

Effects of inorganic nitrogen on taxa-specific cyanobacterial growth and *nifH* expression in a subtropical estuary

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Abstract

The potentially toxic, diazotrophic filamentous cyanobacterium *Cylindrospermopsis raciborskii* has recently become a common component in the summer phytoplankton in the St. Johns River (SJR) estuary, Florida, where *Anabaena* spp. historically dominated. Using a microcosm nutrient enrichment experiment, we investigated the ability of *C. raciborskii* and *Anabaena* spp. to compete under a range of available NO_3^- and NH_4^+ concentrations, to test the hypothesis that *C. raciborskii* benefits from increased dissolved inorganic nitrogen (DIN) availability. TaqMan quantitative polymerase chain reaction (PCR) probes were designed, tested, and applied to target the *nifH* gene in one *C. raciborskii* and two *Anabaena* spp. strains from the SJR. N limitation prevailed, as shown by increased N_2 -fixation rates if no N was added, increased chlorophyll *a* concentrations when DIN was added, and depletion of added DIN. *Anabaena* spp. and *C. raciborskii* showed rapid growth with no DIN additions and were the main taxa responsible for N_2 fixation. Abundances of *C. raciborskii* increased if NH_4^+ was added, but *nifH* was expressed at low levels, suggesting growth was relying on NH_4^+ . *Anabaena* spp. and *C. raciborskii* expressed *nifH* genes when NO_3^- or NH_4^+ were present, but expression was higher with NO_3^- . The *narB* gene sequence was amplified from *Anabaena* spp. and *C. raciborskii* from the SJR, suggesting these taxa are capable of assimilating NO_3^- . However, even small NO_3^- additions blocked the growth of *Anabaena* spp. in the mixed phytoplankton community but not that of *C. raciborskii*. The results suggest that *C. raciborskii* in the SJR is a stronger competitor than *Anabaena* spp. when DIN is present.

The ability to compete and use different nitrogen (N) sources for growth is an important factor in determining the competitive success of individual taxonomic groups in mixed phytoplankton assemblages. Differences in nutrient uptake affinities or storage capabilities can lead to competitive exclusion in controlled laboratory conditions (Tilman 1982), and these models can be applied in nature (Sommer 1993). However, in complex natural phytoplankton assemblages, species composition is difficult to predict and model based on laboratory data, since many different taxa coexist and numerous environmental conditions simultaneously affect growth rates.

Based on laboratory experiments and field observations, under N-replete conditions competition between diazotrophic (N_2 -fixing) and nondiazotrophic cyanobacteria is generally thought to lead to competitive exclusion by nondiazotrophs since they tend to have higher affinities for

dissolved inorganic nitrogen (DIN) (Flores and Herrero 1994) and faster growth rates than diazotrophs. However, some cyanobacterial diazotrophs may be adapted for growth on NH_4^+ or NO_3^- and can effectively compete with nondiazotrophs during time periods when DIN is available (Agawin et al. 2007). In the eutrophic, subtropical St. Johns River (SJR) estuary in Florida, phytoplankton growth tends to be N limited in the summer, manifested as high abundances of diazotrophic cyanobacteria, but short-term and localized inputs of DIN occur (Phlips et al. 2007). Owing to the pulsed nature of the nutrient inputs, the system may locally shift between N-limited and non-N-limited conditions several times during the cyanobacterial growth season. Cyanobacteria that have the ability to fix atmospheric N_2 and effectively compete for combined N sources may have a competitive advantage under these conditions. Although it is known that diazotrophic cyanobacteria can use NH_4^+ and the ability to use NO_3^- is widespread (Flores et al. 2005), little is known about the roles these functions play in an ecological context. In general, NH_4^+ inhibits the function of nitrogenase, the enzyme mediating N_2 fixation, and the magnitude of this inhibition appears taxa specific (Stewart 1973).

In the SJR, *Anabaena* spp., and *Aphanizomenon flos-aquae* have historically dominated diazotroph communities, while *Microcystis* spp. and *Aphanocapsa* spp. are

Acknowledgments

S. Gordon, A. E. Morrison, J. Braddy, A. Joyner, J. Joyner, L. Kelly, K. Rossignol, M. Leonard, and L. A. Cheshire are acknowledged for technical assistance. B. Jenkins, R. Foster, and M. Piehler are acknowledged for discussions. This work was supported by the National Science Foundation (grant DEB-0452324) to P.M. and H.P., and the Gordon and Betty Moore Foundation (J.Z.).

common nondiazotrophic cyanobacteria in the system (Chapman and Schelske 1997). During the past 30 y *Cylindrospermopsis raciborskii*, a potentially toxic cyanobacterium, has appeared in the system and at times can form a significant proportion of diazotrophic cyanobacterial biomass (Phlips et al. 2007). *C. raciborskii* can potentially produce at least three toxins that include the hepatotoxins cylindrospermopsin (Ohtani et al. 1992) and deoxycylindrospermopsin (Norris et al. 1999), and neurotoxic paralytic shellfish poisons (Lagos et al. 1999), of which cylindrospermopsin has been detected in the SJR (Williams et al. 2001). After initial reports of blooms from tropical Australia (Hawkins et al. 1985) and the temperate Lake Balaton, Hungary (Présing et al. 1996), observations of *C. raciborskii* and its toxins have rapidly increased in temperate to tropical environments all over the world, including North America (Chapman and Schelske 1997; Hong et al. 2006), South America (Branco and Senna 1994; Figueredo et al. 2007), Europe (Briand et al. 2002; Fastner et al. 2007), and Africa (Bouvy et al. 2006; Dufour et al. 2006; Mohamed 2007). Several reasons for the success of *C. raciborskii* in eutrophying systems have been proposed, including high affinity for phosphate, preference for high temperatures and vertical stratification, and resistance to grazing (Padisák 1997; Wiedner et al. 2007). We hypothesized that the recent success of *C. raciborskii* in eutrophying freshwaters is promoted by its ability to rapidly shift between N_2 fixation and uptake and assimilation of combined DIN sources and its ability to effectively compete for DIN with nondiazotrophs. The change of N assimilation modes may occur rapidly, as DIN inputs to freshwaters are often episodic and short lived. We compared the competitive strength of native *Anabaena* spp. and *C. raciborskii* from the SJR under variable availabilities of DIN. We hypothesized that *C. raciborskii* can compete more effectively with nondiazotrophic communities than *Anabaena* spp. when DIN is available. This ability would allow *C. raciborskii* to take advantage of periodic DIN pulses and increase its ability to form blooms. To answer these questions, we developed tax-specific real-time quantitative polymerase chain reaction (PCR) probes for quantification of cyanobacterial *nifH* and *narB* genes and gene expression. These genes encode proteins that are central in nitrogen fixation (*nifH*) and assimilatory nitrate reduction (*narB*) of cyanobacteria.

Methods

Field experiment design—A 7-d field experiment was carried out during 14–22 May 2005. Surface water grab samples were collected with 20-liter polyethylene carboys at three stations along the SJR. The coordinates for the three stations were (1) Lake George (Sta. 1), 29.2974°N 81.6417°W (most freshwater station); (2) SJR Sta. 2, 29.3860°N 81.6400°W (St. Johns River, middle station); and (3) SJR Sta. 3, 29.6383°N 81.6212°W (St. Johns River, most estuarine and northernmost station). Water containers were transported from these sites to the incubation site in Palatka, Florida. The sample water was immediately aliquoted to 60 HCl (0.1 mol L⁻¹) washed polyethylene

Cubitainers that served as incubation containers (Hedwin). The Cubitainers are 85% transparent to photosynthetically active irradiance (Paerl et al. 1987). Each station had five treatments, tested as quadruplicate: (1) no nutrients added, (2) single NO_3^- addition (day 0) (20 μ mol L⁻¹ final concentration), (3) repeated NO_3^- additions (400 μ L of 0.2 mol L⁻¹ stock solution) added each day on days 0–6, (4) single NH_4^+ addition (day 0) (20 μ mol L⁻¹ final concentration), or (5) repeated NH_4^+ additions done each day on days 0–6 (400 μ L of 0.2 mol L⁻¹ stock solution). The daily nutrient additions on treatments (3) and (5) were done immediately after subsampling for the daily measurements from the Cubitainers. Additionally, dissolved inorganic carbon (DIC) was replenished daily to all Cubitainers by adding 1 mL of 7.25 g L⁻¹ stock solution, to eliminate potential limitations. The Cubitainers were incubated for 7 d in situ immersed in the river in custom built corrals and covered with screening to alleviate photoinhibition. The screen attenuates 39% of photosynthetically active irradiation. Approximately 800-mL subsamples were taken daily from the Cubitainers for various measurements. The subsampling was carried out at the same time every day, starting around 0730 h. Subsamples were collected for detection of chlorophyll *a* (Chl *a*), N_2 -fixation activity (acetylene reduction assay), nutrients ($NO_3^- + NO_2^-$, NH_4^+ , PO_4^{3-} , total N), DIC, phytoplankton identification and counts, and deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) analyses. Samples for nucleic acids and dissolved inorganic nutrients were collected on days 1, 3, 5, and 7, and for phytoplankton community microscopy and total N on day 7, while the rest of the measurements were made daily.

For DNA and RNA, from 50- to 75-mL water samples were filtered through 3- μ m pore-size polyester filters (Osmonics). The filters were placed in bead beater tubes (BioSpec) with approximately 0.2-g sterile glass beads. The RNA sample tubes additionally contained 1 mL RNawiz solution (Ambion) to stabilize RNA during storage. Both DNA and RNA tubes were immediately frozen in liquid nitrogen. The DNA and RNA samples were shipped to the University of California, Santa Cruz, on dry ice and kept at -80°C until analysis. For Chl *a*, 50 mL of water was filtered through GF/F glass fiber filters (Whatman), and the filtrate was saved for nutrient analyses and stored at -20°C . The GF/F filters were wrapped in aluminum foil and stored at -20°C until Chl *a* was assayed. The acetylene reduction assay (ARA) was used to estimate N_2 -fixation rates. For ARA, 100-mL samples were placed in 120-mL serum vials and capped with rubber sleeve stoppers. Acetylene was generated in the field by adding deionized water to calcium carbide granules (Fisher). Acetylene (10 mL) was added to the serum vials; the vials were shaken by hand for 10–20 s and incubated in situ in the river shaded by one layer of screening ($\sim 30\%$ reduction in incident radiation). After 4-h incubation, the vials were vigorously shaken to equilibrate ethylene between water and gaseous phases, and headspace gas withdrawn and stored in evacuated Vacutainer tubes (Becton-Dickinson). To avoid potential trace ethylene contamination, Vacutainers were opened, aerated overnight, recapped, and

manually reevacuated using a 60-mL syringe prior to use. For the ARA measurement in the culture experiment, 8 mL of culture was incubated for 2 h in the culture incubators in 13-mL Exetainer tubes with 2 mL of acetylene, and 2 mL of gas was subsampled to Vacutainers at the end for determination of ethylene. For determination of DIC, 20-mL glass scintillation vials were filled without an air phase and refrigerated until analysis. For determination of the phytoplankton composition, 125 mL of sample was preserved with Lugol's solution and kept in the dark at room temperature.

Cylindrospermopsis culture experiment—Growth rates and N₂-fixation potential in *C. raciborskii* under different N conditions were studied with culture experiments. A *C. raciborskii* isolate strain CyIL from Lake Griffin (located in central Florida) was grown in Z8 medium (Rippka 1988) with the DIN source replaced with four alternate N sources, each in duplicate 2-liter culture flasks. The cultures were grown for 5 d under constant aeration (air filtered through a 0.2- μ m filter) at 27°C with a 15:9 h light:dark cycle. The N treatments were (1) no combined N, (2) urea, (3) NH₄Cl, and (4) NaNO₃. All N additions were 550 μ mol L⁻¹ final concentration in the culture flasks at the start of the experiment. Cultures were preacclimated to the various N treatments for four growth cycles prior to the experiment (growth until late exponential or stationary phase in each growth cycle). Experimental cultures were inoculated to similar initial Chl *a* concentrations from preacclimated cultures and determined by in vivo fluorescence using a Turner TD-700 fluorometer with narrow band-pass filters for Chl *a* (Welschmeyer 1994). Growth was assayed by Chl *a*, which was collected by filtering from 2 to 8 mL of culture through GF/F filters that were frozen at -20°C for later extraction (*see below*). Subsamples were preserved in Lugol's solution for determination of heterocyst frequencies. A mean of 75 filaments from each culture was calculated, and average and standard deviation between duplicate cultures are reported. Nitrogen fixation (nitrogenase activity) was measured using the acetylene reduction assay (*see above and below*).

Analytical methods—Chl *a* was extracted overnight at -20°C using 90:10 acetone:water mix and the concentration was determined by narrow band-pass fluorometry (Welschmeyer 1994) using a Turner TD-700 fluorometer. For estimation of N₂-fixation (nitrogenase activity), we employed the acetylene reduction assay (Paerl 1998). Acetylene conversion to ethylene was measured using a Shimadzu GC-9A gas chromatograph equipped with a flame ionization detector and a Poropak T stainless steel column at 80°C, using N₂ as a carrier gas. The gas chromatograph was calibrated daily using a standard curve made with ethylene (National Welders). Values for vials incubated with acetylene and deionized water ("blanks") were subtracted from the sample values. Concentrations of DIC were determined using a Shimadzu TOC-5000A analyzer. Nutrient analyses were carried out using a Lachat autoanalyzer (Lachat Instruments). Nutrients were measured using standard methods 4500-NO₃ I, 4500-NH₃ H,

4500-P G, and 4500-N B (Clesceri et al. 1999). Reporting limits for NO_x-N, NH₄⁺-N, PO₄³⁻-P, and total N were 0.26, 0.31, 0.05, and 5.57 μ mol L⁻¹, respectively.

DNA was extracted using the method described by Tillett and Neilan (2000) and the extract was purified using a Plant Minikit (Qiagen). Seven hundred and fifty milliliter xanthogenate buffer (1% potassium ethyl xanthogenate; 100 mmol L⁻¹ Tris-HCl, pH 7.4; 1 mmol L⁻¹ ethylenediaminetetraacetic acid [EDTA], pH 8.0; 1% sodium dodecylsulfate; 800 mmol L⁻¹ ammonium acetate) was added to the filter in a bead beater tube, followed by vortexing and incubation at 70°C for 20 min. Tubes were agitated in a beadbeater (Mini Beadbeater, Biospec) for 3 min and returned to 70°C for 1 h. These two steps were repeated, followed by bead beating for 3 min. The tubes were kept on ice for 30 min, filters were removed using sterile needles, and the tubes spun at 4°C for 10 min at 14,000 \times g. The supernatant was pipetted into new tubes, followed by isopropanol precipitation (750 μ L) for 10 min at room temperature. The tubes were spun for 10 min at 4°C at 14,000 \times g and washed once with 70% ethanol (1 mL). The pellet was dried and dissolved in TE (Tris-EDTA) at 70°C. After the pellet was completely dissolved, the extraction was continued with Qiagen Plant Minikit (Qiagen) purification, following the kit instructions. The final elution volume was 100 μ L of AE buffer, provided in the Plant Minikit.

RNA was extracted using the RNawiz extraction protocol (Ambion) followed by RNeasy Minikit cleaning (Qiagen). First, the tubes with beads and RNawiz were thawed and agitated in the bead beater twice for 2 min, with 1-min cooling period on ice. Filters were removed using sterile needles, and 200 μ L of chloroform was added. The samples were shaken vigorously, incubated for 10 min at room temperature, and then centrifuged at 10,000 \times g for 15 min at 4°C. The colorless aqueous phase was collected into a new tube, and 500 μ L of RNase-free water was added. The sample was divided into two 2-mL tubes, and 500 μ L of isopropanol was mixed with each. Samples were incubated for 10 min at room temperature, and the RNA was pelleted by centrifugation at 10,000 \times g for 15 min at 4°C. The pellet was washed with 1 mL ice cold 75% ethanol and dried for 1 h in a fume hood. Pellets were resuspended to 50 μ L of nuclease-free water, and the contents of the two tubes were combined. The extract was purified with the RNeasy kit (Qiagen). RLT buffer (350 μ L) from the kit was added to the RNA extract, and the rest of the purification was completed through spin columns following the instructions of the manufacturer. An on-column DNase step was included with a 1-h incubation. Final RNA elution volume was 50 μ L, and the extracts were stored at -80°C.

Cyanobacterial cultures isolated from the study area and elsewhere were included in phylogenetic analyses with *nifH* and *narB* genes and in specificity tests for the TaqMan assay. Isolates used in specificity tests are listed in Table 1. For DNA extraction from cultures, 1.8 mL of culture was spun down to a pellet, pellets were frozen at -20°C, and DNA was extracted as described above for the environmental samples. Amplification, cloning, and sequencing of

Table 1. Isolates included in the study. Results of qPCR probe and primer specificity tests are shown. + target detected; – target not detected. FL = Florida, NC = North Carolina.

ID	Species (strain)	Source	Isolation year	<i>nifH</i> Probe		
				7103A	7106A	7111A
7100	unidentified heterocystous LD_B	Lake Dora, FL	1999	–	–	–
7101	<i>Anabaena aphanizomenoides</i> M17	Neuse River, NC	1998	–	–	–
7102	<i>Anabaena ambigua</i> LH_M	Lake Harris, FL	1999	–	–	–
7103	<i>Anabaena</i> sp. LG1	Lake George, FL	2003	+	–	–
7104	<i>Anabaena</i> sp. LG2	Lake George, FL	2003	–	–	–
7105	<i>Anabaena</i> sp. PO1	St Johns River Sta. 2, FL	2003	+	+	–
7106	<i>Anabaena</i> sp. PA1	St Johns River Sta. 3, FL	2003	–	+	–
7107	<i>Anabaena</i> sp. BC1	Bath Creek, NC	2002	–	–	–
7108	<i>Anabaenopsis</i> sp. NRE1	Neuse River, NC	1998	–	–	–
7109	<i>C. raciborskii</i> CylD	Lake Dora, FL	1999	–	–	+
7110	<i>C. raciborskii</i> CylE	Lake Dora, FL	1999	–	–	+
7111	<i>C. raciborskii</i> CylF	Lake Dora, FL	1999	–	–	+
7112	<i>C. raciborskii</i> CylG	Lake Dora, FL	1999	–	–	+
7113	<i>C. raciborskii</i> CylI	Lake Dora, FL	1999	–	–	+
7114	<i>C. raciborskii</i> CylL	Lake Griffin, FL	2000	–	–	+
7115	<i>Nodularia sphaerocarpa</i> UP16a	Baltic Sea	1994	–	–	–
7116	<i>Nodularia sphaerocarpa</i> UP16f	Baltic Sea	1994	–	–	–
7117	<i>Nodularia spumigena</i> Nod31	Baltic Sea	1994	–	–	–
7118	<i>Nodularia spumigena</i> FL2f	Baltic Sea	1994	–	–	–
7119	<i>Nodularia spumigena</i> Nod02_1	Baltic Sea	2002	–	–	–
7127	mixed, <i>Aphanizomenon</i> dominant LG3	Lake George, FL	2005	–	–	–
7128	mixed, <i>Anabaena</i> + <i>Aphanizomenon</i> LG4	Lake George, FL	2005	+	–	–
7129	mixed, nonheterocystous dominant SJR1	SJR, FL	2005	+	–	–
7130	mixed, <i>Anabaena</i> coiled dominant SJR2	SJR, FL	2005	–	–	–
7131	mixed, <i>Anabaena</i> straight dominant LG5	Lake George, FL	2005	–	–	–
7132	mixed, nonheterocystous dominant SJR3	SJR, FL	2005	–	–	–
7133	mixed, nonheterocystous LG6	Lake George, FL	2005	–	–	–
7134	<i>Cylindrospermopsis</i> sp.	the Netherlands	Unknown	–	–	+

nifH and *narB* genes for the isolates were carried out following the same protocols as for the environmental samples (see below). Two microliters of each culture extract were used in qPCR probe and primer specificity tests.

DNA and RNA were quantified using the PicoGreen and RiboGreen quantification methods, respectively (Invitrogen, Molecular Probes). cDNA was made using the Superscript III cDNA kit (Invitrogen), following the protocol from the manufacturer. RNA concentrations were adjusted to be equivalent from all treatments prior to cDNA synthesis, but concentrations varied between sampling sites (4.5–7.8 ng RNA μL^{-1}). The cDNA synthesis reaction was started by incubating 8 μL of the RNA extract with 1 μL of dNTPs (deoxyribonucleotide triphosphate) (stock at 10 mmol L^{-1}) and 1 μL of the inner reverse *nifH* primer (*nifH2*) (stock at 10 $\mu\text{mol L}^{-1}$) for 5 min at 65°C, then chilled on ice. The following were then added: 2 μL of 10 \times buffer, 4 μL of MgCl (stock of 25 mmol L^{-1}), 2 μL of DTT (dithiothreitol) (stock at 0.1 mol L^{-1}), 1 μL of “Rnase out,” and 1 μL of Superscript III reverse transcriptase (RT). Parallel “no RT” treatments were run for all samples with the reverse transcriptase replaced with water. The mixture was incubated for 50 min at 50°C, chilled on ice, then collected by brief centrifugation. One μL Rnase H was added, the mix was incubated for 20 min at 37°C, then stored at –20°C.

The *nifH* and *narB* genes were amplified from the DNA samples, and clone libraries were generated. In addition,

nifH and *narB* genes were amplified and sequenced from cyanobacterial culture isolates (unialgal and mixed) from the SJR, the surrounding lakes, and elsewhere (Table 1). Nested PCR was carried out for amplification of *nifH* as follows (Zehr and Turner 2001). For the first nested reaction, final concentrations of 2.5 mmol L^{-1} MgCl₂, 200 $\mu\text{mol L}^{-1}$ dNTPs (each nucleotide), 1 $\mu\text{mol L}^{-1}$ primers *nifH3* (reverse) and *nifH4* (forward), and 2 U Taq Polymerase (Promega) were included in the reactions. The final volume was adjusted to 50 μL with 5-kDa–filtered nuclease-free water. Two microliters of template DNA was added to each reaction. The first round of amplification was performed with an initial 2 min 94°C denaturation step, followed by 25 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C. The final extension step was for 7 min at 72°C. One microliter of the first round reaction product was used as a template in the second round nested reaction. The PCR mix and conditions in the first and second round reactions were identical, except inner nested primers *nifH1* (forward) and *nifH2* (reverse) (Zehr and Turner 2001) were used in the second round reaction. The *narB* PCR amplification mix had 5 μL of template DNA, MgCl at 3 mmol L^{-1} , dNTPs at 200 $\mu\text{mol L}^{-1}$, forward (ACNG-GYCARCCYAA YGCNATG) and reverse (CKHCKYTCNSWRRTTNGTCAT) *narB* primers (Bird and Wyman 2003) at 0.25 $\mu\text{mol L}^{-1}$, and 2 U Taq polymerase (Promega) with a final volume adjusted to

50 µL with 5-kDa-filtered nuclease-free water. The PCR products were separated on a 1.2% Tris-acetate-EDTA agarose electrophoresis gel, and bands were excised and purified using the Qiagen gel purification kit. The cleaned products were cloned into a pGEM-T vector (Promega), and plasmid DNA was purified using the Montage miniprep kit (Millipore) in a 96-well plate format. Sequencing was performed at the University of California Berkeley sequencing facility (ABI 3730 using 96°C for 1 min, then 25 sequencing cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min using BigDye v.3.1). Most sequences were read to both directions. Sequences were trimmed using the GCG software (Accelrys) and imported to *nifH* and *narB* ARB databases (Ludwig et al. 2004) aligned using a Pfam (<http://www.sanger.ac.uk/Software/Pfam/>) seed alignment and a Hidden Markov Model. The sequences obtained in this study were aligned to the ARB databases using the fast align feature in ARB. If the same sequence was obtained multiple times only a singleton was included in the tree and reported to GenBank.

qPCR probe design and real-time PCR—Primers and probes for the TaqMan 5' nuclease assay were designed for three *nifH* and one *narB* sequence based on sequence data from cultures and samples collected from the field sites during the experiment (Table 2, Figs. 1, 2). For *nifH*, probes for two *Anabaena* spp. sequences (probes 7103A03 and 7106A02) and one *C. raciborskii* strain (probe 7111A01) were designed. A *narB* probe was designed that represented a dominant *narB* sequence type (clone 13106_122Y07) recovered from the system during the experiment. This *narB* sequence clustered with nonheterocystous unicellular cyanobacteria. The primer and probe sets were designed using the Primer Express software (Applied Biosystems). The specificities of the *nifH* primer and probe sets were investigated using the *nifH* database in an ARB database constructed with approximately 8800 *nifH* sequences from GenBank. The primer-probe sets did not match known nonspecific targets from the database with fewer than six mismatches in the primers and probes combined. A *narB* ARB database constructed with sequences from GenBank included 170 sequences covering the region of the amplicon. The 13106_122Y07 probe and primer specificity was checked against the *narB* database and using the culture extracts (Table 1). The *narB* primers and probe detected all sequences in the cluster but did not have significant matches with other sequences in the database. The sequences in the *narB* database always had six or more mismatches with at least one of the primers or the probe, suggesting amplification of nontargets would be highly unlikely. The primers and probes were synthesized at Sigma-Genosys, and the probes were modified with FAM (5') fluorescent reporter and TAMRA (3') quencher. Specificity of each *nifH* and *narB* primer and probe set was tested using 28 cyanobacterial culture extracts from unicyanobacterial and mixed cultures (Table 1). Additional specificity tests were carried out using plasmid preparations of the other probe sets as targets. A dilution series of 10⁻¹ to 10⁻¹¹ of each target sequence was tested with the mismatch primer and probe sets.

Table 2. Quantitative PCR primers and probes developed and applied in the study.

Name	Gene	Culture	Forward primer	Reverse primer	Probe
7103A02	<i>nifH</i>	<i>Anabaena</i> sp.	CCAAGGCTCAAACCACCGTAT	TTCGTGGAGTTCCAATCTTCA	ACACTGGGTGCTGAAAGAGGGGC
7106A03	<i>nifH</i>	<i>Anabaena</i> sp.	TGCTTCACTCCAAGGCTCAA	ACGTCGGGAACCTTTG	CAACCGTCTTCACTTGGCTGTGAAA
7111A01	<i>nifH</i>	<i>C. raciborskii</i>	CCGTTTGATGCTGCACCTCTAAA	GAATCCGGTCAGCATTACTTCTTC	TCAAACCTACCGTATTGCACCTTGGCTGCTG
13106_122Y07	<i>narB</i>	uncultivated	CGGTTATCGTTCCCGTCAAAA	CAGGTAAACCCCAAGCTTGTTTC	CCCCAACACCGGGCCGAAAGT

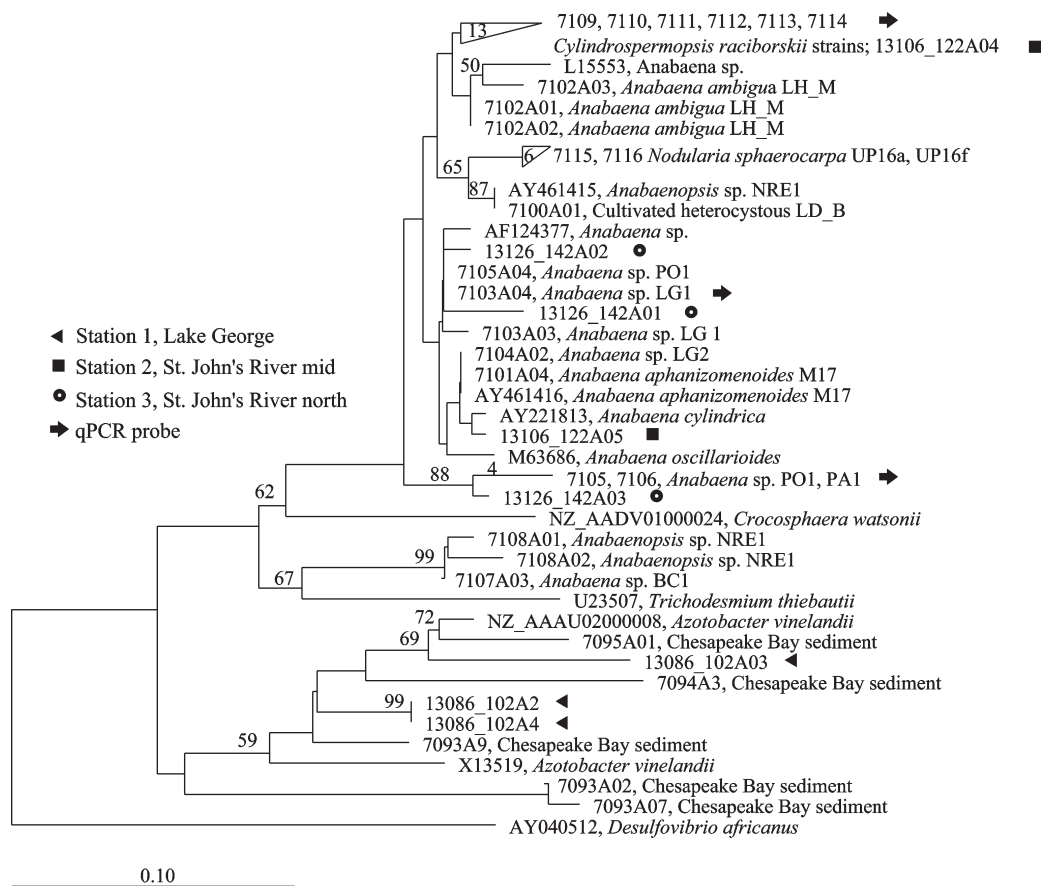


Fig. 1. A neighbor-joining tree of *nifH* amino acid sequences from cyanobacterial isolates and environmental samples. Bootstrap values >50 are shown for 1000 replicates. 7100 to 7116 are cultures sequenced in this study. Sequences to which qPCR probe and primer sets were designed are marked with an arrow. Numbers of sequences in collapsed clusters are indicated. Only unique sequence types are included in the tree.

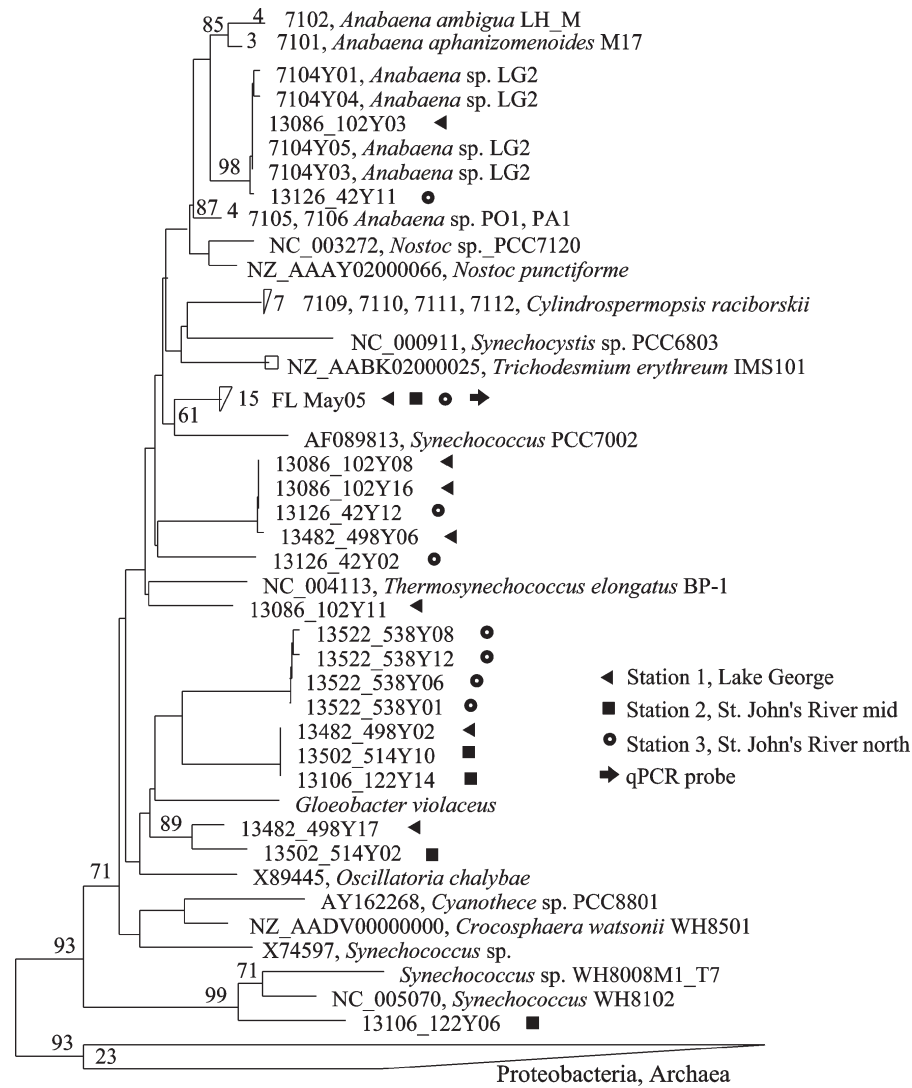
For qPCR, the DNA samples were quantified using the PicoGreen method (Invitrogen), using the Applied Biosystems 7500 real-time PCR system plus/minus assay. Concentration of each DNA sample was adjusted to $0.5 \text{ ng DNA } \mu\text{L}^{-1}$, and $4 \mu\text{L}$ was added to each reaction. In prior studies, 2 ng template DNA produced an optimal signal with the least amount of inhibition (Short and Zehr 2005). For qRT-PCR, $2 \mu\text{L}$ of cDNA was added to each reaction. The reaction mix consisted of $12.5 \mu\text{L}$ Taqman Ready mix containing the buffers, Taq polymerase (AmpliTaq Gold), and AmpErase UNG. Reverse and forward primers ($0.4 \mu\text{mol L}^{-1}$ final concentration) and the TaqMan probe ($0.2 \mu\text{mol L}^{-1}$ final concentration) were added. The final reaction volume was adjusted to $25 \mu\text{L}$ with 5 kDa -filtered nuclease-free water. The analysis was done in an Applied Biosystems 7500 real-time PCR system using the following cycling conditions: initial 2 min at 50°C , then 40 cycles of 15 s at 95°C and 1 min at 60°C . A dilution series comprised of 10^{-1} to 10^{-8} dilutions of linearized plasmids containing inserts of the probe sequences were used as qPCR standards and were included in each run. The number of gene copies per sample was calculated based on the standard curves as described in Short and Zehr (2005). Each treatment from each sampling site was sampled four times during the experiment for qPCR and RT-qPCR. The

“no RT” reactions were analyzed with qPCR in parallel with the RT-qPCR samples. None of the “no RT” reactions amplified in the RT-qPCR. GenBank accession numbers for sequences obtained in this study are EU381318–EU381419.

Statistical analyses—Differences between treatments were tested using a repeated measures ANOVA (RMANOVA) for Chl *a* and N_2 fixation, and one-way ANOVA for gene copy and transcript abundances, with least significant difference (LSD) tests for pairwise comparisons (SPSS 12.0 for Windows). The data were $\log(1 + \text{value})$ transformed if necessary to improve normality and homoscedasticity.

Results

Dissolved inorganic nitrogen concentrations at the beginning of the study were similar at all stations, while phosphate was more variable among stations with lowest concentration at Sta. 2 (Table 3). $\text{NO}_3^- + \text{NO}_2^-$ concentrations were at or below the detection limit of $0.26 \mu\text{mol L}^{-1}$ at all stations, while NH_4^+ was nearly always detectable. Nitrogen concentration trends during the experiment were similar at all three sampling stations.



0.10

Fig. 2. A neighbor-joining tree of *narB* amino acid sequences from cyanobacterial isolates and environmental samples. Bootstrap values >50 are shown for 1000 replicates. 7101 to 7112 are cultures sequenced in this study. Sequence cluster to which a qPCR probe and primer set was designed is marked with an arrow. Numbers of sequences in collapsed clusters are indicated. Only unique sequence types are included in the tree.

In treatments with a single addition of NO_3^- or NH_4^+ , the nutrient pulse was depleted by the third (Lake George) or fifth day (SJR stations) of the experiment. In treatments where DIN addition was repeated every day, DIN accumulated during the experiment (Fig. 3). Phosphate concentrations were reduced during the experiment but remained greater than $0.14 \mu\text{mol L}^{-1}$ through the experiment at all stations and treatments.

Nitrogen limitation of phytoplankton growth was evident from the Chl *a* data (Fig. 4). Chl *a* concentrations increased in all treatments that received DIN additions, with no apparent initial differences resulting from the addition of different N sources. In Sta. 1 and Sta. 3 waters, starting from the fifth or fourth experimental day, respectively, treatments with a single DIN addition had a

reduced Chl *a* concentration compared with those treatments with DIN added every day, whether it was in the form of NH_4^+ or NO_3^- (Fig. 4, Table 4, RMANOVA). Chl *a* under repeated additions of NH_4^+ was significantly

Table 3. Nutrient concentrations ($\mu\text{mol L}^{-1}$) at the beginning of the study (average of four replicate measurements \pm SD).

Station	NO_3^- -N	NH_4^+ -N	PO_4^{3-} -P	TN
Lake George, Sta. 1	BD*	2.69 ± 0.41	0.33 ± 0.01	45 ± 10
St. Johns River, Sta. 2	BD*	2.33 ± 0.50	0.22 ± 0.00	39 ± 9
St. Johns River, Sta. 3	BD*	2.39 ± 0.38	0.48 ± 0.03	38 ± 6

* All or some of the replicate measurements were below the reporting limit of $0.26 \mu\text{mol L}^{-1}$.

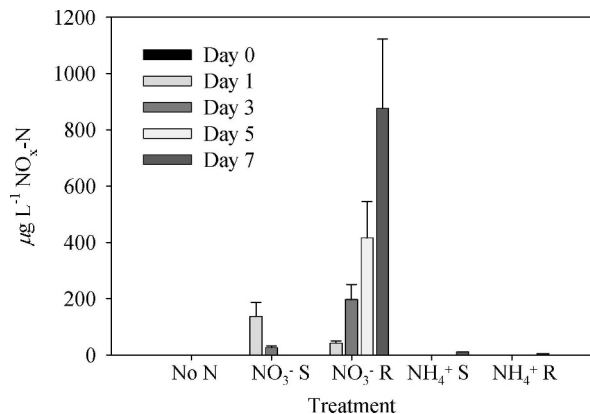


Fig. 3. Typical trends of inorganic nitrogen concentrations in experimental containers. Nitrate + nitrite ($\text{NO}_x\text{-N}$) concentrations in the Lake George experiment are shown. Means and standard deviations of four Cubitainers are shown. NO_3^- S, single NO_3^- addition; NO_3^- R, repeated NO_3^- additions; NH_4^+ S, single NH_4^+ addition; NH_4^+ R, repeated NH_4^+ additions.

different than that in other treatments at these stations, reaching the highest Chl *a* concentration at the end of the experiment. Chl *a* increased in the no N treatment as well but more gradually than in other treatments, and was significantly lower (RMANOVA).

Nitrogen fixation rates increased rapidly in treatments with no N additions, while the rates were low when DIN was added (Fig. 5, Table 4). At Stas. 2 and 3, N_2 -fixation rates were higher on day 7 in treatments that received a single DIN addition compared with those that received DIN every day. Based on RMANOVA that included all sampling points between days 2–7, N_2 -fixation rates in repeated and single DIN additions were different at Stas. 2 and 3 (Table 4). Lake George water had the highest N_2 -fixation rates, while rates were the lowest at the SJR Sta. 2. Dissolved inorganic carbon concentrations were from 12 to 13.8 mg C L⁻¹ at the beginning of the experiment and remained >10 mg C L⁻¹ throughout the experiment in all treatments and stations.

The *nifH* gene was successfully amplified from the cyanobacterial strains (Fig. 1), and a number of *nifH* sequences were obtained directly from the experimental water. Cultures LG1, PO1, and PA1 were isolated from Lake George and the SJR in 2003 (Table 1). Environmental sequences obtained from the experimental water samples clustered closely with sequences from these isolates (Fig. 1). In the *nifH* neighbor-joining tree, *C. raciborskii* formed its own clade, while different *Anabaena* spp. clustered separately. Based on the *nifH* sequence analysis, three sequence types were selected for development of quantitative PCR probes (Fig. 1). Two *Anabaena* spp. *nifH* sequence types were chosen, representing the cyanobacterial strain LG1 from Lake George (clone 7103A03) and strain PA1 from SJR Sta. 3 (clone 7106A02). Two *nifH* sequence types were recovered from strain PO1: one that clusters with strain PA1 and a second one that clusters with strain LG1 (Fig. 4). Presence of both sequence types in this culture was verified by quantitative PCR (see below). Microscopic observations showed that strain LG1 was a

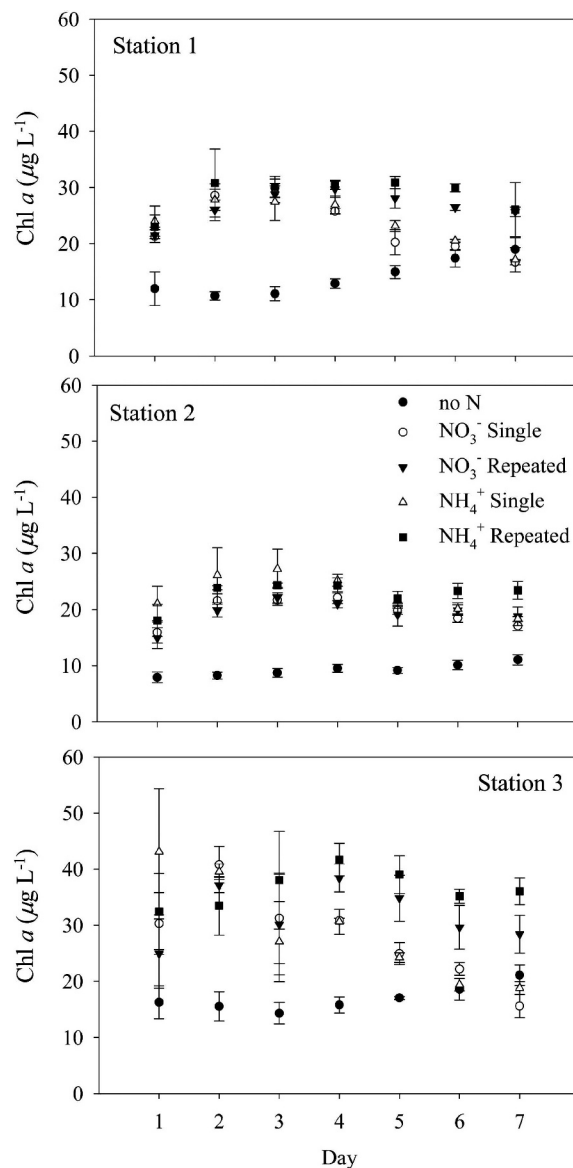


Fig. 4. Chl *a* concentration during the nutrient enrichment experiment. Means and standard deviations of four Cubitainers per treatment and time point are shown.

coiled morphotype *Anabaena* sp., while strain PA1 was a straight morphotype of *Anabaena* sp. Based on microscopic observations, both morphotypes were present at high abundances in the experimental water (data not shown). Neither of these sequence types is an exact match to previously sequenced *Anabaena* spp. The closest cultivated representative for the *nifH* sequence from strain PA1 was *Aphanizomenon* sp. from the Baltic Sea at a 96% nucleotide identity and *Anabaena sphaerica* for strain LG1 at a 94% nucleotide identity. The closest uncultivated representative for the strain LG1 was an environmental sequence from the Chesapeake Bay (Short and Zehr 2007) with a 99% nucleotide identity. Additional environmental sequences from the experimental water clustered with a third *Anabaena* sp. type (strain LG2, sequences starting with 7104A) and γ -Proteobacteria.

Table 4. Differences in nitrogen fixation and Chl *a* between treatments, based on a repeated measures ANOVA, tested separately for each station. Treatments with different letters were different based on LSD post hoc tests ($p \leq 0.05$). Data from four replicates per treatment were included from seven sampling days (day 1–day 7) for Chl *a*, and six sampling days (day 2–day 7) for acetylene reduction. NO₃⁻S, single nitrate addition; NO₃⁻R, daily nitrate addition; NH₄⁺S, single ammonium addition; NH₄⁺R, daily ammonium addition.

	<i>p</i>	No N	NO ₃ ⁻ S	NO ₃ ⁻ R	NH ₄ ⁺ S	NH ₄ ⁺ R
Chl <i>a</i>						
Sta. 1	0.000	a	b	c	b	d
Sta. 2	0.000	a	b	b	c	c
Sta. 3	0.000	a	b	c	bc	d
Acetylene reduction (N ₂ fixation)						
Sta. 1	0.000	a	b	bc	c	c
Sta. 2	0.000	a	b	c	c	d
Sta. 3	0.000	a	b	cd	c	d

The *narB* gene was successfully amplified from several of the cyanobacterial strains (Fig. 2). Sequences from heterocystous diazotrophs clustered together and diazotrophic and nondiazotrophic cyanobacteria formed separate clusters. Sequences from *C. raciborskii* clustered separately from the *Anabaena* spp. sequences. From the experimental water samples, a *narB* sequence type was recovered at a high frequency that clustered most closely with sequences from nondiazotrophic cyanobacteria (*Synechococcus* sp. and *Thermosynechococcus* sp.). A quantitative PCR probe was designed for this sequence type (Fig. 2).

Specificity tests for quantitative PCR *nifH* probe and primer sets with exact match and mismatch DNA extracts showed that the primer-probe sets designed for the heterocystous cyanobacteria selectively detected their target organisms (Table 1). Two targets (7106A02 and 7103A03) were detected in the culture PO1. The two sequence types were detected from this culture by both *nifH* cloning and sequencing, and qPCR (Table 1, Fig. 1). The *C. raciborskii*-specific *nifH* probe and primer set (clone 7111A01) detected *nifH* in all six *C. raciborskii* strains isolated from the system as well as from a *C. raciborskii* strain from the Netherlands (Table 1), but targets did not amplify from any other cultures. In each case where plasmids were used in specificity tests, targets with a few mismatches were also amplified. However, 2–3 orders of magnitude fewer target copies were detected using mismatch plasmids as targets than with exact match targets. The low amplification efficiency from mismatch target plasmids and the fact that *nifH* from nontarget cultures were not detected suggested that cross-hybridization did not affect the results.

Anabaena spp. coiled morphotypes dominated at Stas. 1 and 2 while the straight morphotype dominated at Sta. 3 (Fig. 6). Lake George had the highest *nifH* gene abundances if the abundances of the three diazotrophs were combined, while abundances were lowest at the “midriver” Sta. 2. *Cylindrospermopsis raciborskii* formed a minor proportion of the diazotroph community compared with the two *Anabaena* spp. Abundances of both of the two *Anabaena*

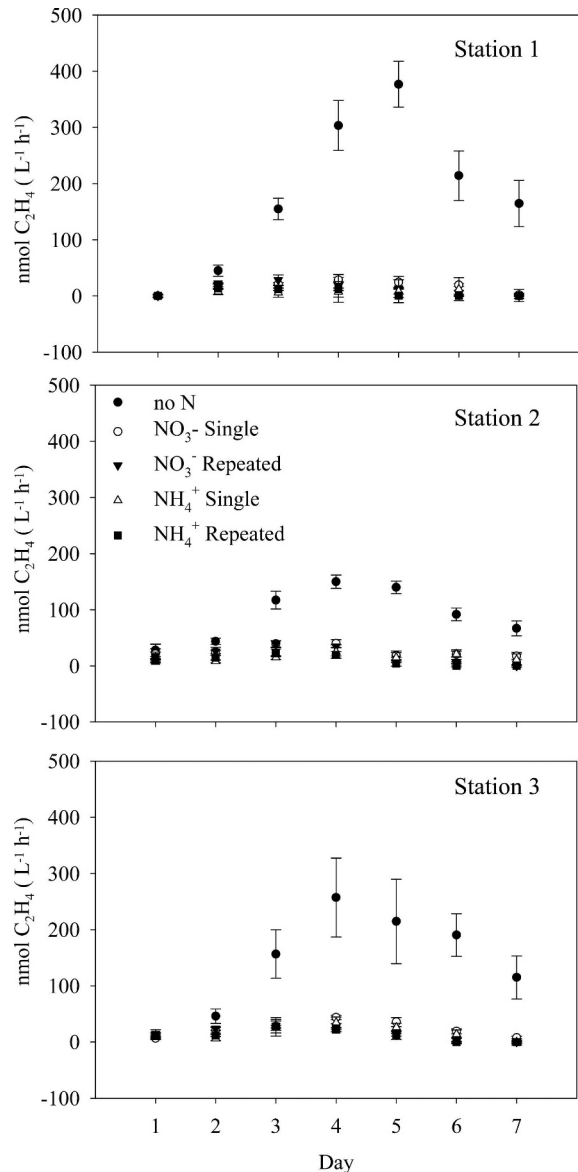


Fig. 5. Nitrogen fixation rates (acetylene reduction) during the nutrient enrichment experiment. Means and standard deviations of four Cubitainers per treatment and time point are shown.

spp. increased in the “no N” treatment at all stations (Fig. 6), while *nifH* gene abundances were lower in the treatments with DIN added (one-way ANOVA). The *nifH* gene abundances generally decreased from day 5 to day 7. Likewise, *C. raciborskii* *nifH* genes appeared to be most abundant in the no N treatment at Stas. 1 and 2, but abundances increased also in the NO₃⁻ and NH₄⁺ additions. The *C. raciborskii* *nifH* gene abundances were not significantly different among treatments (one-way ANOVA).

In the qRT-PCR, *Anabaena* spp. gene expression levels paralleled the abundances of gene copies (Fig. 7). In all cyanobacteria, *nifH* expression was at times present at higher levels in the NO₃⁻ treatments than in the NH₄⁺ treatments. Particularly high *nifH* expression levels with NO₃⁻ were found for *C. raciborskii*. Expression of *nifH* in *C. raciborskii* was very low in the NH₄⁺ treatment at Sta. 3,

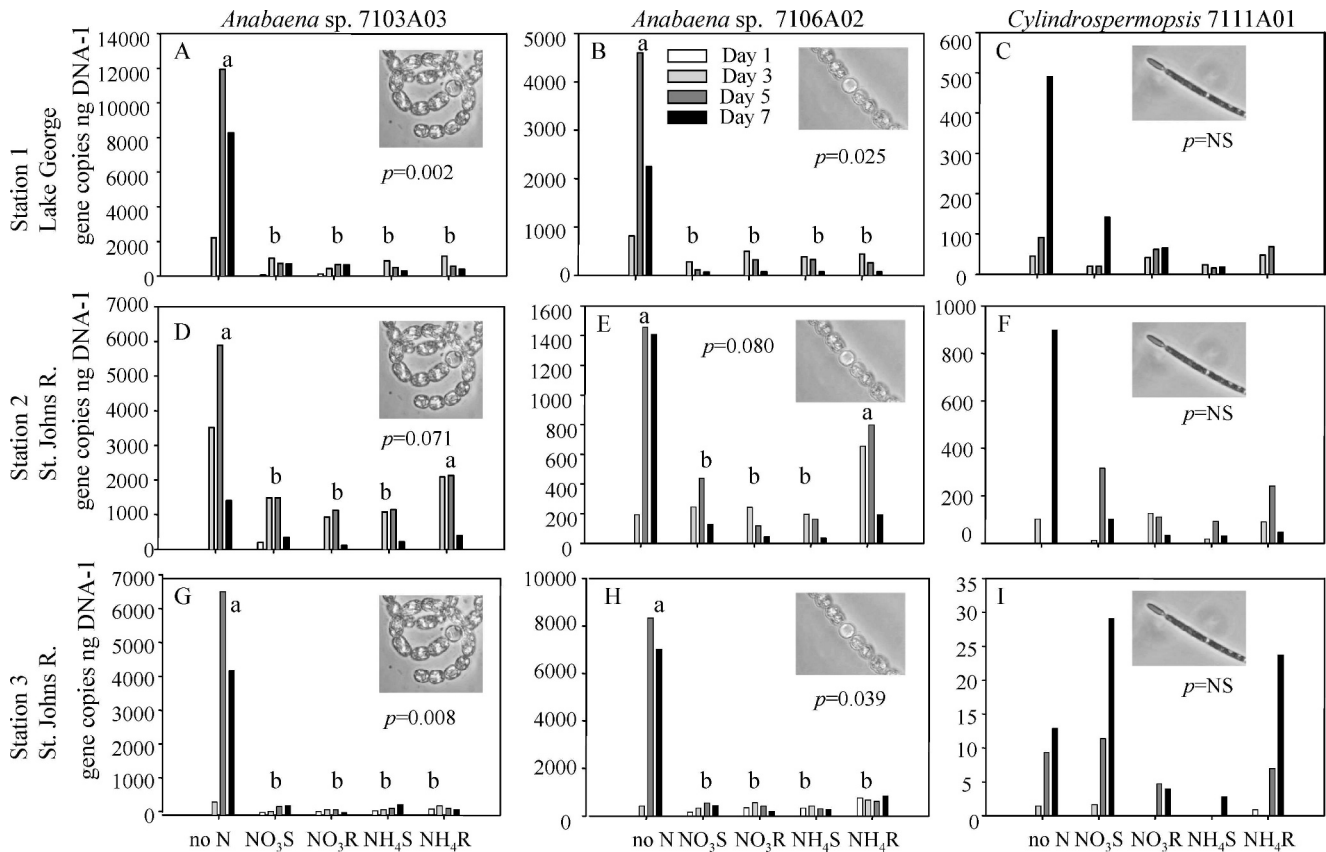


Fig. 6. Abundance of cyanobacterial *nifH* genes in water samples determined by qPCR (gene copies ng DNA⁻¹). (A–C) Lake George; (D–F) St. Johns River Sta. 2; (G–I) St. Johns River Sta. 3. A, D, G, *Anabaena* sp. probe 7103A03 (coiled filament morphotype); B, E, H, *Anabaena* sp. probe 7106A02 (straight filament morphotype); C, F, I, *C. raciborskii* probe 7111A01. NO₃S, single NO₃⁻ addition; NO₃R, repeated NO₃⁻ additions; NH₄S, single NH₄⁺ addition; NH₄R, repeated NH₄⁺ additions. Different letters indicate significant differences (one-way ANOVA, LSD pairwise comparisons).

while *nifH* gene abundance in DNA in this treatment increased, reflecting cell abundances of *C. raciborskii*.

The abundance of the *narB* gene in the uncultivated, potentially unicellular nondiazotrophic cyanobacterium (Fig. 2), had the greatest gene abundances of all targets quantified by qPCR (Fig. 8). The greatest abundances were detected at the SJR Sta. 3 when NH₄⁺ was added; however, differences among treatments were not significant. In Lake George, abundances were lower, with *narB* abundances appearing to increase when NO₃⁻ or NH₄⁺ were added.

The Lake Griffin *C. raciborskii* cultivated isolate (strain L) had the fastest growth rates in batch cultures when it was grown with NH₄⁺ or urea (Fig. 9). Heterocyst frequencies decreased in these two treatments, while they were greater in cultures grown with no combined N or with NO₃⁻ (Fig. 9). High concentrations of NH₄⁺ were released into the culture medium during the course of the experiment in the urea treatment (data not shown). Nitrogen fixation was initially detected in the NH₄⁺ and urea treatments, but by day 4 it was undetectable.

Discussion

The two modes of nutrient additions in the experimental treatments simulated either a short-term or a permanent

removal of N limitation for phytoplankton growth. The single N addition treatment had only a short-term release from N limitation, which was reintroduced soon thereafter (within 3–5 d) due to uptake by the microbial community in the experimental containers (Fig. 3). In contrast, in treatments with repeated N additions, N was not limiting after the initiation of the experiment. The goal of this experiment was to investigate whether diazotrophs have taxa-specific responses to a short-term release from N limitation in comparison with permanent removal of N limitation. We hypothesized that growth of *C. raciborskii* would be less sensitive to both short- and long-term DIN pulses than that of *Anabaena* spp.

Ammonium inhibits synthesis of nitrogenase and heterocyst formation and may inactivate nitrogenase directly through modification of the nitrogenase Fe protein (Flores and Herrero 1994). Nitrate may also inhibit synthesis of nitrogenase in some diazotrophs (Flores and Herrero 1994). Nitrogen fixation in cyanobacteria may continue in the presence of NO₃⁻ or NH₄⁺, but this response is thought to be taxa specific and is dependent on external DIN concentrations (Mulholland et al. 2001; Spröber et al. 2003). The ability of *C. raciborskii* to take up and effectively use different N sources, and the ability to shift between these DIN uptake modes and diazotrophy, are

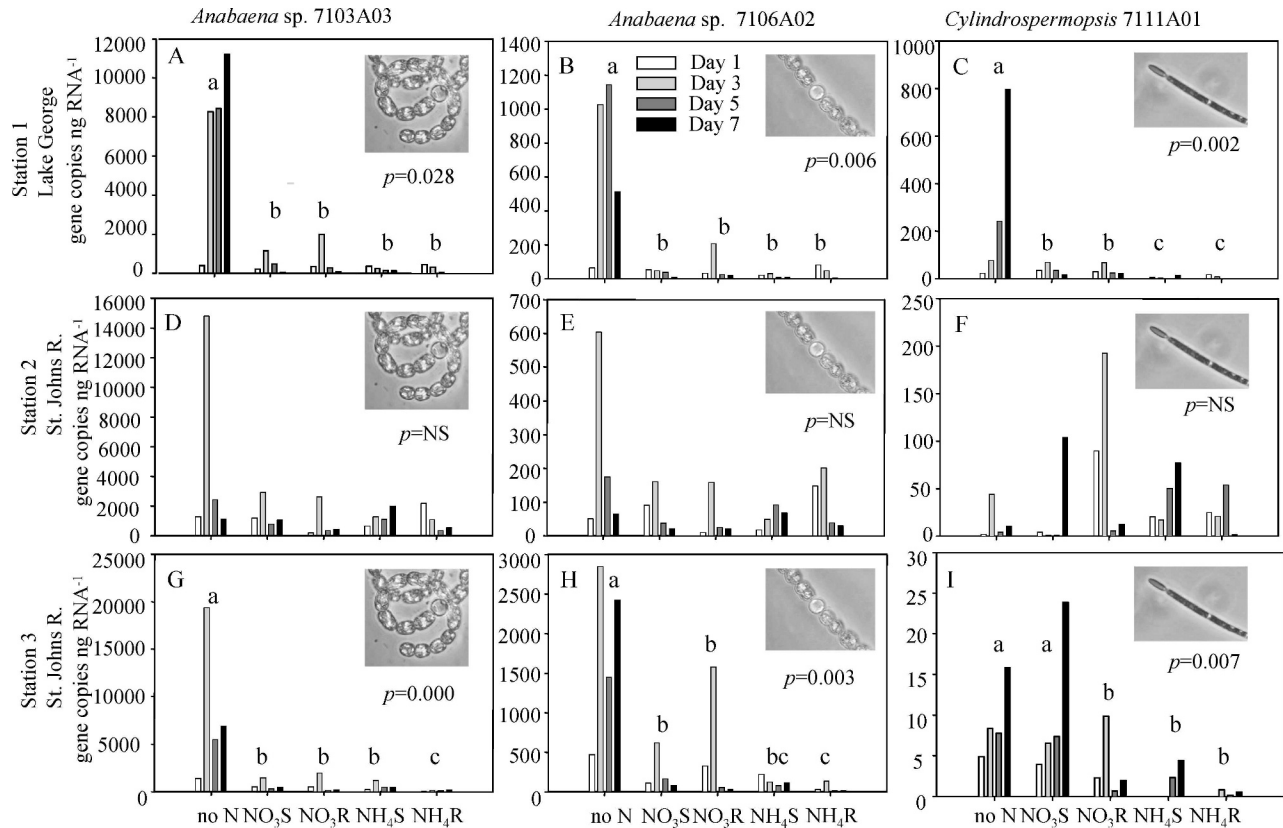


Fig. 7. Expression of *nifH* genes in water samples determined by qPCR (gene copies of cDNA ng RNA⁻¹). (A–C) Lake George; (D–F) St. Johns River Sta. 2; (G–I) St. Johns River Sta. 3. A, D, G, *Anabaena* sp. probe 7103A03; B, E, H, *Anabaena* sp. probe 7106A02; C, F, I, *C. raciborskii* probe 7111A01. NO₃S, single NO₃⁻ addition; NO₃R, repeated NO₃⁻ additions; NH₄S, single NH₄⁺ addition; NH₄R, repeated NH₄⁺ additions. Different letters indicate significant differences (one-way ANOVA, LSD pairwise comparisons).

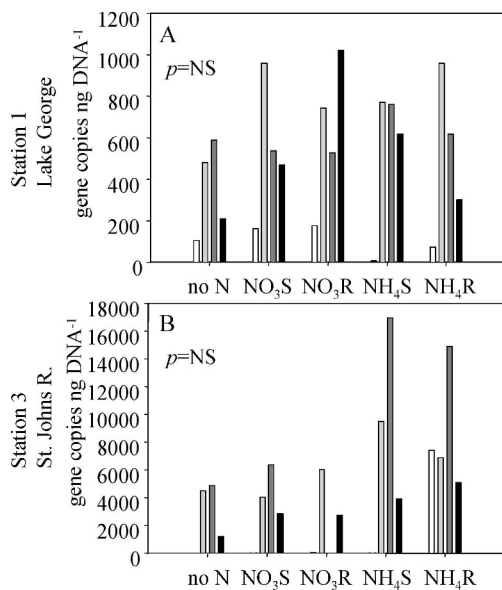


Fig. 8. Abundance of *narB* sequence type 13106_122Y07 genes copies (number of gene copies [ng DNA⁻¹]) in (A) Lake George, (B) St. Johns River Sta. 3. Results from one-way ANOVA are shown.

potentially important factors in determining its competitive success.

The relative responses in abundances of both *Anabaena* spp. (based on the *nifH* qPCR data) were remarkably similar at all of the three experimental sampling stations. Highest *nifH* abundances were detected in the treatment with no N, as evidence of high competitive potential of

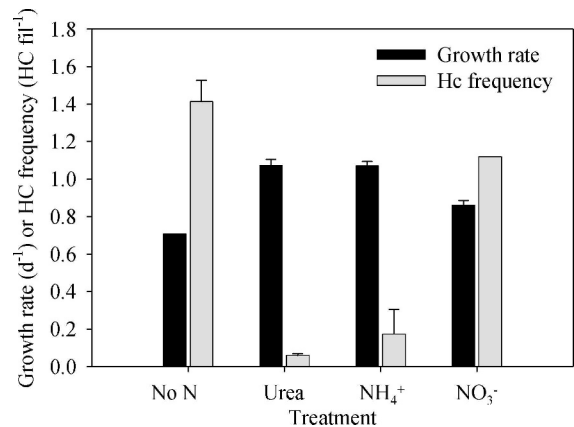


Fig. 9. Growth rates (d⁻¹) and frequencies of heterocysts (hc filament⁻¹) in *C. raciborskii* strain L cultivated under different N sources in the laboratory. Means and standard deviations are shown for duplicate culture flasks for each treatment.

these diazotrophs under the N-limiting conditions. *Anabaena* spp. abundances also increased in response to repeated additions of NH_4^+ at Sta. 2. For *C. raciborskii*, the increases in its *nifH* abundances occurred either to “no N” (all stations), repeated NH_4^+ additions (Sta. 3), or single NO_3^- addition (all stations).

Owing to the great complexity of natural phytoplankton communities, information from monocultures can be highly useful in interpreting field data on how physiological capabilities may contribute to competitive success of individual taxa. The *C. raciborskii* culture growth rate was faster with NH_4^+ than with N_2 , suggesting effective use of this N source. Reduced heterocyst frequencies and low or absent N_2 fixation paralleled the high growth rates with NH_4^+ . In the urea treatment, the appearance of high concentrations of NH_4^+ during the course of the experiment must have been due to biological or chemical breakdown of urea. The high cyanobacterial growth rates with urea therefore reflected growth based on uptake of NH_4^+ (and potentially, uptake of urea). In a prior study, *C. raciborskii* from Lake Balaton continued to fix N_2 under presence of $10 \mu\text{g L}^{-1}$ of NH_4^+ -N (Spröber et al. 2003). Growth of *C. raciborskii* culture with high NO_3^- (6–8 mg NO_3^- -N L^{-1}) in our study relied largely on N_2 , based on high N_2 -fixation rates and heterocyst frequencies. Similar results were found in a field study in which NH_4^+ and N_2 were the preferred N sources in an almost unicyanobacterial bloom of *C. raciborskii* in Lake Balaton (Présing et al. 1996), while NO_3^- uptake was a small fraction of the uptake. Ammonium was also the preferred N source for *C. raciborskii* in a bloom in Australia (Burford et al. 2006). However, in continuous cultures, growth rates were equal under all of these N sources (Spröber et al. 2003), unlike in our study. These differences may reflect either differences in cultivation methods (continuous vs. batch cultures) or in physiological capabilities of *C. raciborskii* strains. In our field study, *C. raciborskii* growth always showed a positive response to N-depleted conditions, but abundances at times also increased in the presence of NH_4^+ or NO_3^- . This was in spite of the fact that *C. raciborskii* was competing for resources with the rest of the diverse phytoplankton, including nondiazotrophic cyanobacteria, eukaryotic phytoplankton, and other bacteria. In contrast, there was low growth of *Anabaena* spp. in the NH_4^+ treatments at Stas. 1 and 3, while at Sta. 2, *nifH* gene abundances of both *Anabaena* spp. were slightly elevated in response to NH_4^+ . These data suggest that both *Anabaena* spp. and *C. raciborskii* can at times compete effectively if NH_4^+ is available at high concentrations. In contrast, a single NH_4^+ pulse at $20 \mu\text{mol L}^{-1}$ followed by N limitation (treatment NH_4^+ S) was inhibitory for growth of both cyanobacteria at all of the three study sites. Although this concentration is clearly not inhibitory for the growth of these cyanobacteria, it is suggested that uptake affinity for NH_4^+ or growth kinetics in these cyanobacteria is lower than other phytoplankton that grew successfully, including the non-diazotrophic unicellular cyanobacteria that were detected by *narB* qPCR at high abundances in the DIN additions.

Using PCR amplification, we verified the presence of the *narB* gene in Florida isolates of *C. raciborskii* and at least

one of the *Anabaena* spp. morphotypes (PA1, PO1) that were targeted with the *nifH* probes. The strains PA1 and PO1 clustered together with both *nifH* and *narB* gene analyses, suggesting that the strains are isolates of the same species. The additional *nifH* sequence type from strain PO1 likely reflects a mixed culture. Despite having the genetic potential for assimilatory NO_3^- reduction, the *Anabaena* spp. PA1-like cyanobacterium (straight morphotype), was a weak competitor under conditions where NO_3^- was provided, even if N limitation developed later (single NO_3^- additions). At all stations, *C. raciborskii* increased in abundance if a single NO_3^- addition was made. These observations suggest that *C. raciborskii* growth in the competitive plankton environment benefited to a greater extent from NO_3^- pulses than native *Anabaena* spp. from the SJR.

The *nifH* genes of all cyanobacteria continued to be expressed in treatments in which NO_3^- or NH_4^+ was added, although at reduced levels, suggesting nitrogenase was active. In addition, expression of *nifH* in the two *Anabaena* spp. and *C. raciborskii* at times continued at elevated levels under NO_3^- compared with NH_4^+ . The high abundance of heterocysts and continued N_2 fixation under NO_3^- treatment in the monoculture experiment support the suggestion that *C. raciborskii* growth in the presence of NO_3^- relied largely on N_2 . Elevated *nifH* expression in NO_3^- treatments suggests that also in the field conditions the cyanobacteria obtain at least part of their N by N_2 fixation when NO_3^- is present. Low but detectable *nifH* expression in the field when NH_4^+ was present is consistent with our observations from the culture experiment where low N_2 -fixation rates were detected when NH_4^+ was present. In *C. raciborskii*, *nifH* was expressed only at very low levels in the NH_4^+ treatment at Sta. 3 where growth occurred, suggesting growth occurred via NH_4^+ assimilation.

Although the overall trends in the abundance and expression of the two *Anabaena* spp. were remarkably similar at the three stations, some differences were observed. There was more variability in *C. raciborskii* than *Anabaena* spp. abundance among stations. Small differences in the phytoplankton community composition and abundances of initial community members may shift the community dynamics under different N amendments. At Sta. 2, *Anabaena* spp. appeared to be able to use NH_4^+ for their growth more effectively than at the other stations, potentially reflecting a different community composition involving competition for resources.

Preferred N sources for growth may differ from species to species and can depend on irradiance intensity (Oliver and Ganf 2000). Diazotroph light requirements are often lower when grown on a combined N source than when grown on N_2 (Agawin et al. 2007). Therefore the success of diazotrophs in situations where they compete with non-diazotrophs can depend on light availability. In these experiments, we were not able to control the effect of light limitation during the course of the experiments; however we wanted to alleviate a potential artifact of photoinhibition by using slight shading. SJR is a blackwater river with high light attenuation (Phlips et al. 2000), and approximately 50% of photosynthetically active irradiation (PAR)

is attenuated every 20 cm of the top water column (Moisander unpubl. data from the St. Johns River, August 2007). The bottles in our incubations experienced a light environment that corresponded to approximately 20–40-cm depth. All experimental bottles were incubated at the surface of the river and covered with similar screening, so there were no biases in the initial light conditions.

The dominant *narB* clone that was recovered from the experiments likely originated from a picocyanobacterium related to *Synechococcus* spp. The qPCR data showed that abundance of this cyanobacterium was at times high in all treatments. This cyanobacterium is therefore likely to be one of the important community members that contributed to the increase in phytoplankton biomass under N enrichment. It is not clear whether the growth of this cyanobacterium under N₂ was due to recycled N from diazotrophs or whether it is in fact a diazotroph.

Although an initial growth spurt occurred in DIN addition treatments, growth reached a plateau relatively quickly, most likely because of depletion of secondary limiting nutrients. Parallel tests showed that additions of P allowed the growth to continue and reach a greater level of biomass (data not shown), as evidence of colimitation of these nutrients in the system. Colimitation by N and P has been observed in this system previously (Piehler et al. in press.). Recently, Elser et al. (2007) showed, using a global meta-analysis of N and P limitation data, that these two nutrients colimit and have synergistic effects on primary productivity across terrestrial, freshwater, and marine systems. SJR does not appear to be an exception.

This is one of the few studies in which *nifH* gene copy and transcript abundances have been quantified and compared among taxa in freshwater or estuarine planktonic field populations. The data from the St. Johns River show that *nifH* expression in heterocystous diazotrophs is not entirely inhibited by DIN in the natural planktonic communities, even at very high DIN concentrations. However, the data suggest that inputs of inorganic N both in the form of NH₄⁺ and NO₃⁻ quickly reduce growth of two major native *Anabaena* spp. in the system and rapidly reduce N₂-fixation rates. The more positive responses of the invasive *C. raciborskii* showed that this microorganism can grow and compete effectively using either diazotrophy or assimilation of fixed DIN. *Cylindrospermopsis* appears to have flexibility in its N utilization mechanisms that allow it to proliferate under conditions when growth limitation in the environment is frequently alternating between nitrogen and other factors.

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Received: 9 January 2008

Accepted: 30 May 2008

Amended: 6 June 2008