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Effects of nitrogenous compounds and phosphorus on the growth of toxic and non-toxic strains of *Microcystis* during cyanobacterial blooms

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ABSTRACT: Since the mid-twentieth century, both nutrient delivery rates and the frequency of harmful algal blooms (HABs) in coastal aquatic ecosystems have intensified. Recent studies have shown that nitrogen (N) or phosphorus (P) can limit primary production in freshwater systems, and *Microcystis* is able to utilize both inorganic and organic forms of N. The present study quantified the microcystin synthetase gene (*mcyD*) and the ribosomal RNA gene (16S) to assess how various nutrient sources affected the growth of toxic and non-toxic strains of *Microcystis* during natural blooms. During the present study, dense *Microcystis* blooms (>10^6 cell equivalents l^{-1}) were observed within 2 contrasting ecosystems in the eastern USA: a tidal tributary and a eutrophic lake. In both systems, all *Microcystis* populations were stimulated by N more frequently than P during nutrient amendment experiments. The abundance of toxic strains of *Microcystis* was enhanced by nutrient enrichment more frequently (83% of experiments) than non-toxic strains (58% of experiments), suggesting that toxic strains may have a greater demand for both nutrients. Furthermore, abundances of toxic strains of *Microcystis* were enhanced by inorganic N more frequently (67% of experiments) than organic N (8% of experiments), while non-toxic strains were stimulated by organic N (50% of experiments) more frequently than inorganic N (25% of experiments). Inorganic P increased abundances of toxic strains of *Microcystis* more frequently than non-toxic strains (42 and 33% of experiments, respectively). Therefore, the dominance of toxic *Microcystis* may be influenced by both the concentration and species of nutrients, with higher concentrations of inorganic N and/or P likely promoting blooms dominated by toxic strains and potentially yielding higher microcystin concentrations.

KEY WORDS: *Microcystis* · Toxic · Non-toxic · Nitrogen · Phosphorus · Inorganic · Organic · *mcyD*

INTRODUCTION

Harmful algal blooms (HABs) are a significant threat to fisheries, public health, and economies around the world. There are strong links between increased nutrient loading and HABs (Anderson et al. 2008, Heisler et al. 2008), particularly within freshwater ecosystems (Paerl 1988, Paerl et al. 2001). Traditionally, rates of primary production in freshwater ecosystems have been thought to be limited by phosphorus (P; Schindler 1977, Smith 1983, Hecky & Kilham 1988), and increases in P-loading (mainly due to anthropogenic influences) have often been associated with blooms of cyanobacteria within these systems (Likens 1972, Paerl 1988). This paradigm is partly based on the assumption that diazotrophic cyanobacteria such as *Anabaena* and *Aphanizomenon* will dominate P-enriched systems that are depleted in nitrogenous nutrients (Paerl 1982, 1988).

Nitrogen (N) may also play an important role in the occurrence of freshwater cyanobacteria blooms, par-

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ticularly for non-diazotrophic cyanobacteria such as *Microcystis*. Laboratory studies have demonstrated that higher N concentrations enhance the growth and toxicity of non-diazotrophic cyanobacteria such as *Microcystis* and *Oscillatoria* (Watanabe & Oishi 1985, Codd & Poon 1988, Utkilen & Gjølme 1995, Orr & Jones 1998). Some freshwater systems that have either large external P supplies or are shallow and have strong benthic mobilization of P from sediments can host levels of dissolved N that limit primary production (Vollenweider & Kerekes 1982, Paerl 2009). Finally, recent field studies have demonstrated that N-loading can promote *Microcystis* blooms (Gobler et al. 2007, Moisander et al. 2009a).

Many cyanobacteria (both diazotrophic and non-diazotrophic) are able to utilize both organic and inorganic forms of N (Paerl 1988). Studies examining ^{15}N assimilation by cyanobacterial blooms have observed uptake rates were highest for ammonium, followed by urea, then nitrate, suggesting that reduced forms of N may promote cyanobacterial blooms (Takamura et al. 1987, Mitamura et al. 1995, Présing et al. 2008). Furthermore, a recent study conducted by Dai et al. (2009) found that a toxic clone of *Microcystis aeruginosa* was able to take up and utilize amino acids, such as alanine, leucine, and arginine, to support growth and toxin production. Overall, these previous studies suggest both organic and inorganic N can be important in promoting blooms of cyanobacteria.

Bloom populations of *Microcystis* are comprised of toxic and non-toxic strains, which are distinguishable only via quantification of the microcystin synthetase gene (*mcyA-J*; Kurmayer & Kutzenberger 2003, Rinta-Kanto et al. 2005, Davis et al. 2009). Previous laboratory studies have found that non-toxic strains of *Microcystis* and *Anabaena* require lower nutrient concentrations to achieve maximal growth rates compared to toxic strains (Rapala et al. 1997, Vézie et al. 2002). Further, laboratory research suggests toxic strains of *Microcystis* are able to outgrow non-toxic strains at high inorganic N concentrations (Vézie et al. 2002). While the role of nutrients in the growth of total *Microcystis* populations in culture and in the field has been studied (Watanabe & Oishi 1985, Codd & Poon 1988, Blomqvist et al. 1994, Fujimoto et al. 1997, Orr & Jones 1998, Baldia et al. 2007, Gobler et al. 2007, Moisander et al. 2009a), the manner in which nutrients may promote toxic and non-toxic strains of *Microcysits* within an ecosystem setting is unknown.

The aim of the present study was to investigate the role of various forms of organic and inorganic nitrogenous compounds, as well as orthophosphate, in the growth of toxic and non-toxic strains of *Microcystis* during natural bloom events. The dynamics of *Microcystis* blooms and nutrients in a lake and a tidal tributary were monitored. Concurrently, nutrient amendment experiments were conducted to investigate how organic and inorganic N as well as orthophosphate affected the dominance of toxic and non-toxic strains of *Microcystis.* This combined observational and experimental approach allowed for a robust assessment of how different forms of N and P may promote toxic *Microcystis* blooms. To our knowledge, this is the first study to specifically quantify the differential responses of toxic and non-toxic strains of *Microcystis* to nutrients during bloom events.

MATERIALS AND METHODS

Sampling-site and water-quality monitoring. During the present study, 2 hydrodynamically different ecosystems were studied in 2008. The Transquaking River (TR; 38° 30′ 45′ N; 75° 58′ 7′ W) is a flowing tributary that empties into the Chesapeake Bay. It spans 37 km along Maryland's (USA) eastern shore, and has previously been host to annual toxic cyanobacterial blooms (Tango & Butler 2008). The seasonal rainfall (May to September) for this system during 2008 was, higher during May and June (14 and 11 cm, respectively) when compared to July through September (11 cm total). Lake Agawam (LA) is a small (0.5 km^2), shallow (4 m maximum depth), closed eutrophic system on Long Island, New York (USA; 40° 52′ 05″ N; 72° 25′ 96″ W) that often experiences dense and toxic cyanobacterial blooms (Gobler et al. 2007). In 2008, rainfall near LA was light in May and October (5.5 and 3.2 cm, respectively) and higher from June through September (13 ± 3.7 cm mo^{-1}). Both systems had similar levels of light transmission (mean Secchi depths = 0.6 ± 0.1 m). During 2008, LA and TR were sampled biweekly to monthly before, during, and after cyanobacterial blooms (May to November). At each site, surface temperature, dissolved oxygen, and pH were measured using a YSI 556 sonde. Twenty liters of surface water was collected in acid-cleaned carboys and taken to the laboratories where triplicate extracted chlorophyll *a* and *in vivo* phycocyanin (as a proxy for total cyanobacteria) were measured with Turner Designs fluorometers using standard techniques (Parsons et al. 1984, Watras & Baker 1988, Lee et al. 1994). For microcystin analysis, whole water was filtered onto triplicate 47 mm glass fiber filters (GF/F) and stored at –20°C until analysis. Duplicate whole water samples were preserved with Lugol's iodine solution (5% final concentration) to characterize and quantify the phytoplankton assemblage. For molecular analysis of *Microcystis*, water was filtered onto triplicate 2 µm polycarbonate filters and immediately placed in CTAB (cetyl trimethylammonium bromide) lysis buffer. The

samples were heated at 50°C for 10 min, then flash frozen in liquid nitrogen, and stored at −80°C until analysis. Triplicate dissolved nutrient samples were collected by filtering lake water through combusted GF/F glass fiber filters and stored at −20°C until analysis. All nutrient analyses were conducted using wet chemistry methods. Nitrate was analyzed by reducing the nitrate to nitrite using spongy cadmium as per Jones (1984). Ammonium, phosphate, and silicate were analyzed according to Parsons et al. (1984). Urea was analyzed following Price & Harrison (1987). Dissolved free amino acids (DFAA) were measured in duplicate by high performance liquid chromatography (HPLC) (Lindroth & Mopper 1979, Cowie & Hedges 1992). Total dissolved nitrogen and phosphorus (TDN and TDP, respectively) were analyzed using persulfate digestion (Valderrama 1981). Dissolved organic N and P (DON and DOP) were determined by subtracting DIN (dissolved inorganic N = nitrate, nitrite, and ammonium) from TDN and by subtracting DIP (orthophosphate) from TDP, respectively. The degree to which individual biological and environmental variables were correlated was evaluated by a Pearson's correlation matrix.

Impacts of inorganic and organic nitrogen on toxic and non-toxic *Microcystis*. Experiments were conducted to assess the impact of increased organic and inorganic N or orthophosphate concentrations on toxic and non-toxic *Microcystis* populations in TR and LA. Sets of triplicate, 1 l bottles (n = 21) were filled with surface water from each experimental site and were either left unamended to serve as a control, or amended with various forms of N: 20 µM nitrate (NO_3^-), 20 µM ammonium (NH_4^+), 10 µM (=20 µM N) urea, 10 µM (=20 µM N), L-glutamine (GA), P (1.25 µM orthophosphate), or a combined treatment of NO_3^- and P. For the experiments conducted in LA, the bottles were placed in Old Fort Pond at the Stony Brook–Southampton marine station located ~1 km west of Lake Agawam. For the experiments conducted in TR, experimental bottles were placed in an incubator (REVCO) with light and temperature levels matching conditions in TR. Light intensity and temperatures during experiments were measured every minute with *in situ* loggers (Onset Computer Corporation) and indicated that incubation temperatures and light levels remained within the same range as those found in each ecosystem. All bottles were gently inverted every 6 to 8 h. After 48 h, samples were filtered as described above to quantify chlorophyll *a* concentrations and densities of total, toxic, and non-toxic *Microcystis* via molecular methods. Using the molecular techniques described below, the densities of total and toxic *Microcystis* in each experimental bottle were quantified and the densities of non-toxic *Microcystis* were determined by difference, providing triplicate densities for each population within each treatment. For the 4 communities measured (total phytoplankton, total *Microcystis*, toxic *Microcystis*, and non-toxic *Microcystis*) differences in abundances among nutrient treatments were compared by means of 1-way ANOVAs or non-parametric Kruskal-Wallis tests, and differences among individual treatments were subsequently assessed with post hoc Tukey multiple comparison tests. For all results the standard variance presented is ± 1 standard error (SE).

Microscopic analysis. Densities of *Microcystis* and other co-occurring cyanobacteria were quantified using gridded Sedgewick-Rafter counting chambers. For all samples, at least 200 cells, colonies, chains, or trichomes were enumerated. *Microcystis*, *Anabaena*, and *Aphanizomenon* were enumerated to the colony, chain, or trichome level, respectively. For eukaryotic plankton such as diatoms, dinoflagellates, and chlorophytes, cells were enumerated. This approach provided good reproducibility (<15% relative standard deviation) among samples.

Microcystin analysis. Filters for microcystin analyses were extracted in 50% methanol containing 1% acetic acid using ultrasound (four 20 s bursts with a 20 s pause between bursts). Previous work has demonstrated that this extraction protocol provides >90% recovery of microcystin-LR from glass fiber filters (Boyer et al. 2004). Following extraction, the methanolic extract was stored at −80°C until analysis. Before analysis, the microcystin extract was diluted to 5% methanol and buffered to a pH of 7 using a 5% Tris-EDTA buffer solution. Microcystin concentrations were measured using a microcystin enzyme-linked immunosorbent assay (Abraxis LLC) following the methodologies of Fischer et al. (2001). This assay is congener-independent as it is sensitive to the ADDA moiety that is found in almost all microcystins. These analyses yielded a detection limit of 0.10 µg l^{-1}, a relative standard deviation of 10 ± 1% for replicated environmental samples, and 99.5 ± 8.2% recovery from environmental samples spiked with 5 µg l^{-1} microcystin-LR, a concentration within the range of samples collected during the present study.

Molecular analyses. Total cellular nucleic acids were extracted from field and experimental samples using methods described in Coyne et al. (2001). Filtered environmental or experimental samples were submersed in CTAB buffer (Dempster et al. 1999) and supplemented with 20 µg l^{-1} pGEM-3z(f+) plasmid (Promega Corporation), which served as an internal control for extraction efficiency and PCR inhibition (Coyne et al. 2005). The filters were then flash frozen using liquid nitrogen and stored at −80°C until extraction. Nucleic acids were extracted after an initial heat-

ing step at 65°C, followed by a double chloroform extraction, and an isopropanol precipitation. Extracted nucleic acids were resuspended in 20 µl of LoTE (3 mmol l^{-1} Tris-HCl [pH 8.0], 0.2 mmol l^{-1} EDTA [pH 8.0]). The quantity and quality of nucleic acids were assessed with a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies).

Two *Microcystis*-specific genetic targets were used during the present study, the *Microcystis* 16S rRNA gene (*Microcystis* 16S rDNA) and *mcyD* gene. The *Microcystis* 16S rRNA gene is specific to the *Microcystis* genus and permitted quantification of the abundance of the total *Microcystis* population. The *mcyD* gene is found within the microcystin synethtase gene operon, which is responsible for the production of microcystin and is only found in toxic strains of *Microcystis* (Tillett et al. 2000), thus allowing us to quantify the toxic population of *Microcystis* (Davis et al. 2009). The non-toxic population was calculated as the difference between the total and toxic population. Quantitative PCR (qPCR) was carried out using an ABI 7300 Real Time PCR instrument using TaqMan labeled probes (Applied Biosystems) and *Microcystis*-specific *mcyD* and 16S rDNA primers (Table 1). Each 10 µl reaction included 5µl of 2× TaqMan Master Mix (Applied Biosystems), 10 µM of each primer (Integrated DNA Technologies), 10 µM TaqMan probe (Table 1), and 1 µl of a 1:25 dilution of the unknown DNA or standard. For amplification of the pGEM and 16S targets, the cycling conditions were 95°C for 10 min, followed by 55 cycles of 95°C for 15 s and 60°C for 1 min. For the *mcyD* gene, the cycling conditions were 95°C for 10 min, followed by 55 cycles of 95°C for 15 s, followed by 50°C for 1 min, then 60°C for 1 min. To prepare standard samples, cultured toxic *Microcystis aeruginosa*, Clone LE-3 (Rinta-Kanto et al. 2005), was enumerated by standard microscopy and collected on polycarbonate filters that were prepared and extracted as described above for field samples. A standard curve of dilutions of the extracted LE-3 genomic DNA was run with each analytical run to serve as a reference for numbers of total and toxic *Microcystis* cells. Such analyses indicate the number of 16S rDNA and *mcyD* genes per LE-3 cell and were not statistically different. Since some *Microcystis* cells may carry varying copies of the 16S rDNA and *mcyD* genes, data were expressed as 'cell equivalents' rather than cell number (Rinta-Kanto et al. 2005). The numbers of toxic and total *Microcystis* cells were determined using the ΔΔCT method (Livak & Schmittgen 2001, Coyne et al. 2005). The difference between the number of *mcyD* cell equivalents (toxic cells) and 16S rDNA cell equivalents (total cells) represented the number of non-toxic cell equivalents (Rinta-Kanto et al. 2005).

Molecular quantification of *Microcystis* has been used in multiple field studies to date (e.g. Rinta-Kanto et al. 2005, Oberholster et al. 2006, Rinta-Kanto & Wilhelm 2006, Hotto et al. 2008, Davis et al. 2009, Ha et al. 2009, Moisander et al. 2009a,b, Rinta-Kanto et al. 2009a,b, Ye et al. 2009, Baxa et al. 2010). Despite the increasingly common use of this method, there are fundamental differences between it and traditional cell counts that could create deviances between cell equivalents and traditional microscopic counts. Traditional microscopic counts can underestimate total *Microcystis* densities. Individual *Microcystis* cells range between 4 and 6 µm in diameter, and in wild field populations both individual cells and colonies are present. However, it is not possible to distinguish between individual *Microcystis* cells and small phytoplankton in natural bloom samples using light microscopy. In contrast, our molecular method quantified all *Microcystis* cells caught on a 2 µm filter. Furthermore, there can be multiple gene copies per cyanobacterium cell, which could also lead to differences between molecular

Table 1. Primers (Integrated DNA Technologies) and probes (Applied Biosystems) used in the qPCR analysis. F: forward primer; R: reverse primer; FAM: 6-Carboxyfluorescein; BHQ-1: Black Hole Quencher-1 (quenching range 480 to 580 nm)

DNA target	Primer	Sequence (5′–3′)	Source
pGEM plasmid DNA	M13F	CCCAGTCACGACGTTGTAAAAACG	Coyne et al. (2005)
	pGEMR	TGTGTGGAATTGTGAGCGGA	Coyne et al. (2005)
	pGEM probe	(Taq) FAM-CACTATAGAATACTCAAGCTTGCAT GCCTGCA-BHQ-1	Coyne et al. (2005)
Microcystis 16S rDNA	184F	GCCGCRAGGTGAAAMCTAA	Neilan et al. (1997)
	431R	AATCCAAARACCTTCCTCCC	Neilan et al. (1997)
	Probe	(Taq) FAM-AAGAGCTTGCGTCTGATTAGCTAGT-BHQ-1	Rinta-Kanto et al. (2005)
Microcystis mcyD	F2	GGTTCGCCTGGTCAAAGTAA	Kaebernick et al. (2000)
	R2	CCTCGCTAAAGAAGGGTTGA	Kaebernick et al. (2000)
	Probe	(Taq) FAM-ATGCTCTAATGCAGCAACGGCAAA-BHQ-1	Rinta-Kanto et al. (2005)

quantification and microscopic counts. Importantly, we have consistently obtained identical cell densities of the toxic *Microcystis* clone LE-3 when quantified via light microscopy or qPCR with the 16S or *mcy*D gene markers (T. W. Davis et al. unpubl. data). Furthermore, prior studies (Davis et al. 2009) have found that toxic (*mcyD*-containing) *Microcystis* cells are consistently (4 ecosystems, multiple years) correlated with microcystin concentrations, whereas non-*mcyD*–containing cell equivalents are not correlated with toxin levels, suggesting that this method accurately enumerates toxic and non-toxic *Microcystis* populations.

RESULTS

Transquaking River cyanobacteria blooms

In 2008, TR hosted dense cyanobacterial blooms co-dominated by *Microcystis* and *Aphanizomenon* (Table 2). Peak algal and cyanobacterial densities occurred on 10 June, while peak *Microcystis* densities, as measured by the 16S rRNA gene ($3.1 \pm 0.63 \times 10^8$ cell equivalents l^{-1}) occurred on 24 June (Fig. 1). Within the total *Microcystis* population, toxic strains dominated from late May through early July with peak densities of $2.8 \pm 0.23 \times 10^8$ cell equivalents l^{-1}, representing 62 ± 20% of the total *Microcystis* population (Fig. 1). After mid-July, dominance shifted towards non-toxic strains of *Microcystis*, which achieved peak densities of $3.6 \pm 0.13 \times 10^7$ cell equivalents l^{-1} in late August (Fig. 1). *Microcystis* colonies in TR on average comprised 47 ± 19 cells. Microcystin concentrations ranged from 0.36 to 12.1 µg l^{-1} and peaked in unison with toxic *Microcystis* densities on 24 June (Fig. 1). Microcystin concentrations were significantly correlated with toxic *Microcystis* densities ($p < 0.01$), but not

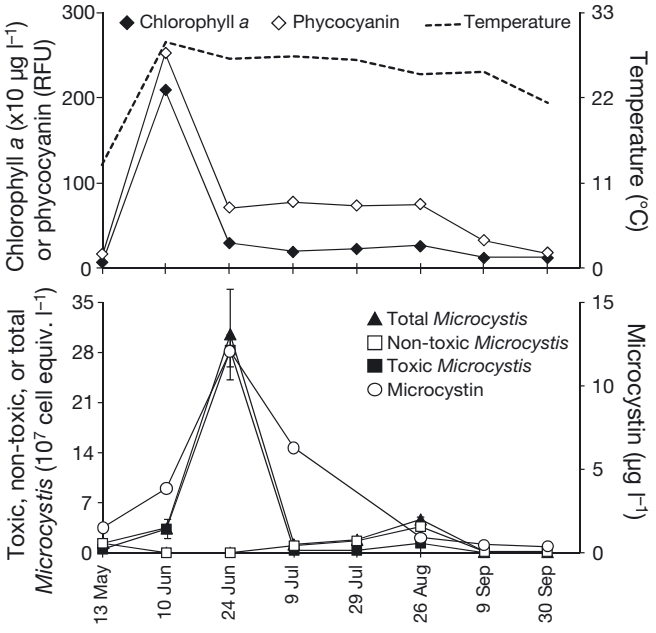

Fig. 1. Time series of parameters measured in the Transquaking River in 2008. Upper panel: levels of total chl *a*, phycocyanin, and temperature. Lower panel: densities of total, non-toxic, and toxic *Microcystis*, as well as concentrations of microcystin. Error bars represent ±1 SE of replicated samples

Table 2. Mean autotrophic plankton densities (SE in parentheses) as quantified via light microscopy for the Transquaking River and Lake Agawam in 2008. Counts are in colonies, chains, and trichomes per milliliter for *Microcystis, Anabaena*, and *Aphanizomenon*, respectively, and in cells per milliliter for the other groups

Sampling date	*Microcystis*	*Anabaena*	*Aphanizomenon*	Diatoms	Chlorophytes	Dinoflagellates
Transquaking River						
13 May	325 (25)	0 (0)	0 (0)	1100 (25)	125 (30)	0 (0)
10 Jun	400 (27)	35000 (110)	0 (0)	130 (9)	0 (0)	210 (80)
24 Jun	4900 (100)	700 (100)	51000 (2800)	2600 (220)	1100 (100)	3000 (180)
9 Jul	7350 (250)	125 (125)	120000 (7100)	16000 (1100)	3900 (580)	125 (130)
29 Jul	4800 (80)	15 (15)	20000 (1400)	1400 (10)	990 (50)	210 (70)
26 Aug	11000 (95)	240 (30)	21000 (600)	1500 (55)	15 (15)	100 (10)
9 Sep	3300 (24)	845 (12)	44000 (1100)	2800 (12)	4800 (210)	155 (10)
30 Sep	1000 (45)	0 (0)	24000 (182)	1600 (68)	3000 (50)	95 (5)
Lake Agawam						
3 Jun	952 (95)	0 (0)	0 (0)	200 (20)	540 (50)	0 (0)
5 Jun	1420 (142)	0 (0)	0 (0)	170 (20)	250 (30)	0 (0)
1 Jul	813 (81)	27 (3)	13 (1)	20 (2)	67 (7)	0 (0)
23 Jul	1300 (130)	380 (38)	820 (82)	180 (20)	160 (0)	0 (0)
18 Aug	1800 (180)	0 (0)	30 (30	0 (0)	40 (4)	0 (0)
23 Sep	5000(500)	47 (5)	80 (8)	890 (90)	20 (2)	0 (0)
15 Oct	4000 (400)	0 (0)	40 (4)	170 (20)	27 (3)	0 (0)
30 Oct	4900 (490)	67 (7)	220 (22)	290 (30)	40 (40)	0 (0)

non-toxic *Microcystis* densities, chlorophyll *a*, phycocyanin, or the total cyanobacterial densities ($p > 0.05$). Concentrations of inorganic nitrogen were highest during May and June (>1 µM), but dropped to <1 µM during the summer and early fall (Table 2), while DIN:DIP ratios were chronically low (3.2 ± 1.8). Silicate levels were high in TR (25 ± 8.3 µM), while urea and DFAA concentrations were quite low (0.3 ± 0.1 and 0.1 ± 0.01 µM, respectively). DON was the largest aqueous N pool, with concentrations ranging from 14.1 ± 6.59 to 44.8 ± 1.85 µM (Table 3), while DOP concentrations ranged from 0.4 to 1.4 µM (Table 3). Non-toxic strains of *Microcystis* were significantly correlated with DON concentrations from June through October ($p < 0.01$). Temperatures in TR rose from 13.4°C in May to 29.2°C in July and dropped to 21.4°C by October (Fig. 1).

Lake Agawam cyanobacteria blooms

LA hosted cyanobacterial blooms that differed from those in TR in community composition and intensity (Table 2, Fig. 2). The LA blooms were dominated by *Microcystis* on every date sampled (Table 2). Total *Microcystis* densities ranged from 1.59 ± 0.09 to 17.3 ± 0.10 × 10^6 cell equivalents l^{-1}, with peak densities occurring on 15 October (Fig. 2). Unlike TR, the total *Microcystis* community was dominated by non-toxic strains throughout the 2008 field season (83 ± 4% of total *Microcystis* cells; Fig. 2). Non-toxic strains of *Microcystis* ranged from 1.42 ± 0.06 to 15.3 ± 0.06 × 10^6 cell equivalents l^{-1} (Fig. 2), while toxic *Microcystis* strains ranged from 0.7 ± 0.3 to 2.81 ± 0.20 × 10^6 cell equivalents l^{-1}, with peak densities occurring on

Table 3. Mean dissolved inorganic and organic nutrient concentrations (µM with SE in parentheses) for the Transquaking River and Lake Agawam, 2008. DIN: dissolved inorganic nitrogen; DIP: dissolved inorganic phosphate; DON: dissolved organic nitrogen; DOP: dissolved organic phosphorus; DFAA: dissolved free amino acids

Sampling date	Inorganic nutrients				
	Nitrate	Ammonium	Silicate	DIN	DIP
Transquaking River					
13 May	10.25 (0)	6.36 (0.13)	16.0 (3.28)	16.6 (0.23)	3.87 (0.38)
10 Jun	1.99 (0.68)	3.56 (0.02)	1.23 (0.04)	3.77 (0.35)	2.92 (0.39)
24 Jun	0.08 (0.02)	0.84 (0.13)	48.5 (7.21)	0.92 (0.12)	1.09 (0.06)
9 Jul	0.17 (0.10)	0.74 (0.07)	71.6 (3.02)	0.91 (0.06)	0.97 (0.08)
29 Jul	0.07 (0.02)	0.57 (0.03)	11.9 (0.27)	0.65 (0.04)	0.63 (0.05)
26 Aug	0.30 (0.16)	0.62 (0.02)	10.0 (1.07)	0.91 (0.17)	0.37 (0.04)
9 Sep	0.59 (0.03)	0.57 (0.03)	27.6 (4.99)	0.82 (0.23)	0.41 (0.05)
30 Sep	0.26 (0.08)	0.57 (0.08)	15.8 (0.03)	0.69 (0.20)	0.39 (0.01)
Lake Agawam					
5 Jun	5.48 (0.13)	2.45 (0.28)	22.6 (0.01)	7.94 (0.34)	0.06 (0.00)
1 Jul	1.18 (0.08)	3.44 (0.77)	20.7 (1.23)	4.62 (0.70)	0.38 (0.05)
23 Jul	1.25 (0.03)	1.23 (0.15)	45.9 (10.7)	2.06 (0.57)	0.12 (0.01)
18 Aug	0.43 (0.25)	0.48 (0.01)	59.4 (2.79)	0.78 (0.33)	0.30 (0.08)
23 Sep	4.14 (0.12)	0.47 (0.04)	68.5 (1.30)	4.61 (0.15)	0.21 (0.10)
15 Oct	6.85 (0.14)	0.68 (0.04)	68.1 (5.80)	7.53 (0.14)	0.18 (0.02)

Sampling date	Organic nutrients				Ratios	
	Urea	DFAA	DON	DOP	DIN:DIP	DON:DOP
Transquaking River						
13 May	0.38 (0.02)	0.1 (0.002)	14.1 (6.59)	0.61 (0.06)	4	23
10 Jun	0.42 (0.01)	0.28 (0.005)	19.4 (1.78)	1.06 (0.05)	1	18
24 Jun	0.45 (0.01)	0.1 (0.002)	24.1 (1.44)	1.05 (0.16)	1	23
9 Jul	0.31 (0.01)	0.06 (0.002)	28.5 (2.82)	0.86 (0.27)	1	33
29 Jul	0.38 (0.09)	0.07 (0.002)	21.9 (0.99)	0.31 (0.06)	1	71
26 Aug	0.13 (0.02)	0.09 (0.006)	44.8 (1.85)	0.56 (0.10)	2	79
9 Sep	0.27 (0.05)	0.05 (0.002)	24.6 (1.71)	0.47 (0.09)	2	52
30 Sep	0.14 (0.08)	0.07 (0.002)	23.9 (6.69)	0.55 (0.25)	2	43
Lake Agawam						
5 Jun	0.06 (0.06)	0.13 (0.003)	17.0 (8.27)	1.09 (0.37)	262	16
1 Jul	0.05 (0.02)	0.18 (0.01)	13.4 (2.81)	0.68 (0.13)	12	20
23 Jul	0.04 (0.00)	0.10 (0.003)	14.0 (2.66)	1.15 (0.10)	17	12
18 Aug	0.06 (0.04)	0.05 (0.002)	7.94 (0.78)	0.60 (0.06)	3	13
23 Sep	0.35 (0.02)	0.09 (0.002)	12.6 (1.59)	0.72 (0.23)	22	17
15 Oct	0.26 (0.14)	0.11 (0.003)	4.91 (1.15)	0.97 (0.29)	42	5

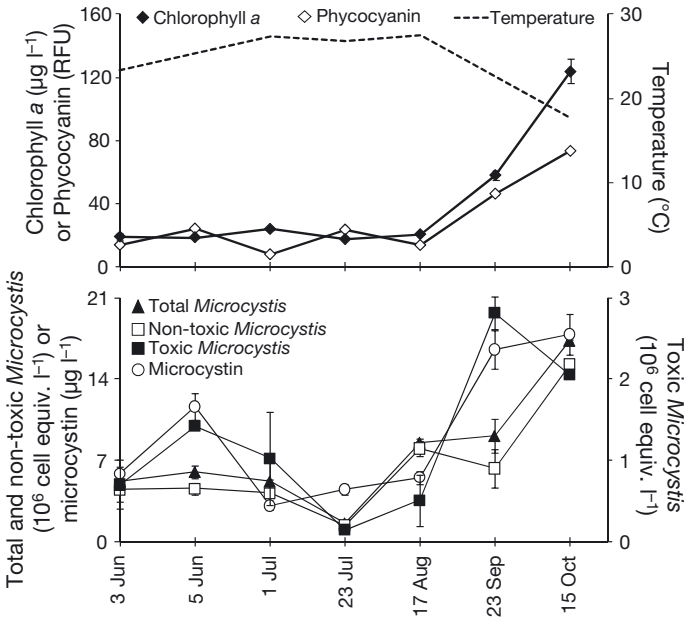

Fig. 2. Time series of parameters measured in Lake Agawam in 2008. Further details as in Fig. 1

23 September (Fig. 2). *Microcystis* colonies in LA on average comprised 65 ± 9 cells. As was the case in TR, microcystin concentrations (3.1 to 17.8 µg l^{-1}) were significantly correlated with toxic *Microcystis* cell equivalents ($p < 0.01$), but not with total or non-toxic *Microcystis* cell equivalents ($p > 0.05$). DIN concentrations in LA were high in the early summer (4 to 7 µM), lower in July and August (<2 µM), and elevated again in the fall (4 to 7 µM; Table 3). In contrast, silicate levels were always high (20 to 70 µM) in LA (Table 3), while DIP, urea, and DFAA were always low (<0.4, 0.1, and 0.1 µM, respectively; Table 3). DON and DOP were the largest dissolved N and P pools in LA, with mean concentrations of 10 ± 2 and 1.2 ± 0.2 µM (Table 3). Temperatures in LA rose from 23.3°C in June to 27.5°C in August and dropped to 10.3°C by the end of October (Fig. 2).

Growth responses of toxic and non-toxic strains of *Microcystis* during nutrient amendment experiments

Transquaking River

Biomass of the total phytoplankton community in TR was significantly increased by one form of N in every experiment conducted and was most consistently stimulated by nitrate ($p < 0.05$; Table 4). Similarly, the total *Microcystis* population in TR was stimulated by N through most of the field season (5 of 6 experiments; $p < 0.05$; Table 4, Fig. 3). Interestingly, the total *Microcystis* community was more frequently stimulated by urea (50% of experiments) than by nitrate (33% of experiments), and was never stimulated by ammonium ($p < 0.05$; Table 4; Fig. 3). GA also stimulated the total *Microcystis* population in a third of the experiments conducted, doubling cell equivalents above the control in the final 2 experiments ($p < 0.05$; Table 4, Fig. 3). Increased phosphate concentrations yielded significantly increased *Microcystis* cell equivalents in 2 experiments conducted in June and early July ($p < 0.05$; Table 4, Fig. 3).

Toxic strains of *Microcystis* were stimulated more often by N enrichment (83% of experiments) than their

Table 4. Treatments that significantly stimulated the total cyanobacterial community, total *Microcystis* community, non-toxic *Microcystis*, and toxic *Microcystis* relative to control treatments ($p < 0.05$) during nutrient amendment experiments in the Transquaking River and Lake Agawam in 2008. NO_3: nitrate; NH_4: ammonium; N + P: nitrate and orthophosphate; P: orthophosphate; U: urea; GA: L-glutamine. A dash indicates no treatment significantly increased the population over the control

Sampling date	Total phytoplankton	Total *Microcystis*	Non-toxic *Microcystis*	Toxic *Microcystis*
Transquaking River				
10 Jun	NO_3	NO_3, U, P	–	NO_3, U, P
24 Jun	N+P	–	U, GA, N+P	NO_3, NH_4
9 Jul	NO_3, N+P	NO_3, P	–	NO_3, P
29 Jul	NO_3, NH_4, U, GA	P, N+P	P	NO_3, NH_4, N+P
26 Aug	NO_3, NH_4, U, GA, N+P	U, GA	U, GA	–
9 Sep	NO_3, GA, N+P	U, GA, N+P	U, GA	N+P
Lake Agawam				
5 Jun	–	–	–	P
1 Jul	NH_4, P, N+P	–	–	NH_4, P, N+P
23 Jul	N+P	NO_3, NH_4, U, P	NO_3, NH_4, U, P	NO_3
18 Aug	NO_3, U, GA, N+P	NO_3, U, P, N+P	NO_3, U, P, N+P	–
23 Sep	N+P	–	–	NO_3, NH_4
15 Oct	–	NO_3, NH_4, U, GA, P, N+P	NO_3, NH_4, U, GA, P, N+P	NO_3, NH_4, P

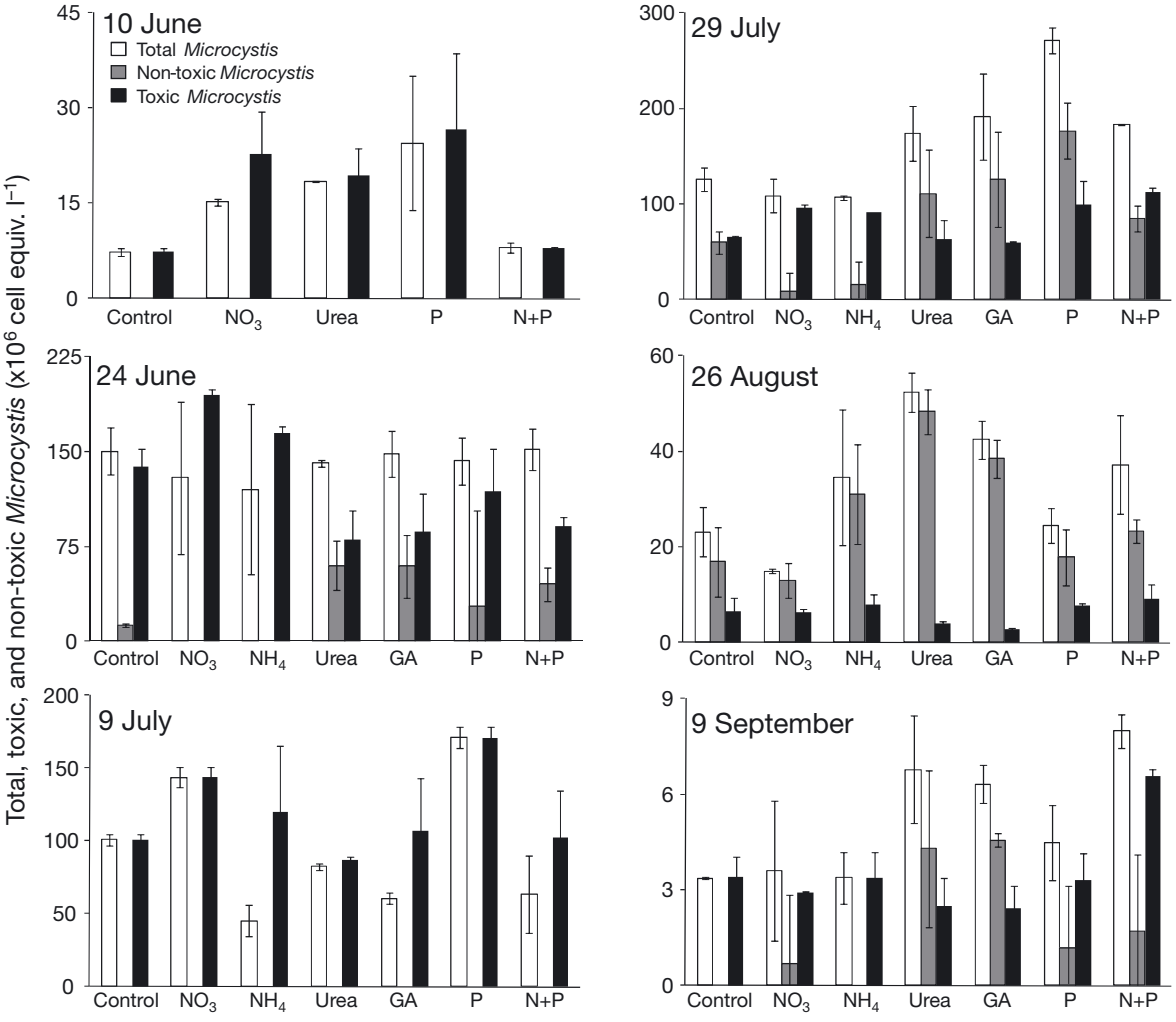

Fig. 3. Total, non-toxic, and toxic *Microcystis* densities during nutrient amendment experiments conducted in the Transquaking River during the summer of 2008. Error bars represent ±1 SE of triplicate experimental bottles. NO_3: nitrate; P: orthophosphate; N + P: nitrate and orthophosphate; NH_4: ammonium; GA: L-glutamine. During several experiments and treatments, the non-toxic *Microcystis* population was below methodological detection limits

non-toxic counterparts (66% of experiments; $p < 0.05$; Table 4, Fig. 3). Toxic strains were stimulated by inorganic forms of N during most experiments (83%), increasing cell equivalents from 1 to 4 times above the control ($p < 0.05$; Table 4, Fig. 3). In contrast, cell equivalents of toxic strains were enhanced by urea in only 1 experiment, but were never affected by GA (Table 4, Fig. 3). P additions yielded increased toxic *Microcystis* in one-third of the all TR experiments, increasing cell equivalents, on average, 2-fold beyond the control ($p < 0.05$; Table 4, Fig. 3). Finally, on 2 dates (29 July and 9 September), the combined addition of N and P stimulated toxic *Microcystis* populations ($p < 0.05$; Table 4, Fig. 3). In a manner nearly the opposite of toxic strains, non-toxic strains of *Microcystis* were never significantly affected by the addition of any individual inorganic N compound ($p > 0.05$; Table 4, Fig. 3). These strains were stimulated, however, by organic N compounds (urea and GA) during half of the experiments, displaying cell equivalents between 2 and 6 times higher than the control ($p < 0.05$; Table 4, Fig. 3). Finally, non-toxic strains were significantly enhanced above the control by P in 1 experiment and by the dual addition of N and P in another experiment ($p < 0.05$; Table 4, Fig. 3).

Lake Agawam

None of the phytoplankton populations monitored were limited by any form of N in LA in June (Table 4, Fig. 4). From early July to late September, the total

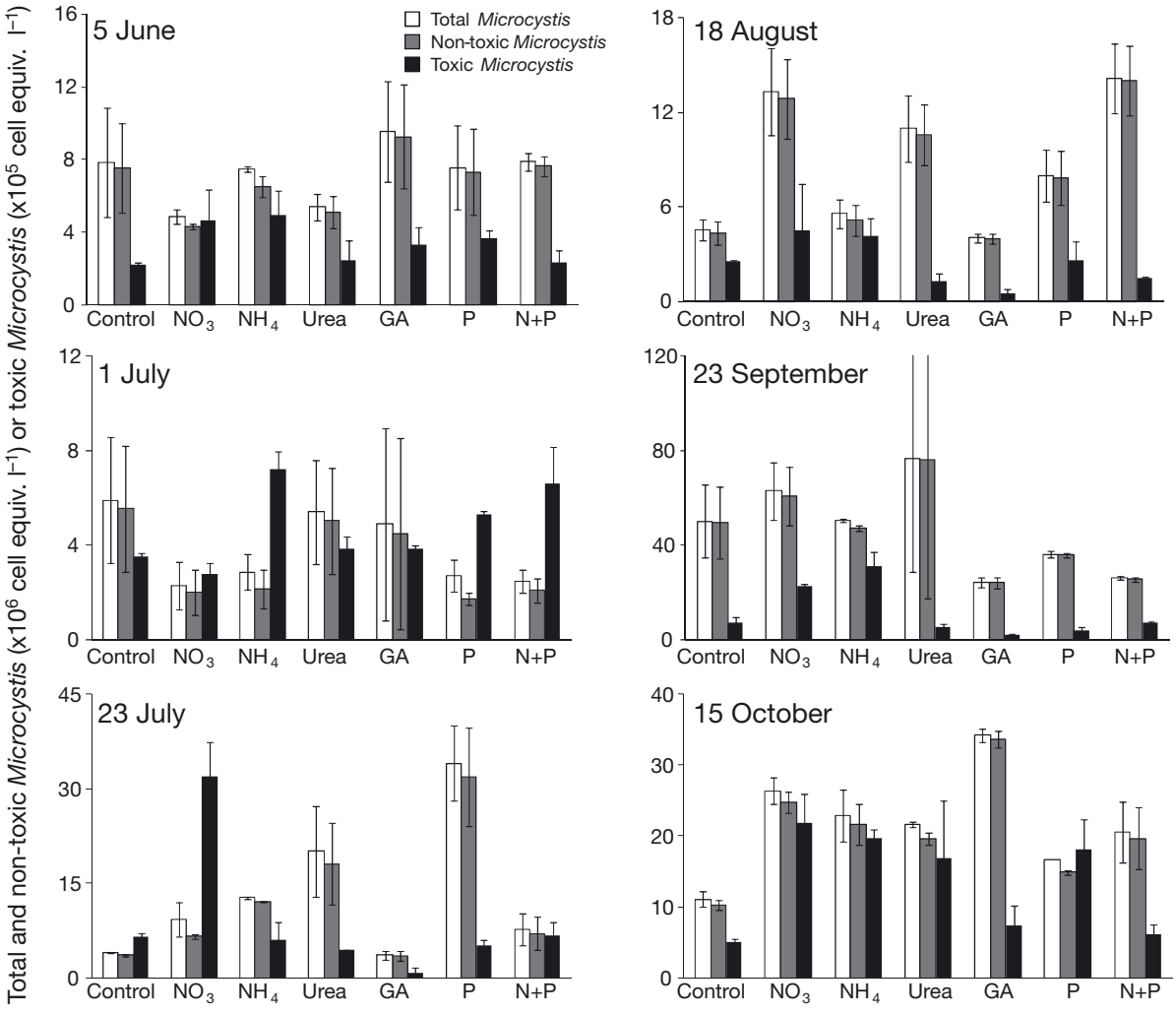

Fig. 4. Total, non-toxic, and toxic *Microcystis* densities during nutrient amendment experiments conducted in Lake Agawam during the summer of 2008. Note that the scale for the toxic cells is 1 order of magnitude less than that of the total and non-toxic cell scale. Error bars represent ±1 SE of triplicate experimental bottles. NO_3: nitrate; NH_4: ammonium; GA: L-glutamine; P: orthophosphate; N + P: nitrate and orthophosphate

phytoplankton community was stimulated by both inorganic and organic forms of N ($p < 0.05$; Table 4). Since the non-toxic strains of *Microcystis* comprised most (84%) of the total *Microcystis* community in LA, their growth responses to nutrients were generally the same as those in the total *Microcystis* population. The densities of the total and non-toxic *Microcystis* cells were not affected by nutrients until late July, when both inorganic and organic N additions yielded densities significantly higher than the control treatment ($p < 0.05$; Table 4, Fig. 4). In a manner similar to TR, the total *Microcystis* and non-toxic populations were stimulated by urea and nitrate more frequently than ammonium, displaying cell equivalents 2 to 5 times beyond the control ($p < 0.05$; Table 4, Fig. 4). The addition of P significantly enhanced total and non-toxic *Microcystis* abundances in half of the experiments conducted, increasing densities 2 to 9 times above the control ($p < 0.05$; Table 4, Fig. 4).

The response of toxic strains of *Microcystis* to nutrient enrichment differed from that of other algal populations. The abundance of toxic *Microcystis* cell equivalents was significantly enhanced by nutrient enrichment over the controls ($p < 0.05$) more frequently (83% of experiments) than that of the non-toxic population (50% of experiments; Table 4, Fig. 4). Unlike their non-toxic counterparts, toxic *Microcystis* in LA was enhanced only by inorganic forms of N, with nitrate and ammonium yielding densities that were between 2 to 5 times greater than that of the control ($p < 0.05$; Table 4, Fig. 4). Toxic *Microcystis* was also enhanced by P additions in half of the experiments, as

P increased cell equivalents 2 to 4 times beyond those of the controls ($p < 0.05$; Table 4, Fig. 4). Finally, on 1 date (1 July), the combined addition of N and P yielded toxic *Microcystis* densities 2 times above that of the control ($p < 0.05$; Table 4, Fig. 4).

DISCUSSION

Eutrophication is considered a primary cause of many HABs (Paerl 1997, Anderson et al. 2008, Heisler et al. 2008), particularly within freshwater ecosystems (Paerl 1988, 2008, Paerl et al. 2001). Because freshwater ecosystems are traditionally viewed as P-limited, management plans for such systems are commonly aimed toward reducing P loads (Schindler et al. 2008). The present study provides new insight into the role of nutrients in the occurrence of toxic and non-toxic *Microcystis* blooms. Specifically, the findings demonstrate that N enrichment can promote blooms of *Microcystis* more frequently than P and that inorganic nutrients may favor toxic strains over those which cannot produce microcystin.

Microcystis community composition differed between the 2 ecosystems studied. Toxic strains of *Microcystis* dominated the TR community during the early summer months (May and June) before non-toxic strains became more abundant, whereas non-toxic strains dominated the LA *Microcystis* community (83 ± 4%) on every date sampled. The dominance of toxic strains in TR but not in LA could be related to the availability of P in each system. Vézie et al. (2002) reported that the growth rates of toxic *Microcystis* exceeded those of non-toxic strains under high P concentrations, and concentrations of P were nearly an order of magnitude higher in TR (1.3 µM) compared to in LA (0.19 µM; $p < 0.05$; *t*-test). Changes in the dominance of toxic strains throughout the year further implicated the importance of P for this population in TR, as P concentrations were significantly higher from May through mid-July (2.2 ± 0.71 µM), when toxic strains dominated the *Microcystis* population (62 ± 20%), than in late July through October (0.45 ± 0.06 µM; $p < 0.01$; *t*-test), when non-toxic strains were most abundant (77 ± 7%; Table 3, Fig. 1). In addition, toxic *Microcystis* abundances were enhanced by P more frequently than non-toxic strains, a finding consistent with our prior work on lakes in the northeast United States (Davis et al. 2009). Oh et al. (2000) reported that P-limitation can enhance the levels of microcystin per cell in *Microcystis*. In contrast, we found significant ($p < 0.05$) linear co-variance between densities of toxic cells and concentrations of microcystin in each ecosystem studied, suggesting cellular microcystin content did not change substantially as P levels changed, perhaps in part because non-toxic *Microcystis* cells succeeded toxic cells when P concentrations declined. The association of toxic *Microcystis* with high DIP and non-toxic strains with lower DIP may be due to several factors. Hesse et al. (2001) reported that a microcystin-producing *Microcystis* strain had a higher content of light-harvesting pigments than non-toxic mutant strains. Hence, the RNA and DNA required for the synthesis of both light-harvesting pigments and microcystin by toxic strains of *Microcystis* may represent a significant P requirement.

Toxic strains of *Microcystis* were stimulated by N enrichment more frequently than the non-toxic strains during the present study (Table 5). This difference was strongest in TR, where DIN concentrations and DIN:DIP ratios were lowest and N enrichment enhanced the abundance of toxic *Microcystis* in all but a single experiment, but did so for the non-toxic strains in only half of the experiments (Table 4, Fig. 4). The stronger response of toxic *Microcystis* to N is consistent with laboratory studies, which have reported that toxic strains of *Microcystis* and *Anabaena* require higher N concentrations to achieve maximal growth rates compared to non-toxic strains (Rapala et al. 1997, Vézie et al. 2002). Furthermore, laboratory experiments have established clear relationships between DIN supply and microcystin production by toxic strains of *Microcystis* (Orr & Jones 1998, Long et al. 2001), which is consistent with the N requirements for microcystin synthesis, as microcystin is a N-rich compound (10 N atoms per molecule), and studies have found that microcystin can represent up to 2% of the cellular dry weight of *Microcystis* (Nagata et al. 1997). Beyond this N in the toxin, toxic *Microcystis* strains will have additional N requirements associated with the enzymes involved in the synthesis of microcystin (Tillett et al. 2000), as well as with additional light-harvesting pigments they may possess (Hesse et al. 2001). Although the precise mechanism is unclear, toxic *Microcystis* cells seem to have a higher N requirement than non-toxic cells (Rapala et al. 1997, Vézie et al. 2002, present study).

The species of N employed during field experiments strongly influenced whether toxic or non-toxic strains dominated *Microcystis* populations. For example, in every experiment where toxic strains of *Microcystis* were stimulated by individual N compounds ($n = 8$), they were always stimulated by at least 1 form of inorganic N (Table 4). On the other hand, this population was enhanced by organic N in only 1 experiment (Table 4). Conversely, when non-toxic strains were stimulated by individual N forms ($n = 6$), this happened more frequently via organic N compounds ($n = 6$) than inorganic N compounds ($n = 2$; Tables 4 & 5). These findings are consistent with the results of Vézie et al.

Table 5. The percentage of experiments in which N compounds significantly increased the density of the total phytoplankton community, total *Microcystis* community, non-toxic *Microcystis*, and toxic *Microcystis* relative to control treatments ($p < 0.05$) during nutrient amendment experiments. Percentages and number of significant treatments out of total number of experiments (in parentheses) shown

Compound	Experiments (%)			
	Total phytoplankton	Total *Microcystis*	Non-toxic *Microcystis*	Toxic *Microcystis*
Any N compound	83 (10/12)	67 (8/12)	50 (6/12)	75 (9/12)
Nitrate	50 (6/12)	42 (5/12)	25 (3/12)	58 (7/12)
Ammonium	25 (3/12)	17 (2/12)	17 (2/12)	42 (5/12)
Inorganic N	58 (7/12)	42 (5/12)	25 (3/12)	67 (8/12)
Urea	25 (3/12)	50 (6/12)	50 (6/12)	8 (1/12)
L-glutamine	33 (4/12)	25 (3/12)	33 (4/12)	0 (0/12)
Organic N	33 (4/12)	50 (6/12)	50 (6/12)	8 (1/12)
Orthophosphate	8 (1/12)	50 (6/12)	33 (4/12)	42 (5/12)

(2002), who found that increases in nitrate concentrations yielded faster growth rates for toxic *Microcystis* cultures compared to non-toxic cultures. While the biochemical mechanism(s) responsible for these trends are unclear, there was a clear differentiation regarding the response of toxic and non-toxic *Microcystis* populations to organic and inorganic N during the present study.

For both study sites, all *Microcystis* populations were more frequently enhanced by N enrichment than by P enrichment, indicating that, in both systems, these populations were more N- than P-limited (Tables 4 & 5). This finding is contrary to the long held view that freshwater ecosystems are exclusively P-limited (Schindler 1977, Smith 1983, Hecky & Kilham 1988, Schindler et al. 2008), but consistent with more recent laboratory (Vézie et al. 2002) and field (Gobler et al. 2007, Moisander et al. 2009a) studies of *Microcystis*. Importantly, during all of the experiments conducted across both sites, N *or* P increased the abundance of 1 or more of the *Microcystis* populations relative to control treatments, and there were 3 occasions at each site when increases in N *and* P concentrations did so, suggesting these populations were occasionally co-limited by N and P (Table 4, Figs. 3 & 4). As such, the dual management of N and P will be required to control the future occurrence of toxic *Microcystis* blooms in these, and likely other, systems (Howarth & Paerl 2008, Lewis & Wurtsbaugh 2008, Conley et al. 2009, Paerl 2009, Xu et al. 2010).

The differing responses of toxic and non-toxic strains of *Microcystis* during N and P enrichment, and our observations of these populations and nutrients in TR, provide evidence for a hypothesis that may partly account for the seasonal dynamics of these strains in temperate ecosystems. In TR, we observed a seasonal transition from higher inorganic nutrients, lower organic (DON, DOP, urea, DFAA) nutrients (Table 3), and dominance by toxic *Microcystis* strains during early summer (May to July; Fig. 1), followed by a depletion of inorganic nutrients, an elevation in organic nitrogen, and dominance by non-toxic *Microcystis* in late summer (Table 3, Fig. 1). This pattern of nutrients is common for aquatic ecosystems, as warming summer temperatures bring decreases in freshwater-based nutrient delivery rates (Gobler & Sañudo-Wilhelmy 2001) and increases in phytoplankton nutrient assimilation rates (Goldman & Carpenter 1974). Thus, while toxic *Microcystis* thrives on the abundant sources of inorganic nutrients during early summer, the depletion of these nutrients by mid-summer, potentially due to the decreased rainfall and/or groundwater flow, likely contributes to the demise of this population, a pattern observed in TR. The remineralization of dead cells and higher pelagic and benthic remineralization rates within warmer summer waters (Boynton et al. 1995) both likely contribute towards a water column that is enriched in DON during late summer. The present study demonstrated that non-toxic strains of *Microcystis* experience increased growth rates after enrichment with organic compounds, which could partially account for their dominance during late summer in TR. This population shift between toxic and non-toxic strains concurrent with decreases in DIN has been found in other systems, such as Lake Ronkonkoma, New York (Davis et al. 2009). Furthermore, Briand et al. (2009) found a similar seasonal shift in genotypes from toxic strains to non-toxic strains in a French reservoir. This trend was not seen in LA possibly due to the low P (mean = 0.19 µM DIP) concentrations, which may have inhibited toxic strains from becoming dominant within this system due to their high P requirement (Vézie et al. 2002); toxic strains are always a minor component of the total *Microcystis* community in this system (Davis et al. 2009, present study).

In conclusion, *Microcystis* populations in LA and TR were frequently stimulated by N and, to a lesser

extent, P. Toxic strains of *Microcystis* were more frequently promoted by N than non-toxic strains, but non-toxic strains were more frequently stimulated by organic N than toxic strains were. Therefore, dominance of toxic *Microcystis* and, ultimately, the toxicity of *Microcystis* blooms may be influenced by both the concentration and species of available nutrients, with increases in inorganic N- and/or P-loading likely promoting blooms dominated by toxic strains and potentially yielding higher microcystin concentrations.

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