RESEARCH ARTICLE

Facultative diazotrophy increases *Cylindrospermopsis raciborskii* competitiveness under fluctuating nitrogen availability

Pia H. Moisander[1,2], Lou Anne Cheshire[3], Jeremy Braddy[3], Elizabeth S. Calandrino[3], Melissa Hoffman[3], Michael F. Piehler[3] & Hans W. Paerl[3]

[1]Ocean Sciences Department, University of California Santa Cruz, Santa Cruz, CA, USA; [2]Department of Biology, University of Massachusetts Dartmouth, North Dartmouth, MA, USA; and [3]Institute of Marine Sciences, University of North Carolina at Chapel Hill, Morehead City, NC, USA

Correspondence: Pia H. Moisander, Department of Biology, University of Massachusetts Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747, USA.
Tel.: +1 508 999 8222;
fax: +1 508 999 8196;
e-mail: pmoisander@umassd.edu

Received 11 June 2011; revised 15 November 2011; accepted 17 November 2011.

DOI: 10.1111/j.1574-6941.2011.01264.x

Editor: Riks Laanbroek

Keywords
Anabaena; cyanobacterial blooms; cyanotoxin; C/N; N/P; N_2 fixation; nutrient limitation.

Abstract

Relative fitness of three bloom-forming and potentially toxic cyanobacteria from the subtropical St. John's River, Florida was investigated under a range of nutrient conditions, during a bloom dominated by *Cylindrospermopsis raciborskii*. Nitrogen (N) was the primary nutrient limiting phytoplankton primary productivity and biomass. Phytoplankton biomass was also enhanced by phosphorus (P) added either alone or jointly with N, suggesting different components of the phytoplankton experienced distinct nutrient limitations. Based on quantitative PCR, the diazotrophic cyanobacteria *Anabaena* sp. and *C. raciborskii* were responsible for the primary production response to P additions, while the nondiazotrophic *Microcystis aeruginosa* appeared to benefit from N released from the diazotrophs. *Cylindrospermopsis raciborskii* maintained high net growth rates under diazotrophic and nondiazotrophic conditions, while *Anabaena* sp. growth was significantly reduced under DIN enrichment. *C. raciborskii* appears to be a generalist with regard to N source, a lifestyle traditionally not considered a viable ecological strategy among diazotrophs. Using facultative diazotrophy, *C. raciborskii* gains a growth advantage under fluctuating DIN conditions, such as systems that are under the influence of anthropogenic N loading events. The described niche differentiation may be a key factor explaining the recent global expansion of *C. raciborskii*.

Introduction

Phytoplankton community composition shifts in response to environmental factors, and with constant changes in the environment, high diversity is maintained (Hutchinson, 1961). Predictable periodicity in community composition at the taxonomic group level is observed because of seasonal changes in temperature, water column stratification, and nutrient availability. For example, shifts from spring diatom- and dinoflagellate-dominated communities to summer nitrogen (N_2)-fixing or non-N_2-fixing cyanobacterial blooms, are frequently observed phenomenon in many freshwater systems (Reynolds, 2006). Prediction of the species composition at lower taxonomic level, such as which cyanobacterial species dominate blooms each year, remains a challenge, largely attributed to limited knowledge of different physiologies and genetic capabilities among the numerous phytoplankton species and strains making up the community. Our ability to predict and understand factors driving phytoplankton species composition and dominance is of particular interest for cyanobacteria, because many species have the capacity to produce a range of secondary metabolites that act as toxins for vertebrates and invertebrates (Chorus & Bartram, 1999). In recent decades, the potentially toxin-producing cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju (Woloszynska, 1912) has been increasingly reported in freshwaters around the world (Padisak, 1997). The genus was first thought to be associated with a toxic bloom in Australia (Francis, 1878). Recently, *C. raciborskii* has been

FEMS MICROBIOLOGY ECOLOGY

reported in bloom densities in numerous tropical, subtropical, and temperate systems around the world, including Australia (Fabbro & Duivenvoorden, 1996), North (Chapman & Schelske, 1997) and South America (Lagos *et al.*, 1999), Europe (Shafik *et al.*, 2001; Valerio *et al.*, 2005; Fastner *et al.*, 2007; Moustaka-Gouni *et al.*, 2009), and Africa (Bouvy *et al.*, 2006). The species produces several toxins, including cylindrospermopsin, deoxy-cylindrospermopsin, and saxitoxin (Hawkins *et al.*, 1997; Chorus & Bartram, 1999), and these toxins have been shown to accumulate in higher organisms (Saker & Eaglesham, 1999) and traced to drinking water sources (Hawkins *et al.*, 1985). The apparently rapid expansion of geographic range and potential ecosystem and human health concerns associated with occurrences of the species have prompted much interest in understanding *C. raciborskii* ecology. Various hypotheses have been proposed to explain this recent expansion (Briand *et al.*, 2004). High affinity and storage capability for phosphorus (P) (Istvanovics *et al.*, 2000), preference for low light levels (Moustaka-Gouni *et al.*, 2010), ability to compete well under high pH (Padisak, 1997) and high temperatures (Saker & Griffiths, 2000; Briand *et al.*, 2002), and allelopathy (Figueredo *et al.*, 2007) have all been proposed as reasons for enhancing fitness of *C. raciborskii*. Although the species is capable of fixing atmospheric nitrogen (N_2), some studies have suggested that combined N was an important N source supporting blooms (Présing *et al.*, 1996; Burford *et al.*, 2006; Piehler *et al.*, 2009; Calandrino & Paerl, 2011), and thus suggesting that *C. raciborskii* may compete well under nondiazotrophic conditions. A low frequency of heterocysts, specialized cells that are sites of N_2 fixation (Branco & Senna, 1994), also supported the idea that *C. raciborskii* growth may be supported by N sources other than N_2. In a recent study on subtropical estuarine waters in the United States, St. John's River watershed (SJR), Florida, we showed that *C. raciborskii* may be able to compete more effectively than the native diazotrophic cyanobacteria (*Anabaena* spp.) under conditions in which dissolved inorganic nitrogen (DIN) was periodically present (Moisander *et al.*, 2008). Both *Anabaena* spp. and *C. raciborskii* grow in laboratory cultures if DIN is provided, and the growth rate of *C. raciborskii* cultures under NH_4^+ or NO_3^- exceeds growth rates of those under diazotrophic conditions (Hawkins *et al.*, 2001; Moisander *et al.*, 2008). The objective of this study was to experimentally test the hypothesis that *C. raciborskii* has exceptionally high fitness under nondiazotrophic conditions, when diazotrophs are generally not thought to compete effectively under such conditions (Fogg *et al.*, 1973; Paerl, 1990; Reynolds, 2006).

We investigated taxa-specific fitness of three cyanobacterial genera under a range of nutrient conditions in the SJR watershed in Florida, United States. The ecosystem experiences seasonality and periodic loading events of N and P that are reflected as summertime blooms of cyanobacteria with annual variability in magnitude and species composition (Phlips *et al.*, 2007). Over the recent decade, *C. raciborskii* has emerged as a significant component of this late summer phytoplankton in the SJR (Chapman & Schelske, 1997). Cylindrospermopsin is frequently detected and appears to originate from both *C. raciborskii* and *Aphanizomenon ovalisporum* in the system (Yilmaz *et al.*, 2008; Yilmaz & Phlips, 2011). We conducted nutrient enrichment bioassay experiments on water collected at two sites along the river and investigated responses in total phytoplankton community and the cyanobacteria using taxa-specific molecular detection (quantitative real-time PCR) of abundances of the cyanobacteria, N_2 fixation and primary productivity rate measurements, total phytoplankton biomass, and trends in concentrations and ratios in dissolved and particulate N and P.

We hypothesized that *C. raciborskii* is an example of a cyanobacterium with unusual facultative diazotrophic lifestyle, which allows selective and effective use of either N_2 fixation or DIN assimilation, depending on environmental conditions. We propose that this strategy is a significant factor in explaining the apparent recent expansion of *C. raciborskii* in eutrophying freshwaters worldwide.

Materials and methods

Experimental design

Nutrient enrichment bioassays were conducted in the SJR estuary, Florida, in August, 2006. Water for experiments was collected at two sites: Lake George (LG) (N29.3459 W81.6160) and a SJR site (SJ) further downstream in Palatka (N29.6376 W81.6212). LG is located at the headwaters of the river and is estimated to provide approximately 50% of the water to the lower river, while several large tributaries enter the river between LG and SJ site (Phlips *et al.*, 2007). Water (320 L per site) was collected from the surface to acid-washed (1% hydrochloric acid) polyethylene carboys from each site and transported to the incubation site in a shoreline station of SJR in Palatka. Eight liters of water was poured into low-density polyethylene containers (cubitainers) that had been acid washed followed by a rinse with sample water. The cubitainers were then amended with nutrients. Nitrogen sources were added either at the beginning (T0) of the experiment (NH_4^+ L, NO_3^- L; low DIN) or daily (NH_4^+ H, NO_3^- H; high DIN), and P additions were performed daily after subsampling for various tests. Low DIN additions were 20 μM at the initiation of the experiment. High DIN additions were 400 μL of a 0.2 M stock solution per day (amended concentration in cubitainers increasing over

the course of experiments because of decreasing sample volume). Dissolved inorganic phosphorus (DIP) was added to approximately 2 μM final concentration per day to all P treatments. Cubitainers were incubated in the river under one layer of neutral density screening (reducing incident irradiance by approximately 30%) to reduce photoinhibition. For 7 days, subsamples for various measurements were collected in the morning from the cubitainers. Subsamples were collected each day for determination of N_2 fixation rates (acetylene reduction assay) and for total phytoplankton biomass, determined by chlorophyll *a* (Chl *a*) concentration. Every other day, samples were additionally collected for determination of gene copy numbers of the dominant cyanobacteria (samples filtered for molecular analyses) and dissolved inorganic carbon (DIC), and primary productivity rate measurement (PP) was conducted. On days 0, 3, and 7, subsamples were additionally collected for determination of particulate organic carbon and nitrogen (PC, PN). Nutrients were amended to the cubitainers ('H' treatments, and all treatments with P) each day after subsamples were collected. Total volume of water withdrawn from the cubitainers when the experiment was ongoing was 3.6 L.

Analytical methods

For determination of the N_2 fixation rates, 60 mL of sample was transferred to 72-mL glass serum vials that were then sealed with red rubber stoppers, and 7 mL of headspace was replaced with acetylene, generated from calcium carbide. Serum vials were shaken vigorously to equilibrate the gases and incubated in the river for 4 h. At the termination of the incubation, serum vials were again shaken, and 3.6 mL of gas was withdrawn into Vacutainers (Beckton Dickinson). Vacutainers were transported to the UNC-CH Institute of Marine Sciences (UNC-CH IMS) for measurement. Ethylene concentration was determined by a Shimadzu GC-14 gas chromatograph, equipped with a flame ionization detector, and Poropak T column as described previously (Moisander *et al.*, 2008). Two millilitres of gas was withdrawn from the Vacutainers by replacing the contents with water and transferred into the gas chromatograph autosampler vials.

For determination of Chl *a*, 50 mL of sample water was filtered on GF/F glass–fiber filters (Whatman, Piscataway, NJ). The filters were blotted dry, folded, wrapped in aluminum foil, and frozen at −20 °C until determination by fluorometry within the next month. Five millilitres of 90% aqueous acetone was added to the filter in a 15-mL centrifuge tube. Filters were sonicated in an ice bath, in the dark for 10 min, and placed

Table 1. Quantitative PCR primers and 5′ nuclease probes applied in the study

Name	Gene	Target	Forward primer	Reverse primer	Probe	Ref
7103A02	*nifH*	*Anabaena* sp.	CCAAGGCTCAAACCACCGTAT	TTCGTGGAGTTCCAAATCTTCA	ACACTTGGCTGCTGAAAGAGGCGC	Moisander *et al.* (2008)
7111A01	*nifH*	*C. raciborskii*	CCGTTTGATGCTGCACTCTAAA	GAATCCGGTCAGCATTACTTCTTC	TCAAACTACCGTATTGCACTTGGCTGCTG	Moisander *et al.* (2008)
188F, 254R	*cpcBA*	*Microcystis aeruginosa*	GCTACTTCGACCGCGCC	TCCTACGGTTTAATTGAGACTAGCC	CCGCTGCTGTCGCCTAGTCCCTG	Kurmeyer and Kutzenberger (2003)

in −20 °C for 24 h. The extract was then cleared through a GF/F filter in a Swinnex (Millipore) holder, and Chl *a* fluorescence was determined with a Turner TD-700 fluorometer using the nonacidification method (Welschmeyer, 1994).

DIN (NH_4^+ + and NO_x^-), total dissolved N (Total N), and PO_4^{3-} concentrations were determined on glass–fiber (GF/F)-filtered subsamples using high-sensitivity automated colorimetric flow injection analyses (Lachat QuickChem 8000, Lachat Instruments, Milwaukee, WI). Primary productivity was estimated using the ^{14}C method (Paerl, 1993) as follows. In the morning (~10:00 hours) following sample collection, quadruplicate light and a single dark 76-mL polyethylene bottles containing sample water were injected with 0.3 mL of $NaH^{14}CO_3$ (ICN Radiochemicals; specific activity 58 mCi $mmol^{-1}$) and incubated and covered with neutral density screening just below the water surface for 4 h. At the completion of incubation, the particulates in each bottle were collected onto 25-mm diameter GF/F filters, dried, and fumed with hydrochloric acid vapors for 24 h to remove abiotically precipitated ^{14}C. Dried filters were transported to the laboratory, then placed in vials containing scintillation cocktail (Cytoscint), and run in a Beckman-Coulter LS 6500 liquid scintillation counter. Counts were converted to total CO_2 fixed using the method of (Paerl, 1993). For determination of DIC, 20-mL Pyrex glass vials were filled with sample water and stored in a refrigerator until experiment was completed, and total DIC was determined at the UNC-CH IMS using a Shimadzu TOC-5000A carbon analyzer.

For measurement of total particulate organic carbon and nitrogen, 100 mL of sample was filtered on precombusted (450 °C for 4 h) glass–fiber filters (GF/F, Whatman). Filters were placed in small sterile petri dishes and then frozen at −20 °C until analysis. The sample filters were fumed for 6 h with concentrated hydrochloric acid to remove inorganic carbon and then dried at 60 °C. The fumed and dried filters were analyzed for organic C and N content using a Perkin Elmer CHN analyzer (Model 2400 Series II) standardized with acetanilide.

For determination of abundances of the cyanobacteria by quantitative PCR (qPCR), 50 mL of sample water was filtered on 0.2-μm Supor filters (Pall Gelman). Filters were folded and placed in sterile bead beater tubes, then immediately frozen in liquid nitrogen, and stored at −80 °C upon arrival into laboratory. DNA was isolated using a modified protocol of the GeneRite extraction kit DNA-EZ RW03C (North Brunswick, NJ). Each filter was inserted into a 2-mL screw-cap tube preloaded with glass beads (Sigma G-1277), and 500 μL of lysis buffer was added. The tubes were secured in a bead beater (Biospec) and homogenized for 2 min. The tubes were removed and centrifuged in a microcentrifuge for 1 min. The crude supernatant was removed and added to a 1.5-mL microcentrifuge tube and spun to pellet the debris. Approximately 400 μL of supernatant was removed to a new 1.5-mL tube, and 1200 μL of binding buffer was added. The solution was mixed and transferred to a DNAsure column and spun for 1 min, then discarded. The column was washed twice with 500 μL of EZ-wash buffer, and an extra spin was performed to remove excess liquid. The spin column was placed in a new collection tube, and 25 μL DNA Elution Buffer, heated to 70 °C, was added. After 1 min, the sample was centrifuged, and the elution step was repeated to obtain a total volume of 50 μL. The DNA was stored at −20 °C until analysis. Quantitative real-time PCR 5′-nuclease assay (TaqMan assay) was used for determination of abundances of cyanobacteria at station LG water. Primers and probes were previously designed and are listed in Table 1. For qPCR standards that were included in each qPCR plate, we used serial dilutions of linearized plasmid standards ($10–10^7$ gene copies per reaction) with cloned inserts of the target gene. These standards were created in previous studies (Moisander *et al.*, 2008, 2009). Duplicate experimental bottles were processed for real-time PCR analyses, with analytical duplicates.

Statistical analyses

Statistical comparisons between treatments were conducted using the repeated measures ANOVA (PASW Statistics 18 or 19). Data normality and homoscedasticity were improved by log transformations. Observations during the 7-day incubation period suggested that in some cases, the phytoplankton biomass or rates 'crashed' at the last

Table 2. Physico-chemical conditions at the beginning of the experiment. Molar nutrient ratios are shown. POC particulate organic carbon; PON particulate organic nitrogen

	LG	SJR
Temperature (deg C)	30.1	31.7
Salinity	0.69	0.65
O_2 (%)	78.3	106.6
O_2 (mg L^{-1})	5.89	7.8
pH	8.04	8.47
Chl *a* (μg L^{-1})	20.0	16.1
POC (μg L^{-1})	4806.1	4677.2
PON (μg L^{-1})	509.9	560.5
TPC : TPN	11.0	9.7
NO_x^- (μg L^{-1})	4.13	4.11
NH_4^+ (μg L^{-1})	20.55	23.35
SRP (μg L^{-1})	7.47	5.15
Total dissolved N (μg L^{-1})	380.5	440.0
DIN/DIP	1.5	2.4

time points. In such cases, we used the logarithmic and stationary periods in statistical comparisons by repeated measures ANOVA with Bonferroni post hoc tests. The time points included in the statistical tests are indicated where results are shown.

Results

The nutrient responses suggested that different components of the phytoplankton were limited by distinct nutrients or nutrient combinations. In experimental incubations from both study sites, addition of P had the greatest influence on the magnitude of total phytoplankton biomass (Fig. 1). A positive response in total phytoplankton biomass to increasing level of N was observed (LG water, DIN additions without P) (Fig. 1), and addition of N jointly with P further increased biomass; however, the highest biomass was reached when P was added alone. The rate of increase in biomass was slower when P was added alone than under P + N. Thus, DIN was the primary nutrient limiting the nondiazotrophic phytoplankton biomass, also evidenced as high densities of actively N_2-fixing cyanobacteria in the system. There was a decrease in total phytoplankton biomass after day 5 or 6 in some, but not all treatments. Results at the SJ site had similar general trends as at LG, with the greatest increases in biomass seen in response to P added alone or jointly with N. As in LG water, there was a small, yet significant increase in biomass in response to one of the N-alone treatments (NO_3^- H), as evidence of N-limited conditions (Table 1, Table S1). At SJ, Chl *a* increased in P and P + N treatments and 'crashed' abruptly after day 4 in P + N treatments. The peak in Chl *a* abundance in the P-only treatment at SJ was offset from the peak in the P + N treatments, occurring two days later, when biomass in the P + N treatments had already started to decrease. Concentration of Chl *a* in P + N treatments reached a maximum at the LG site on day 5–7, and on day 4 at the SJ site. Peak in the P-only treatment occurred on days 5 and 6 at LG and SJ, respectively.

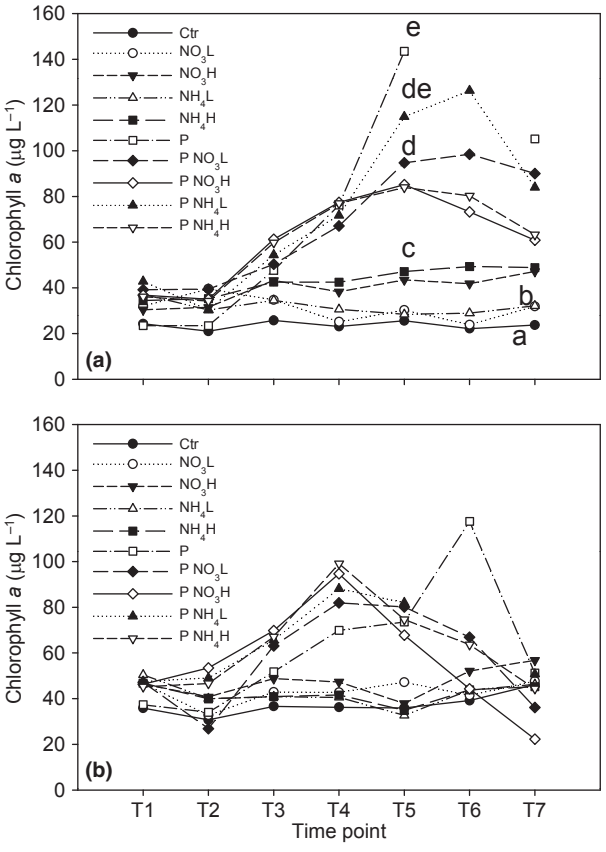

Fig. 1. Total phytoplankton biomass (chlorophyll *a*, μg L^{-1}) during the bioassay experiments on water from (a) LG and (b) SJR. Differences among treatments ($P < 0.05$) based on repeated measures ANOVA are shown as a < b < c < d < e for LG. Results for statistical comparisons for SJ are shown in Table S1. Time points T3–T5 and T2–T4 were included in the tests for LG and SJ, respectively.

Primary production (PP) responses to nutrients were generally similar at LG and SJ sites. Addition of N (NO_3^- or NO_4^+ high or low additions) elevated PP slightly, yet significantly, over the control (Fig. 2, Table S1), supporting observations from total phytoplankton biomass responses. Nitrogen was thus the primary limiting nutrient for the net phytoplankton CO_2 fixation. Primary productivity increased further if P was added, either with or without parallel N addition and was not significantly different among P vs. P + N treatments. At SJ, PP reached its maximum and started to decrease faster in P + N treatments with high N than with low N additions.

Even under DIN additions, N_2 fixation was detected in all treatments through the experiment (Fig. 3). The rates peaked around the days 4–5 and then decreased back to ambient levels on day 7. The maximum N_2 fixation rates measured over the course of the experiments were in P-addition treatments, with highest N_2 fixation detected in the P-only treatments, but these highest rates were reached relatively slowly and were not significantly different from P + N treatments in which unexpectedly high rates of N_2 fixation were detected. Rates of N_2 fixation increased most rapidly at both sites in $P + NH_4^+$ L, $P + NH_4^+$ H, or $P + NO_3^-$ H, while N_2 fixation increased more slowly in the P-only and $P + NO_3^-$ L treatments. The peak N_2 fixation in the P treatment was detected on day 4 at the LG and on day 4 or 5 at the SJ site. There appeared to be a slight increase over time in the N_2 fixation rates in the control and the NH_4^+ L and NO_3^- L treatments, all without P; this trend was seen more clearly at the SJ site. However, the N_2 fixation rates in the control were not significantly different from 'N only' treatments at LG and SJ.

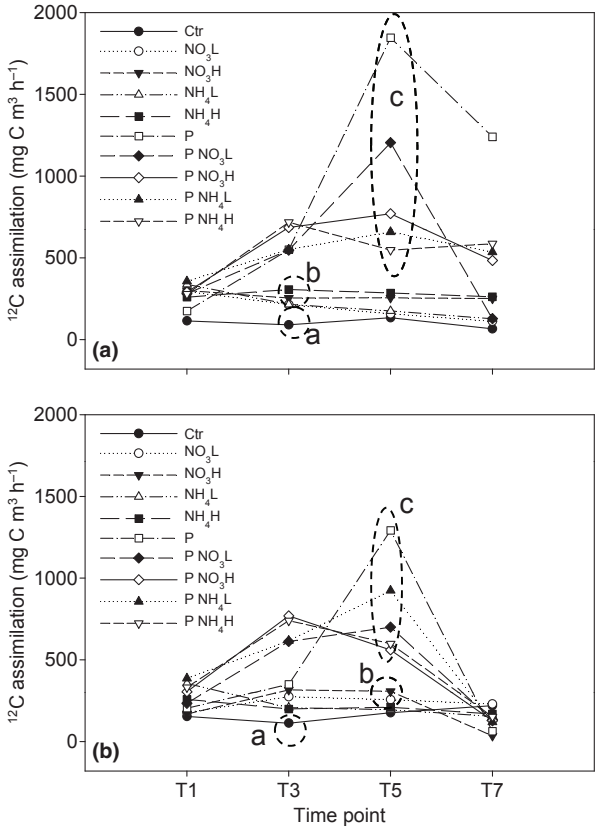

Fig. 2. Primary production (mg C m^3 h^{-1}) during the bioassay experiments on water from (a) LG and (b) SJR. Dashed lines separate treatments that were significantly different ($P < 0.05$) based on repeated measures ANOVA over the time points T1–T5. Differences among treatments are shown as a < b.

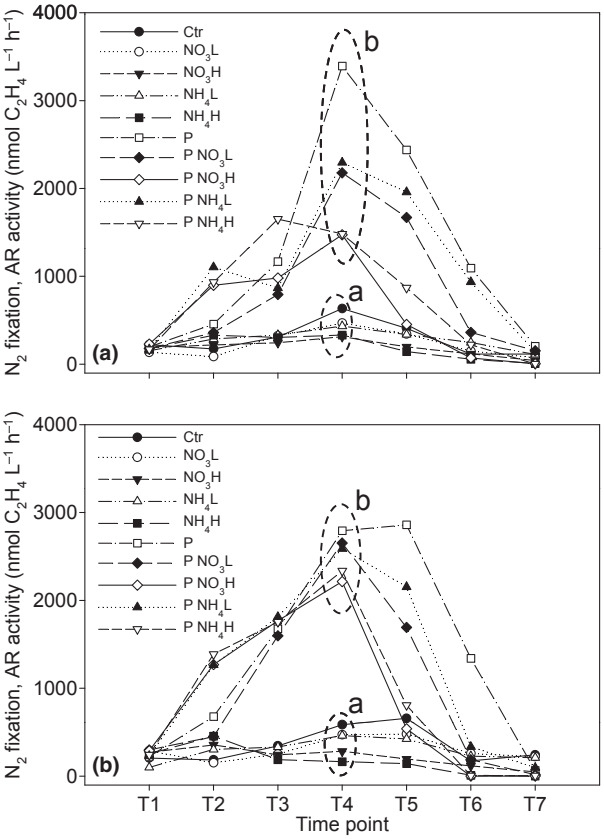

Fig. 3. Nitrogen fixation (nmol ethylene L^{-1} h^{-1}) during the bioassay experiments on water from (a) LG and (b) St. Johns River. Dashed lines separate treatments that were significantly different ($P < 0.05$) based on repeated measures ANOVA over the time points T1–T5. Differences among treatments are shown as a < b.

Relative abundances of *Anabaena* sp., *C. raciborskii*, and *Microcystis* were determined by quantitative PCR at the LG site over the course of the experiment (Fig. 4). Abundances of the diazotrophic cyanobacteria remained at the level of the control in the 'N only' additions in all cyanobacteria. Abundances of both diazotrophic (*Anabaena* and *C. raciborskii*) and nondiazotrophic (*Microcystis*) cyanobacteria increased when P was added either alone or jointly with N. For *Anabaena*, addition of 'P only' resulted in the greatest increase in abundance and the fastest net growth rate (0.87 day^{-1}) (Fig. 5). Addition of DIN had a negative influence on *Anabaena*, reducing growth rates to 0.1–0.38 day^{-1}. In the high DIN additions ($P + NH_4^+$ H and $P + NO_3^-$ H), *Anabaena* abundance decreased after the day 3, after a small initial increase; while in the low DIN additions ($P + NH_4^+$ L and $P + NO_3^-$ L), *Anabaena* abundance increased slowly for 5 days. For *Microcystis*, the increase in abundance under P addition was small, but there was a statistically significant difference from control (Fig. 4).

Responses in net growth were different between *Anabaena* and *C. raciborskii*. Presence of P was required for growth stimulation in both (Fig. 4), but responses were different under P + N. In *C. raciborskii*, addition of P with or without DIN (high and low additions) resulted in abundances that were not significantly different, and thus negative impact from DIN was not observed. Net growth rates of *C. raciborskii* under P and P + N treatments ranged from 0.56 to 0.74 day^{-1}, and the fastest net growth rate was measured under $P + NH_4^+$ H (Fig. 5). At the end of the experiment, *C. raciborskii* abundances remained high, and in some treatments, abundances were still increasing.

For the non-N_2-fixing *Microcystis*, DIN at its highest availability added with P resulted in the greatest increases in abundances (net growth rate 0.45 day^{-1}). Unexpectedly, P added alone also resulted in a small increase in cell abundances, while daily additions of either $P + NH_4^+$ or $P + NO_3^-$ resulted in fastest apparent growth rates. Under low DIN availability even with presence of P, net

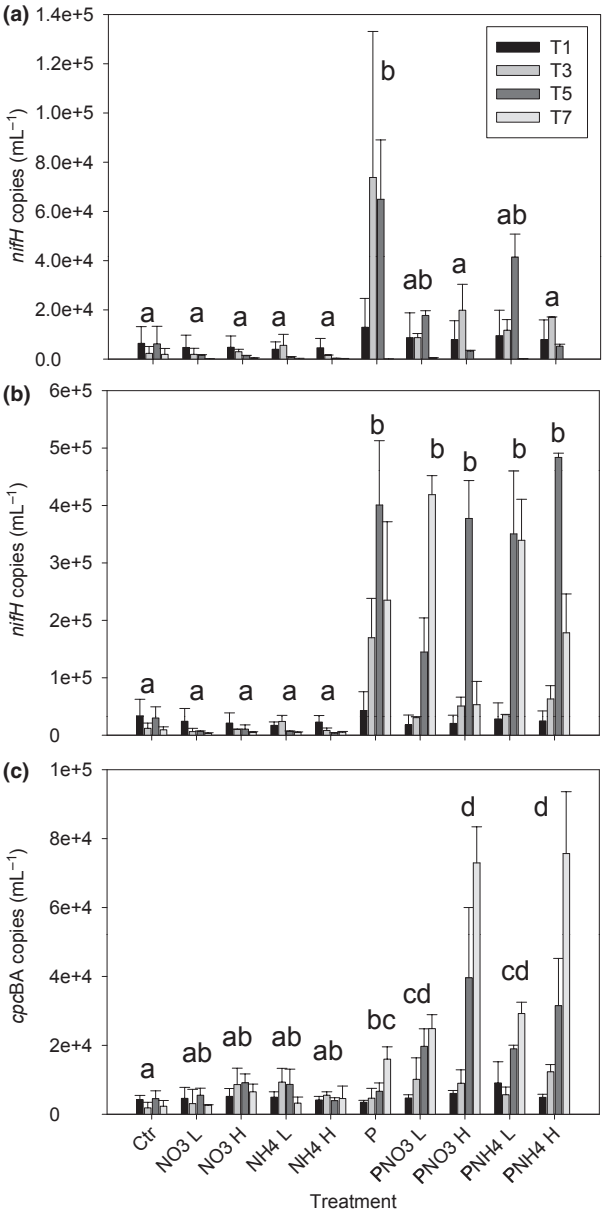

Fig. 4. Abundances of cyanobacteria (gene targets mL^{-1}) in the experiment from LG. (a), *Anabaena* sp.; (b), *Cylindrospermopsis raciborskii*; (c), *Microcystis aeruginosa*. Statistically significant differences ($P < 0.05$) based on repeated measures ANOVA are shown as a < b < c < d. Time points included in ANOVA were T3–T5, T1–T7, and T5–T7 for *Anabaena* sp., *C. raciborskii*, and *M. aeruginosa*, respectively.

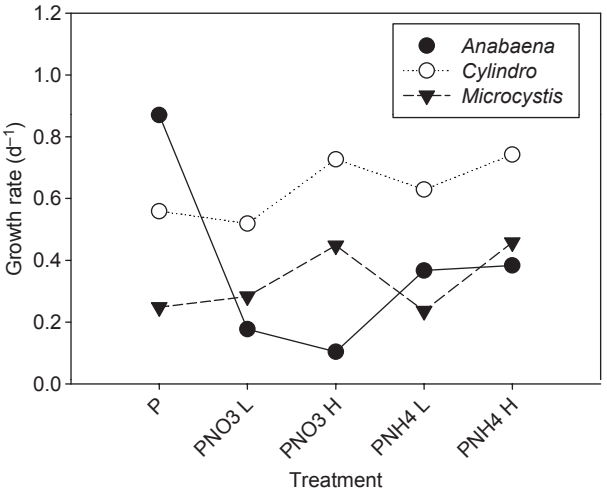

Fig. 5. Growth rates (day^{-1}) of cyanobacteria in the bioassay for water from LG.

growth rates increased only slightly from control and were not significantly different from the P-only treatment (net growth rates 0.24–0.28 day^{-1}).

Nutrient measurements were conducted on the incubation containers to estimate nutrient availabilities, to assure that concentrations did not reach inhibitory levels, and to detect potential cross-contamination. Samples for dissolved nutrient measurements were always collected approximately 24 h after the additions. Ammonium concentration was at the same level (approximately 10–19 μg L^{-1}) in treatments with or without NH_4^+ for the first 5 days, indicating that all added NH_4^+ was taken up after 24 h (Table 1, Table S2). Only on day 7 did NH_4^+ start to accumulate in NH_4^+ H treatments. Nitrate remained at the level of the ambient concentrations in all treatments into which NO_3^- was not added (0.4–4.1 μg L^{-1}), suggesting no or negligible cross-contamination. Experimental additions resulted in accumulation of NO_3^- following daily additions, with accumulation on day 7 under P + NO_3^- H additions. Soluble reactive phosphorus (SRP) remained at the level of ambient nutrients in treatments into which P was not added experimentally (5.2–16 μg L^{-1}). Excess SRP was detected on day 7 at the SJ site (30–52 μg L^{-1}) and the LG site (14.6–19 μg L^{-1}) in all of the treatments with experimental P additions (but not in the 'no P' treatments).

Total particulate organic C and N increased most profoundly in P treatments. The molar ratio of C/N decreased in all P + N treatments over the duration of the experiment, with lowest molar ratio in the 'P-only' treatment (Fig. 6). C/N also decreased from control levels when DIN alone was added at high concentrations. C/N remained close to the initial ambient conditions or increased in the control and low DIN additions.

Discussion

The wide range of genetic and physiological strategies in diverse phytoplankton species allows the different species to access resources differently and find a niche under

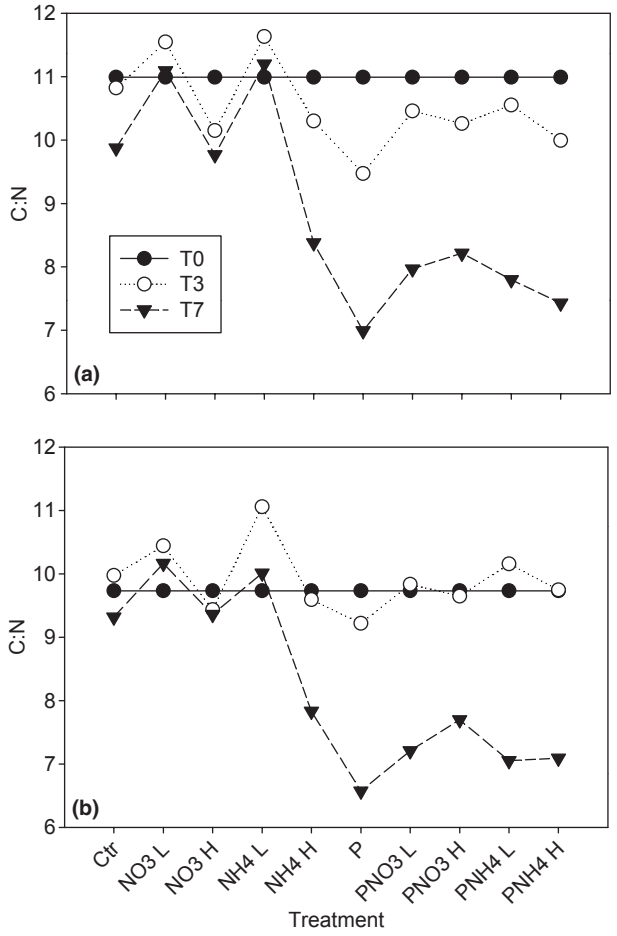

Fig. 6. C/N ratio over time as a function of treatment in the bioassay experiments from LG (a) and SJR (b).

changing environmental conditions. The diversity in phytoplankton physiological capabilities (such as nutrient acquisition and storage mechanisms) becomes evident under experimental nutrient enrichments, where different components of phytoplankton respond to nutrients differently. Understanding competitiveness of diazotrophic and toxin-producing cyanobacteria in mixed phytoplankton communities has been subject to much study over the past decades (Reynolds, 1984; Paerl, 1988), because of their importance from the perspectives of biogeochemical cycles and ecosystem and human health. The low relative ratio of N to P was identified early as an important factor promoting growth of diazotrophs in various environments (Fogg, 1969; Paerl, 1990), and classically, poor competitive success of diazotrophs is predicted under DIN-enriched conditions. While diazotrophs are never in principle, N limited, the nondiazotrophic phytoplankton experience either N or P limitation in many freshwater systems. Thus, the challenge in interpretation of a simple nutrient amendment experiment is that the nutrient response observed at the community level is not a representation of nutrient limitation of any individual species, but a composite of diverse responses in all components of the phytoplankton.

Phytoplankton growth in the SJR estuary has previously been shown to periodically experience either N or P limitation (Moisander *et al.*, 2008; Piehler *et al.*, 2009), with widespread presence of diazotrophic cyanobacterial blooms as evidence for frequent N limitation (Phlips *et al.*, 2003). Our study occurred during the peak temperatures that are supporting growth of bloom-forming cyanobacteria (Phlips *et al.*, 2007). The SJR has a high humic acid content, and phytoplankton production is thought to be limited by both light and temperature during the winter months (Phlips *et al.*, 2000, 2007).

The primary N limitation of phytoplankton growth in this study was observed as an increase in primary productivity and phytoplankton biomass in response to N added alone, a response that appears to have been because of nondiazotrophic growth (*Microcystis* being one of the genera contributing to this trend). N limitation was also suggested by the observed reduction of total particulate organic C/N under NH_4^+ H and NO_3^- H additions (with no accompanying P), caused by the incorporation of N to phytoplankton biomass.

Quantitative PCR is a high throughput method for determination of microbial abundances, and in this study was especially useful as the lowest cyanobacterial abundances would have been difficult to capture by microscopy. The gene copy numbers reported can be used as approximations of numbers of cells, with an assumption that there is one gene copy (or intergenic region in the case of *cpc*BA) per genome and one genome per cell. Under the N-limiting conditions, N_2-fixing cyanobacteria could take advantage of the provided P and quickly initiate growth under P amendments. Therefore, pulses of P loading alone to the SJR under N-limiting conditions such as the ones observed during the experiment would benefit net growth of both *Anabaena* sp. and *C. raciborskii*. Such response in diazotrophic cyanobacteria to P additions is expected and often observed in systems where N is limiting (Moisander *et al.*, 2003; Posselt *et al.*, 2009). *Cylindrospermopsis* is thought to have a particularly high affinity for phosphate (Istvanovics *et al.*, 2000); however, in our experiments, *Anabaena* was a better competitor under diazotrophic and P-replete conditions. The ability of both *Anabaena* sp. and *C. raciborskii* to initiate exponential growth in the mixed population under these conditions suggests these taxa are partitioning resources in ways that allow for co-existence. The two genera have very distinct niches with regard to utilization of DIN, however.

Although diazotrophic cyanobacteria are commonly able to assimilate combined N, their relative competitiveness in the environment is generally thought to be reduced in DIN replete conditions, because of the slow growth rates of diazotrophs (Postgate, 1990). Regulatory pathways (including expression of *nifA* and *nifL* genes) of the nitrogenase enzyme synthesis are inhibited by NH_4^+, and thus nitrogenase is not expected to be active in high NH_4^+ environments (Howard & Rees, 1994; Dixon & Kahn, 2004). Indeed, studies from freshwaters generally show a negative correlation between availability of DIN and abundance of N_2 fixers (Reynolds, 2006; Smith, 2006). While a negative relationship between NH_4^+ and N_2 fixation is frequently observed, presence of NO_3^- often does not entirely inhibit the nitrogenase activity (Postgate, 1990). Possible reasons for this include lack of assimilatory pathways for NO_3^-, the more oxidized state of the molecule that makes NO_3^- assimilation more energy demanding, or the distinct effects of NH_4^+ and NO_3^- on heterocyst differentiation (Renstrom-Kellner *et al.*, 1990). The nitrogenase enzyme continues to be synthesized, however, if the environmental DIN does not provide enough N for the cell's needs (Postgate, 1990). This appeared to be the case in all our N treatments, in which N_2 fixation continued, although at reduced levels, when NH_4^+ or NO_3^- was provided at subsaturating concentrations (in both L and H treatments, shown by disappearance of the added NH_4^+ on a daily basis). Low DIN pulses, thus, do not inhibit N_2 fixation or growth of certain cyanobacterial diazotroph groups in SJR.

Phosphorus additions resulted in largest increases in phytoplankton biomass, including diazotrophs. Highest biomass was reached under conditions in which diazotrophs had a competitive advantage, that is, when external N remained limiting. Combined activity and growth of *Anabaena* spp. and *C. raciborskii* were responsible for the high production under the P sufficient, but N-limiting treatments. C/N ratio in these conditions was close to the Redfield ratio at both study sites at the end of the experiment (6.7 and 6.9 in SJ and LG, respectively), suggesting actively growing phytoplankton. However, the competitive advantage of *Anabaena* sp. growth was lost when combined N was added. The results show clearly that *Anabaena* sp. loses the competitive edge under NH_4^+ and NO_3^- enrichment even under low N availability. *Anabaena* sp., thus, could be considered a specialist in a broad ecological sense regarding N enrichment in our incubations. In contrast, *C. raciborskii* had high competitive strength under a broad range of N conditions, spanning from fully diazotrophic situation (P added but no N) to heavily N-enriched conditions (NH_4^+ H, with P). This cyanobacterium can be considered a generalist in our incubations, with high metabolic flexibility under a broad range of N regimes. Thus, *C. raciborskii* appears to be an efficient facultative diazotroph, with high competitive strength under both low and high DIN enrichments. *Anabaena* sp. had initially a faster growth rate than *C. raciborskii* in the treatment into which P only was added. However, at the 7th day time point, *Anabaena* sp. biomass 'crashed', when both *C. raciborskii* and *Microcystis* continued to increase biomass. Simultaneously, the N_2 fixation activity returned to very low levels on day 7. The high and increasing biomass of *C. raciborskii* at this time point suggests that *C. raciborskii* growth was relying on alternative N sources. Reasons for such sudden decline in *Anabaena* abundance at the end of the experiment are not clear, but as an alternative to growth inhibition because of shortage of nutrients may have been caused by species-specific viral lysis. Targeted cyanophage-mediated control of select cyanobacterial species is a factor that could potentially have influenced bloom community development (Ortmann *et al.*, 2002). A longer lag phase, yet slow increase in *Microcystis* biomass in the P-only treatment, suggests that this nondiazotrophic cyanobacterium may have benefited from the new N released from the diazotrophs (Agawin *et al.*, 2007).

Some *C. raciborskii* strains appear to have lost their N_2 fixation capability and the ability to form heterocysts, along with some other genetic elements, and have been described as the morphologically distinct, nondiazotrophic *Raphidiopsis* spp. (Stucken *et al.*, 2010). The *Cylindrospermopsis* and *Raphidiopsis* morphotypes are closely similar phylogenetically and are currently considered the same species (Moustaka-Gouni *et al.*, 2009). Although our study suggests that facultative diazotrophy allows *C. raciborskii* to compete more effectively under conditions where there are large fluctuations in DIN availability, the genome streamlining found in the *Raphidiopsis* morphotype suggests that maintaining N_2 fixation capability in the genome is not always beneficial, presumably when DIN is available more consistently (Stucken *et al.*, 2010).

Although the greatest biomass accumulated under diazotrophic conditions, it was observed that the growth rates were slower without N addition than if N was provided with P, supporting the argument that growth of diazotrophs is slower than that of nondiazotrophic phytoplankton, because of energy expenditures spent for N_2 fixation. It took as long as 6 days for the diazotrophic community to catch up with the other N treatments to reach the maximum biomass. Such differences in growth rates have been inferred before and were thought to explain why formation of diazotrophic cyanobacterial blooms, including *C. raciborskii* (Padisak, 1997), require calm weather and long residence times (Fogg, 1969; Paerl, 1990). LG is an impoundment with a relatively long residence time, thus supporting higher densities of

cyanobacterial blooms than the faster moving sections downstream (Phlips *et al.*, 2007).

Nutrient loading to the SJR has a seasonal and episodic nature, with intermittent periods of nutrient limitation (Phlips *et al.*, 2007). The goal of this study was to elucidate how the fluctuation in nutrient availability may impact community composition and abundances of potentially toxic diazotrophic and nondiazotrophic cyanobacteria, as well as the overall production and trophic status of the ecosystem. The results of this study highlight several aspects of the cyanobacterial community dynamics. The results show that (1) different cyanobacterial communities gain benefit under distinct N/P scenarios during the cyanobacterial bloom period; (2) The historically dominant *Anabaena* sp. has low fitness a.k.a. survival success under DIN enrichment and highest fitness under diazotrophic conditions; (3) *C. raciborskii* has high fitness under both diazotrophic and nondiazotrophic conditions, and is thus employing facultative diazotrophy; and (4) *Microcystis aeruginosa* benefits from the most nutrient-enriched conditions, and it's growth rates in SJR are lower than *C. raciborskii* under all N/P scenarios when nutrients are not saturating, possibly controlled by factors not measured in this study.

These observations help in characterizing the evolving picture of *C. raciborskii* ecophysiology in SJR and other freshwaters worldwide. With its unique physiological flexibility, *C. raciborskii* has a competitive edge in ecosystems that frequently fluctuate between N-enriched and N-limiting conditions.

Acknowledgements

We thank St. Johns River Water Management District for assistance in the field and J. Zehr (UCSC) for facility support. M. Ochiai and L. Kelly are acknowledged for technical assistance. This work was supported by the National Science Foundation DEB award (0452324) for P.M., M.P., and H.P.

References

Agawin NSR, Rabouille S, Veldhuis MJW, Servatius L, Hol S, van Overzee HMJ & Huisman J (2007) Competition and facilitation between unicellular nitrogen-fixing cyanobacteria and non-nitrogen-fixing phytoplankton species. *Limnol Oceanogr* **52**: 2233–2248.

Bouvy M, Ba N, Ka S, Sane S, Pagano M & Arfi R (2006) Phytoplankton community structure and species assemblage succession in a shallow tropical lake (Lake Guiers, Senegal). *Aquat Microb Ecol* **45**: 147–161.

Branco CWC & Senna PAC (1994) Factors influencing the development of *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa* in the Paranoá Reservoir, Brasília, Brazil. *Algol Stud* **75**: 85–96.

Briand JF, Robillot C, Quiblier-Lloberas C, Humbert JF, Coute A & Bernard C (2002) Environmental context of *Cylindrospermopsis raciborskii* (Cyanobacteria) blooms in a shallow pond in France. *Water Res* **36**: 3183–3192.

Briand JF, Leboulanger C, Humbert JF, Bernard C & Dufour P (2004) *Cylindrospermopsis raciborskii* (Cyanobacteria) invasion at mid-latitudes: selection, wide physiological tolerance, or global warming? *J Phycol* **40**: 231–238.

Burford MA, McNeale KL & McKenzie-Smith FJ (2006) The role of nitrogen in promoting the toxic cyanophyte *Cylindrospermopsis raciborskii* in a subtropical water reservoir. *Freshw Biol* **51**: 2143–2153.

Calandrino ES & Paerl HW (2011) Determining the potential for the proliferation of the harmful cyanobacterium *Cylindrospermopsis raciborskii* in Currituck Sound, North Carolina. *Harmful Algae* **11**: 1–9.

Chapman AD & Schelske CL (1997) Recent appearance of *Cylindrospermopsis* (Cyanobacteria) in five hypereutrophic Florida lakes. *J Phycol* **33**: 191–195.

Chorus I & Bartram J (1999) *Toxic Cyanobacteria in Water: A Guide to Public Health Significance, Monitoring, and Management*. Chapman & Hall, London.

Dixon R & Kahn D (2004) Genetic regulation of biological nitrogen fixation. *Nat Rev Microbiol* **2**: 621–631.

Fabbro LD & Duivenvoorden LJ (1996) Profile of a bloom of the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju in the Fitzroy River in tropical central Queensland. *Mar Freshw Res* **47**: 685–694.

Fastner J, Rucker J, Stuken A *et al.* (2007) Occurrence of the cyanobacterial toxin cylindrospermopsin in northeast Germany. *Environ Toxicol* **22**: 26–32.

Figueredo CC, Giani A & Bird DF (2007) Does allelopathy contribute to *Cylindrospermopsis raciborskii* (cyanobacteria) bloom occurrence and geographic expansion? *J Phycol* **43**: 256–265.

Fogg GE (1969) The physiology of an algal nuisance. *Proc R Soc B* **173**: 175–189.

Fogg GE, Stewart WDP, Fay P & Walsby AE (1973) *The Blue-Green Algae*. Academic Press, London.

Francis G (1878) Poisonous Australian lake. *Nature* **18**: 11–12.

Hawkins P, R. , Runnegar MTC, Jackson ARB & Falconer IR (1985) Severe hepatotoxicity caused by the tropical cyanobacterium (blue-green alga) *Cylindrospermopsis raciborskii* (Woloszynska) Seenaya and Subba Raju isolated from a domestic water supply reservoir. *Appl Environ Microbiol* **50**: 1292–1295.

Hawkins PR, Chandrasena NR, Jones GJ, Humpage AR & Falconer IR (1997) Isolation and toxicity of *Cylindrospermopsis raciborskii* from an ornamental lake. *Toxicon* **35**: 341–346.

Hawkins PR, Putt E, Falconer I & Humpage A (2001) Phenotypical variation in a toxic strain of the phytoplankter, *Cylindrospermopsis racibirskii* (Nostocales,

Cyanophyceae) during batch culture. *Environ Toxicol* **16**: 460–467.

Howard JB & Rees DC (1994) Nitrogenase: A nucleotide-dependent molecular switch. *Annu Rev Biochem* **63**: 235–264.

Hutchinson GE (1961) The paradox of plankton. *Am Nat* **XCV**: 21–27.

Istvanovics V, Shafik HM, Presing M & Juhos S (2000) Growth and phosphate uptake kinetics of the cyanobacterium, *Cylindrospermopsis raciborskii* (Cyanophyceae) in throughflow cultures. *Freshw Biol* **43**: 257–275.

Kurmeyer R, Kutzenberger T (2003) Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium. *Microcystis sp. Appl. Environ. Microbiol* **69**: 6723–6730.

Lagos N, Onodera H, Zagatto PA, Andrinolo D, Azevedo S & Oshima Y (1999) The first evidence of paralytic shellfish toxins in the freshwater cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil. *Toxicon* **37**: 1359–1373.

Moisander PH, Steppe TF, Hall NS, Kuparinen J & Paerl HW (2003) Variability in nitrogen and phosphorus limitation for Baltic Sea phytoplankton during nitrogen-fixing cyanobacterial blooms. *Mar Ecol Prog Ser* **262**: 81–95.

Moisander PH, Paerl HW & Zehr JP (2008) Effects of inorganic nitrogen on taxa-specific cyanobacterial growth and *nifH* expression in a subtropical estuary. *Limnol Oceanogr* **53**: 2519–2532.

Moisander PH, Ochiai M & Lincoff A (2009) Nutrient limitation of *Microcystis aeruginosa* in northern California Klamath River reservoirs. *Harmful Algae* **8**: 889–897.

Moustaka-Gouni M, Kormas KA, Vardaka E, Katsiapi M & Gkelis S (2009) Raphidiopsis mediterranea SKUJA represents non-heterocystous life-cycle stages of *Cylindrospermopsis raciborskii* (WOLOSZYNSKA) SEENAYYA et SUBBA RAJU in Lake Kastoria (Greece), its type locality: evidence by morphological and phylogenetic analysis. *Harmful Algae* **8**: 864–872.

Moustaka-Gouni M, Kormas KA, Polykarpou P, Gkelis S, Bobori DC & Vardaka E (2010) Polyphasic evaluation of *Aphanizomenon issatschenkoi* and *Raphidiopsis mediterranea* in a Mediterranean lake. *J Plankton Res* **32**: 927–936.

Ortmann AC, Lawrence JE & Suttle CA (2002) Lysogeny and viral production during a bloom of the cyanobacterium *Synechococcus* spp. *Microb Ecol* **43**: 225–231.

Padisak J (1997) *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: Worldwide distribution and review of its ecology. *Arch Hydrobiol Suppl* **107**: 563–593.

Paerl HW (1988) Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. *Limnol Oceanogr* **33**: 823–847.

Paerl HW (1990) Physiological ecology and regulation of N_2 fixation in natural waters. *Advances in Microbial Ecology* **11**: 305–344.

Paerl HW (1993) Interaction of nitrogen and carbon cycles in the marine environment. *Aquatic Microbiology – An Ecological Approach*, (Ford TE, ed), pp. 343–381. Blackwell Scientific Publications, Boston, MA.

Phlips EJ, Cichra M, Aldridge FJ, Jembeck J, Hendrickson J & Brody R (2000) Light availability and variations in phytoplankton standing crops in a nutrient-rich blackwater river. *Limnol Oceanogr* **45**: 916–929.

Phlips EJ, Bledsoe E, Cichra M & Badylak S (2003) *The Distribution of Potentially Toxic Cyanobacteria in Florida*. (Johnson D & Harbison RD, eds), pp. 22–36. Florida Department of Health and the Center for Disease Control, St. Petersburg, FL.

Phlips EJ, Hendrickson J, Quinlan EL & Cichra M (2007) Meteorological influences on algal bloom potential in a nutrient-rich blackwater river. *Freshw Biol* **52**: 2141–2155.

Piehler MF, Dyble J, Moisander PH, Chapman AD, Hendrickson J & Paerl HW (2009) Interactions between nitrogen dynamics and the phytoplankton community in Lake George, Florida, USA. *Lake Reserv Manag* **25**: 1–14.

Posselt AJ, Burford MA & Shaw G (2009) Pulses of phosphate promote dominance of the toxic cyanophyte *Cylindrospermopsis raciborskii* in a subtropical water reservoir. *J Phycol* **45**: 540–546.

Postgate J (1990) *Nitrogen Fixation*. Edward Arnold, Cambridge University Press, Cambridge, UK.

Présing M, Herodek S, Vörös L & Kóbor I (1996) Nitrogen fixation, ammonium and nitrate uptake during a bloom of *Cylindrospermopsis raciborskii* in Lake Balaton. *Arch Hydrobiol* **136**: 553–562.

Renstrom-Kellner E, Rai AN & Bergman B (1990) Correlation between nitroganse and glutamine synthetase expression in the cyanobacterium *Anabaena cylindrica*. *Physiol Plant* **80**: 12–19.

Reynolds CS (1984) *The Ecology of Freshwater Phytoplankton*. Cambridge University Press, Cambridge, UK.

Reynolds CS (2006) *Ecology of Phytoplankton – Ecology, Biology, and Conservation*. Cambridge University Press, Cambridge, UK.

Saker ML & Eaglesham GK (1999) The accumulation of cylindrospermopsin from the cyanobacterium *Cylindrospermopsis raciborskii* in tissues of the Redclaw crayfish *Cheraz quadricarinatus*. *Toxicon* **37**: 1065–1077.

Saker ML & Griffiths DJ (2000) The effect of temperature on growth and cylindrospermopsin content of seven isolates of *Cylindrospermopsis raciborskii* (Nostocales, Cyanophyceae) from water bodies in northern Australia. *Phycologia* **39**: 349–354.

Shafik HM, Herodek S, Presing M & Voros L (2001) Factors effecting growth and cell composition of cyanoprokaryote *Cylindrospermopsis raciborskii* (Woloszynska) Sheenayya et Subba Raju. *Algol Stud* **102**: 75–93.

Smith VH (2006) Responses of estuarine and coastal marine phytoplankton to nitrogen and phosphorus enrichment. *Limnol Oceanogr* **51**: 377–384.

Stucken K, John U, Cembella A *et al.* (2010) The smallest known genomes of multicellular and toxic cyanobacteria: Comparison, minimal gene sets for linked traits and the evolutionary implications. *PLoS ONE* **5**: e9235.

Valerio E, Pereira P, Saker ML, Franca S & Tenreiro R (2005) Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. *Harmful Algae* **4**: 1044–1052.

Welschmeyer NA (1994) Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. *Limnol Oceanogr* **39**: 1985–1992.

Woloszynska J (1912) Das Phytoplankton einiger javanisher Seen, mit Berucksichtigung des Sawa-Planktons. *Bull Acad Sci Cracovie Mat Nat Bull Ser B* **1912**: 649–709.

Yilmaz M & Phlips EJ (2011) Diversity of and selection acting on cylindrospermopsin *cyrB* gene adenylation domain sequences in Florida. *Appl Environ Microbiol* **77**: 2502–2507.

Yilmaz M, Phlips EJ, Szabo NJ & Badylak S (2008) A comparative study of Florida strains of *Cylindrospermopsis* and *Aphanizomenon* for cylindrospermopsin production. *Toxicon* **51**: 130–139.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of statistical differences between treatments based on Repeated Measures ANOVA.
Table S2. Dissolved nutrient concentrations in the experimental incubations over the duration of the experiments from Lake George and St. John's River.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.