0001
GL Aug 6	Alanine	NA	NA	0.001	0.854	0.0004
MB Sept 1	Nitrate	0.911	0.155	0.170	0.552	0.0279
MB Sept 1	Ammonium	1.628	0.236	0.145	0.879	0.0020
MB Sept 1	Urea	3.626	0.224	0.062	0.950	<0.0001
MB Sept 1	Alanine	1.649	0.169	0.102	0.959	<0.0001

K_s is the half saturation coefficient and μ_{max} is the maximum growth rate. Alpha is the ratio of μmax to K_s or the linear regression slope. Linear regressions were fit to Sandusky Bay (*SB*) and the Grand Lake St Marys (*GL*), thus K_s and μ_{max} could not be obtained

^{15}N assimilation

The $\delta^{15}N$ values for most ^{15}N enrichments after the 1-h incubation were greater than initial and zero N enrichment control and indicate that the cyanobacteria were assimilating the ^{15}N molecules (Fig. 3). ANOVA indicated significant differences among treatments in all four experiments ($p < 0.001$). The formalin-killed treatment resulted in $\delta^{15}N$ values similar to control, which indicates that abiotic uptake was not a factor in the experiment. Enrichment of 5 μmol L^{-1} NH_4^+ or enrichments of all four ^{15}N forms resulted in the greatest $\delta^{15}N$ values for all experiment. $\delta^{15}N$ values for NH_4^+ enrichment were between 1,269 (Maumee Bay September 1) and 11,116‰ (Sandusky Bay) for all experiments, and these values were 1.5 (Sandusky Bay) to 4.6 times (Maumee Bay September 1) greater than the next highest $\delta^{15}N$ values, which were associated with urea enrichment. Urea (10 μmol L^{-1}) enrichment resulted in the next highest $\delta^{15}N$ for experiments Maumee Bay July 14 (Fig. 3a) and GLSM (Fig. 3c), while urea and NO_3^- (30 μmol L^{-1}) had similar $\delta^{15}N$ for the Sandusky Bay experiment (Fig. 3b). Alanine enrichment (10 μmol L^{-1}) resulted in similar $\delta^{15}N$ as NO_3^- for the two Maumee Bay experiments, and alanine resulted in the lowest $\delta^{15}N$ of the Sandusky Bay and GLSM experiments.

N preference index (ρ) significantly differed among treatments in all four experiments ($p < 0.001$). Ammonium had the highest N ρ in all four experiments, while NO_3^- had the lowest ρ (Fig. 4). Urea had the second highest ρ and alanine was intermediate. The two Maumee Bay experiments had the largest difference between the NH_4^+-ρ and the urea-ρ. There was less difference between NH_4^+-ρ and the urea-ρ for the Sandusky Bay and GLSM experiments.

The $\delta^{15}N$ values for any ^{15}N form were greater when that ^{15}N compound was enriched signally, and $\delta^{15}N$ values decreased for that ^{15}N compound, when the other forms of ^{14}N were added (Fig. 5). For example, the $\delta^{15}N$ values for 30 μmol L^{-1} $^{15}NO_3^-$ were 7,767‰ when no other N was added, but the $\delta^{15}N$ values decreased ($p < 0.001$) to 689‰

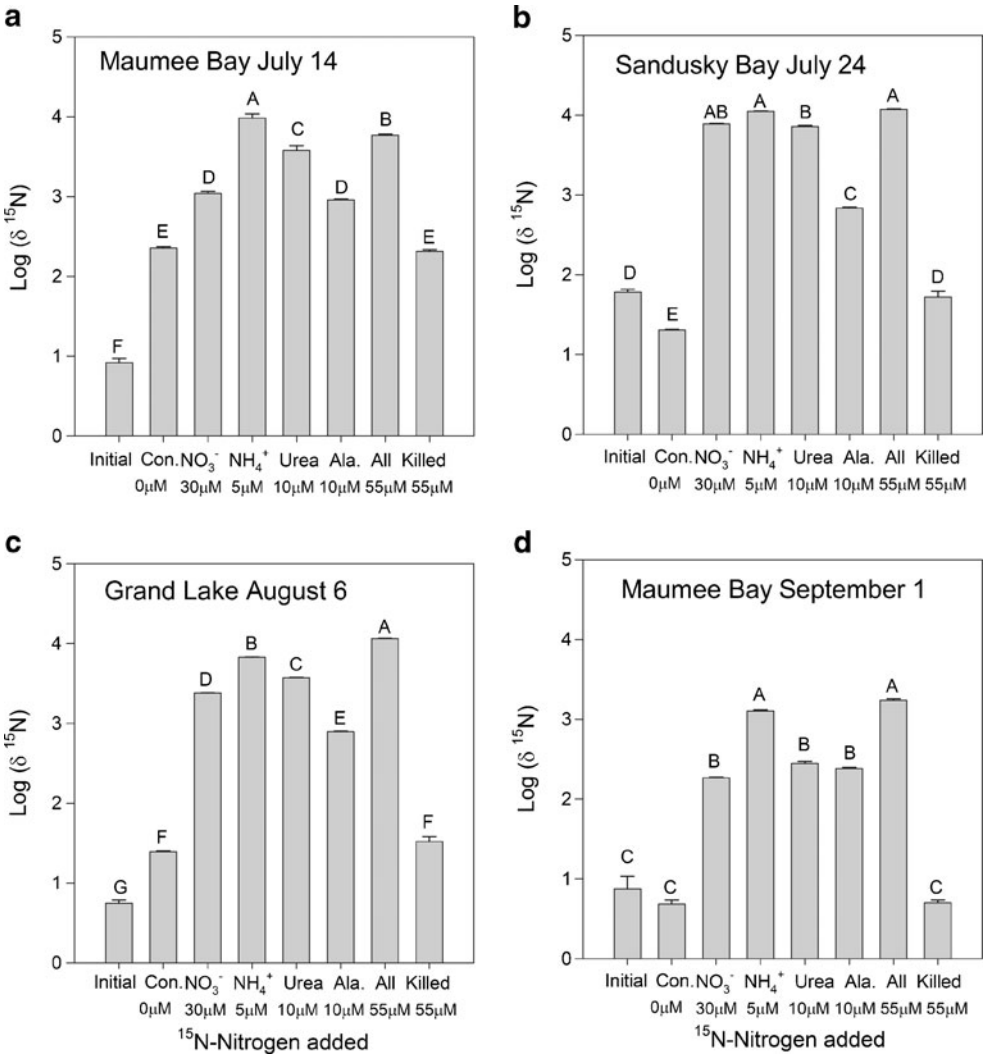

Fig. 3 Log $\delta^{15}N$ values (‰, average±SE) of four cyanobacteria blooms following ^{15}N enrichment after 1-h incubation. Letters above the bar indicate results of the Tukey test and the means of different letters are significantly different ($p < 0.05$). The "killed" treatment represents formalin-killed treatment in which all of the ^{15}N forms were added. *Con*. zero N enrichment control, *Ala*. L-alanine

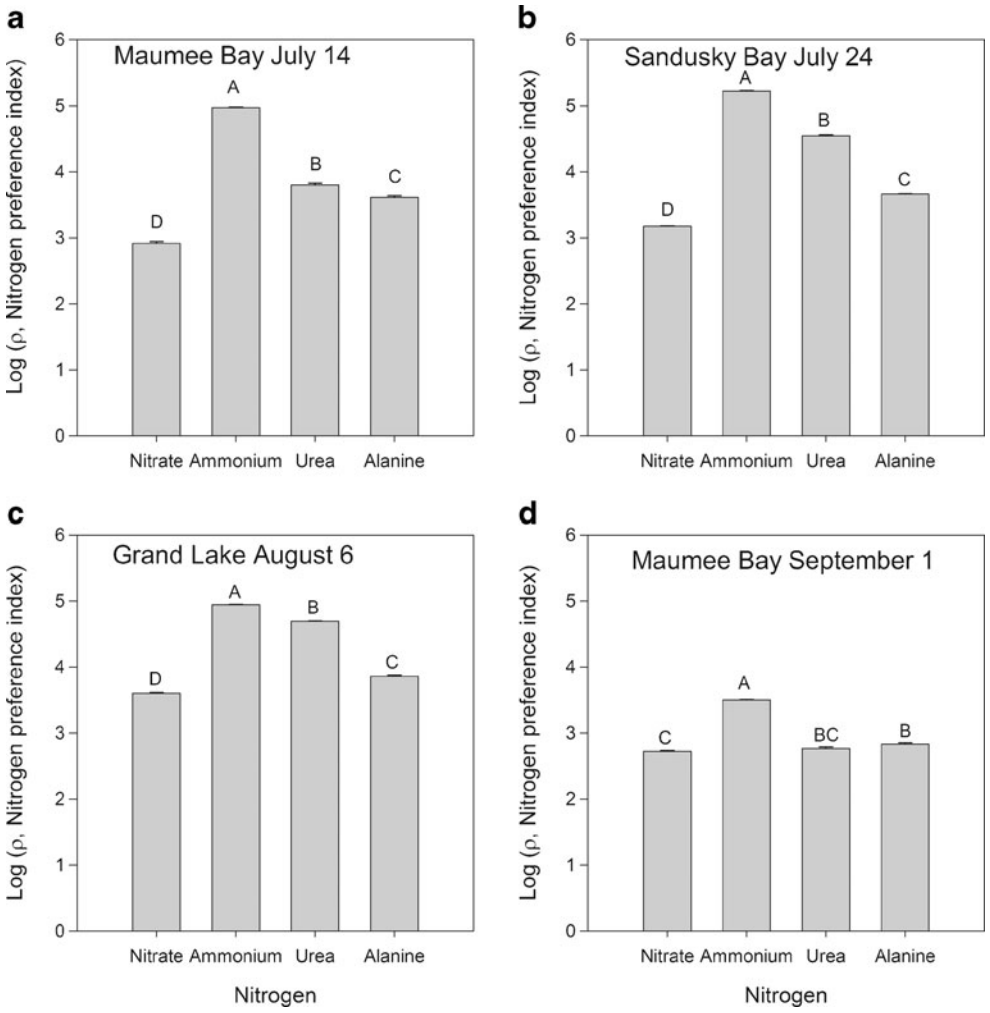

Fig. 4 Log of the nitrogen preference index (average±SE) for ^{15}N nitrate, ammonium, urea, and L-alanine. Note the difference in the *y*-axis scale

when 30 μmol L^{-1} $^{15}NO_3^-$ were added with 5 μmol L^{-1} $^{14}NH_4^+$, 10 μmol L^{-1} urea-^{14}N, and 10 μmol L^{-1} L-alanine-^{14}N (Fig. 5b).

Discussion

The ambient concentrations of bioavailable N measured in these three lakes during a cyanobacterial bloom were very low, especially NO_3^- (Table 2). Furthermore, the addition of N stimulated the growth rate of the cyanobacteria within the blooms (Fig. 2). Therefore, the growth of the cyanobacteria during these blooms was limited by the low concentrations of bioavailable N.

Growth rate versus N concentration followed the expected Monod growth pattern in the two Maumee Bay experiments, which were mainly *Microcystis*. However, growth rate versus N concentration was linear for the Sandusky Bay and GLSM experiments, which were dominated by *Planktothrix*. Although the two different taxa showed a different response to N enrichment concentration, the likely difference is due to the initial abundance. The initial chl *a* level in the two Maumee Bay experiments was 11.4 and 82.8 μg L^{-1}, and growth saturation was reached within 100 μmol L^{-1} of N addition. The initial chl *a* level in the Sandusky Bay experiment was 158.9 μg L^{-1}, and the GLSM initial chl *a* was 565.3 μg L^{-1}. Because of the high initial chl *a* at Sandusky Bay and GLSM, growth saturation was not reached within 100 μmol L^{-1} enrichment (Fig. 2). If higher N concentrations were added, it would be expected that growth saturation would be reached. However, given the high biomasses in the flasks, the cyanobacteria may be self-shading and become light-limited. Carbon limitation may also be a problem in bottle assays with very high phytoplankton abundances. Thus, the actual maximum growth rates may not be obtained, which is evident in the GLSM experiment because growth at 100 μmol L^{-1} N was 0.23 day^{-1}, while the Sandusky Bay growth rate at 100 μmol L^{-1} N was 0.44 day^{-1}.

As hypothesized, growth rate of both *Microcystis* blooms was stimulated by all four forms of N; however, μ_{max} differed

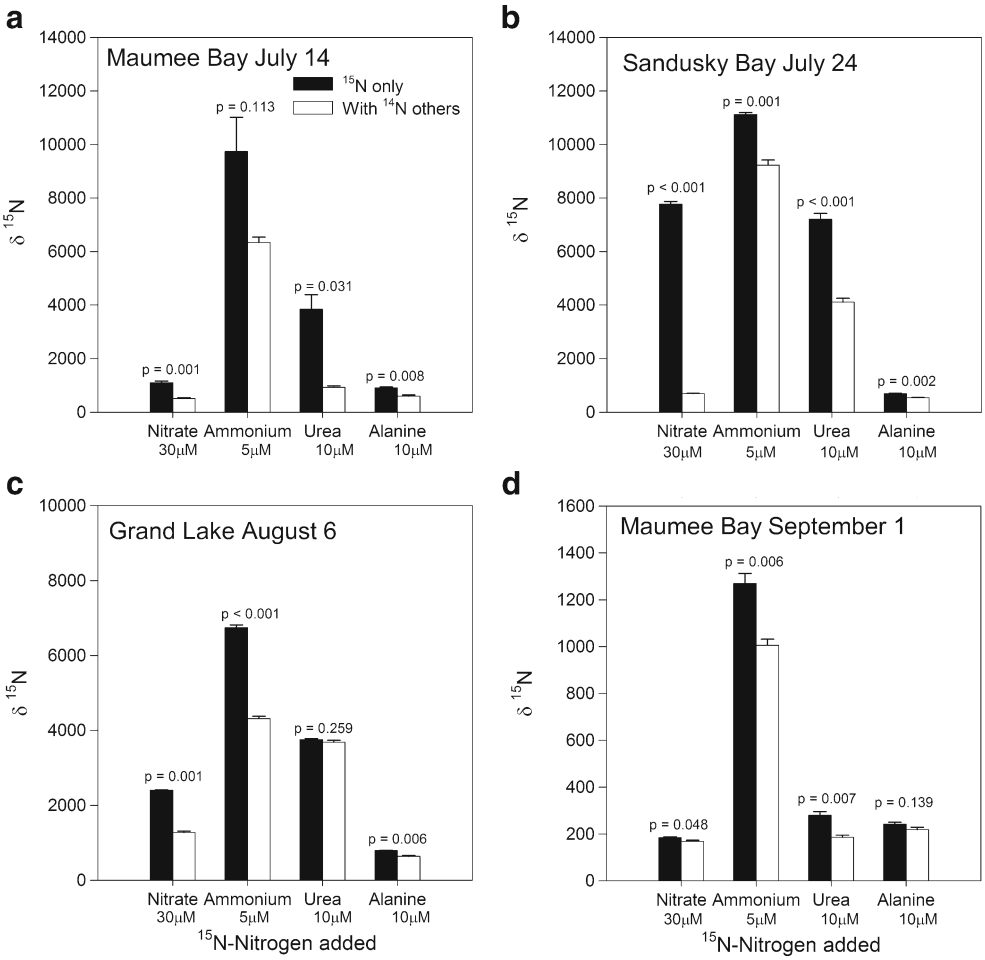

Fig. 5 Comparison of $\delta^{15}N$ values (‰, average±SE) when a ^{15}N form was added signally (*black bars*) to when that ^{15}N form was added with all the other ^{14}N forms. *P* values above each pair represent the *p* value of the Student's *t* test

among the N forms (Table 3). Ammonium resulted in the greatest μ_{max}, NO_3^- gave the lowest μ_{max}, while organic N (urea and alanine) were intermediate. Therefore, the order which stimulated the greatest growth was NH_4^+>organic N> NO_3^-. Stimulation of *Microcystis* growth by N enrichment during bloom conditions is not a novel finding, but there are some different findings among this study and others. N-limited *Microcystis* blooms of Western California, USA showed the same growth yield to additions of NH_4^+, NO_3^-, and urea (Moisander et al. 2009). In Eastern US lakes, enrichment of urea and NO_3^- stimulated *Microcystis* growth more frequently than did NH_4^+ or the amino acid glutamine (Davis et al. 2010). In Wascana Lake of Southern Saskatchewan, Canada, non-heterocystous cyanobacterial blooms (*Microcystis* and *Planktothrix*) growth and toxin production was stimulated by NH_4^+ and urea more so than NO_3^- (Donald et al. 2011). Our findings and those of experiments elsewhere demonstrated that additional N pollution (of any bioavailable form) will exacerbate the problems associated with *Microcystis* during bloom conditions. However, the degree to which blooms are stimulated may depend on which N form is enriched and may be system specific.

The amino acid alanine stimulated growth of the two *Microcystis* blooms (Maumee Bay July 14 and September 1), while the *Planktothrix* blooms did not show growth response to alanine. Furthermore, ^{15}N-alanine resulted in the lowest $\delta^{15}N$ values for the *Planktothrix* blooms, while ^{15}N-alanine gave similar $\delta^{15}N$ values to $^{15}NO_3^-$ for the *Microcystis* blooms. It is likely that *Microcystis* directly used alanine for growth, while *Planktothrix* did not. Previous research has shown that axenic cultures of *M. aeruginosa* can assimilate alanine (as well as leucine and arginine) to support growth (Dai et al. 2009). *Planktothrix* may not have assimilated alanine and/or the microbes in Sandusky Bay and GLSM did not convert alanine into a useable N form. Previous research has shown that *P. agardhii* can utilize the amino acids leucine and arginine for production of the cyanotoxin microcystin (Tonk et al. 2008), and *Planktothrix rubescens* can utilize several amino acids, including alanine, to support growth (Walsby and Juttner 2006). Thus, it is rather surprising that Sandusky Bay and GLSM *P. agardhii* was unable to utilize alanine to support growth, especially because of low ambient bioavailable N concentration at time of enrichment.

Shallow, well-mixed, turbid lakes and their embayments, such as Sandusky Bay and GLSM, are typically dominated by filamentous cyanobacteria like *Planktothrix* (Scheffer 1998). Nutrient status of the lake can drive the competitive outcome between filamentous cyanobacteria. In culture experiments, *P. agardhii* outcompeted *Limnothrix redekei* under N-limiting conditions, while the opposite was true under P-limitation (Rücker et al. 1997). Furthermore, *P. agardhii* was dominant in shallow well-mixed lakes that had low bioavailable N concentrations and low N/P ratios, which indicates N-limitation (Rücker et al. 1997). *L. redekei* was dominant in shallow well-mixed lakes that had low dissolved reactive P levels (Rücker et al. 1997). The data presented here clearly indicate that Sandusky Bay and GLSM are N-limited because of the low bioavailable N levels and the growth stimulation by the addition of N. Hence, the reason why *P. agardhii* dominates these lakes. However, previous cyanobacterial blooms in GLSM have been the buoyant N-fixing cyanobacterium *Aphanizomenon gracile* (Davenport and Drake 2011). Low TN/TP ratio (<16 mol) will favor *Aphanizomenon* over *Planktothrix* in shallow eutrophic lakes (Teubner et al. 1999). The presence of combined N will inhibit the nitrogenase enzyme needed for N-fixation (Flores and Herrero 2005). A likely explanation for the *Planktothrix* bloom in 2012 in GLSM is that the NH_4^+ or urea levels were high enough to suppress the nitrogenase enzyme but low enough for N-limitation of growth and TN/TP ratios were greater than 16 (Table 2).

The ^{15}N experiments show that NH_4^+ was the preferred N form of N-stressed cyanobacteria, which is not a novel idea (Dugdale and Goering 1967). However, the ^{15}N experiments did show that the N-stressed cyanobacteria utilized any N form when that one form is singly provided, (except *Planktothrix* did not assimilate alanine). Furthermore, $\delta^{15}N$ of any particular N form was less when all forms were concurrently enriched (Fig. 5), and highest $\delta^{15}N$ values occurred in enrichments with all four ^{15}N labeled forms (Fig. 3). These results suggest that N-stressed cyanobacteria will assimilate multiple forms of N at once, although cyanobacteria showed highest preference for NH_4^+. It has been suggested that NH_4^+ concentrations greater than 1 μmol L^{-1} will inhibit uptake of other N forms by repressing the *ntcA* gene (Lindell and Post 2001); however, this statement may not be entirely true. The ^{15}N data presented here suggests that N-stressed cyanobacteria will keep N uptake systems ready in case non-NH_4^+-N is supplied. The results here confirm the finding of Dortch (1990) that suggested phytoplankton favor NH_4^+ over other N forms, but uptake of other N forms may continue even when ambient NH_4^+ concentrations are greater than 1 μmol L^{-1}.

The Maumee Bay September 1 experiment had $\delta^{15}N$ values that were overall one-fifth that of the other three experiments (Fig. 3). The ambient dissolved N concentrations were greater than the other three experiments (Table 2) and were likely supporting growth because *Microcystis* was able to maintain positive growth (although low growth) without additional N. Thus, the low $\delta^{15}N$ values for the Maumee Bay September 1 were likely due to the higher ambient dissolved N concentrations or an intercellular storage of nutrients that were able to support growth and lessen the need to assimilate the enriched ^{15}N.

The growth of many cyanobacteria during bloom conditions is constrained by low levels of bioavailable N (Jeppesen et al. 2005; Xu et al. 2010). Thus, the need to regulate N inputs to control eutrophication has been touted by many researchers lately (Conley et al. 2009; Paerl et al. 2011); however, P abatement is paramount (Schindler et al. 2008). The results from this study suggest during N-limitation inputs of all forms of bioavailable N can stimulate growth and N uptake, but those rates will differ among cyanobacteria and N form. If lake managers decide that N regulation is needed to alleviate problems associated with cyanobacteria, then all forms of bioavailable N need to be targeted. However, without concurrent reductions of external P loading, these lakes will likely remain eutrophic (Schindler et al. 2008).

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