SUPREME COURT OF THE STATE OF NEW YORK
COUNTY OF YATES

In the Matter of the Application of

SIERRA CLUB, COMMITTEE TO PRESERVE THE FINGER LAKES by and in the name of PETER GAMBA, its President; and COALITION TO PROTECT NEW YORK by and in the name of KATHRYN BARTHOLOMEW, its Treasurer; and SENECA LAKE GUARDIAN, A WATERKEEPER AFFILIATE by and in the name of YVONNE TAYLOR, its Vice President,

Petitioners,

For a Judgment Pursuant to Article 78 of the Civil Practice Law and Rules,

–against–

NEW YORK STATE DEPARTMENT OF ENVIRONMENTAL CONSERVATION, BASIL SEGGOS, COMMISSIONER, GREENIDGE GENERATION, LLC and LOCKWOOD HILLS, LLC,

Respondents.

AFFIDAVIT OF GREGORY BOYER

Index No. 2017-0232

State of New York,
County of Onondaga, ss.:

GREGORY BOYER, being duly sworn, deposes and says:

I have reviewed the attached affidavit I gave last year and I continue to be of the opinion that adding large volumes of heated water in the Dresden bay area of Seneca Lake could result in increased HABs outbreaks in that area and that this issue deserves further study.

GREGORY BOYER

Sworn to before me this
26th day of April, 2018

Amy B. Hirsh
Notary Public

AMY B. HIRSH
Notary Public, State of New York
Qualified in Onondaga County
No. 01HI6003191
Commission Expires Feb. 23, 2022

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SIERRA CLUB, COMMITTEE TO PRESERVE THE FINGER LAKES by and in the name of PETER GAMBA, its President; and COALITION TO PROTECT NEW YORK by and in the name of KATHRYN BARTHOLOMEW, its Treasurer,

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NEW YORK STATE DEPARTMENT OF ENVIRONMENTAL CONSERVATION, BASIL SEGGOS, COMMISSIONER, GREENIDGE GENERATION, LLC, GREENIDGE PIPELINE, LLC, GREENIDGE PIPELINE PROPERTIES CORPORATION and LOCKWOOD HILLS, LLC,

Respondents.

AFFIDAVIT OF GREGORY BOYER IN SUPPORT OF PETITIONERS' MEMORANDUM OF LAW IN OPPOSITION TO THE MOTIONS TO DISMISS

Index No. 2016-0165

State of New York,
County of Onondaga, ss.:

GREGORY BOYER, being duly sworn, deposes and says:

1. I am Professor of Biochemistry and Director, Great Lakes Research Consortium at the State University of New York, College of Environmental Science and Forestry in Syracuse, New York.

2. I received my B.A. Degree in Biochemistry from the University of California at Berkeley and my Ph.D. degree in Biochemistry from the University of Wisconsin. After postdoctoral fellowships at the Plant Research Labs at Michigan State University and in the Department of Oceanography at the University of British Columbia, I joined the Faculty of Chemistry at SUNY-ESF in 1985. My expertise is with toxins and other compounds produced by marine and freshwater algae. I was director of NOAA's

Lower Great Lakes project to develop monitoring strategies for toxic blue-green algae in the lower great lakes and currently assist the NYS Department of Environmental Conservation with their Citizen State Lake Assessment Program with algal issues. I have authored over 130 publications on bioactive natural products, served as a principal investigator on over $10 million in research funding, served as chair of the Department of Chemistry at SUNY-ESF for four years and am an advisor to the International Joint Commission on issues regarding Harmful Algal Blooms (HABs) within the Great Lakes. I am the current director of New York's Great Lakes Research Consortium. The GLRC consists of 18 New York Universities and nine Canadian Universities, almost 300 scientists in total, working on all aspects of Great Lakes Science, education and outreach. My educational background and professional experience are more fully set forth in my Curriculum Vitae, attached as Exhibit A.

3. This affidavit addresses the impact increasing water temperature at the mouth of the Keuka Outlet might have on occurrences of HABs in Seneca Lake near the Outlet.

4. HABs are algal blooms composed of cyanobacteria known to naturally produce biotoxins. They can occur when certain types of microscopic algae grow quickly in water.

5. Toxic cyanobacteria are a global public health and environmental concern.

6. Toxic blooms are most commonly formed by Microcystis, a well-known producer of the hepatotoxin, microcystin

7. Long term exposure to microcystin has been associated with severe human health effects, including liver and colorectal cancers.

8. While it is clear that the occurrence of toxic cyanobacteria blooms around the world have increased during recent decades, the underlying causes of such blooms and

the factors influencing the dynamics of toxic and non-toxic strains within them are poorly understood.

9. My colleagues and I have studied the effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of Microcystis during cyanobacteria blooms in Lake Agawam, Lake Ronkonkoma, Lake Champlain, and Mill Pond, Long Island. In a research paper published in 2009, Timothy W. Davis, Dianna L. Berry, Christopher J. Gobler and I described the results of our study. Davies et al, *Harmful Algae* 8 (2009) 715–725. A copy of this paper is attached as Exhibit B. Our study found that in Mill Pond, Long Island in June 2006 temperature was a significant treatment effect which increased the growth rate of toxic Microcysits by 22% relative to the control. Higher phosphorus concentrations also increased the growth rate of toxic Microcystis (by 33% relative to the control). However, the additive interaction between temperature and phosphorus yielded a growth rate for toxic Microcystis that was 63% higher than the control and was the highest growth rate of either population in any treatment. The other measurements reported in the study showed similarly that the highest levels of toxic Microcystis were measured when the highest levels of both temperature and nutrients were observed.

10. Reports of HABs are increasing in New York State as a whole.

11. In the last few years, reports of HABs in the Finger Lakes have increased.

12. HABs outbreaks were discovered in Seneca Lake in 2015 and 2016, including one just south of the Keuka Outlet on Perry Point that was analyzed by my lab in August 2016. The concencentration of the liver toxin microcystins in two bloom samples collected from Seneca Lake in September of 2016 exceeded the US-EPA guideline values for microcystin in drinking water by more than 10-fold, and exceeded the proposed US-EPA guidelines for recreational contact with microcystin containing blooms by 5-8 fold.

13. It is my understanding that the Greenidge Generating Station in Dresden, New York is scheduled to resume operations this spring after a six year hiatus and that when those operations begin the plant will begin to discharge very large volumes of heated condenser cooling water into the Keuka Outlet a short distance before the Outlet discharges into Seneca Lake.

14. These discharges are likely to result in increases in the water temperature of the Dresden bay area of Seneca Lake surrounding the mouth the Outlet.

15. It is known that the Keuka Outlet carries agricultural runoff and discharges from the Penn Yan wastewater treatment plant which may result in significant increased nutrient concentrations in the Outlet. See the results of sampling conducted by members of the Seneca Lake Pure Waters Association (SLPWA) at various sites along the Outlet in July, August and September 2015 attached as Exhibit C. These results are posted on the SLPWA website at http://senecalake.org/wp-content/uploads/2016/06/KeukaOutletFSD-052816.pdf .

16. In these circumstances, I am of the opinion that increasing water temperatures in the Dresden bay area of Seneca Lake could result in increased HABs outbreaks in that area and that this issue deserves further study before proceeding forward.

GREGORY BOYER

Sworn to before me this
14 day of January, 2017

Notary Public

SHERRY RICHARDSON
Notary Public, State of New York
Qualified in Onondaga County
Reg. No. 01RI6223246
Commission Expires June 7, 2019

Exhibit A

Gregory L. Boyer
Faculty of Chemistry, State University of New York
College of Environmental Science and Forestry
Syracuse, NY 13210
Contact: Email: glboyer@esf.edu Telephone: (315) 470-6825
FAX: (315) 470-6855

RESEARCH INTERESTS

The chemistry and biochemistry of biologically active natural products from plants and algae including toxins, siderophores, allelopathic agents, and growth regulators. Special interests include the chemistry / ecology of marine and freshwater harmful algal blooms such as cyanobacteria in large lake ecosystems, automated monitoring systems for these blooms, rapid detection methods for toxic cyanobacteria and paralytic shellfish poisoning (PSP) toxins, large scale algal culture facilities for bioproducts productions, and the biochemistry of iron in forest and aquatic (marine and freshwater) ecosystems.

EDUCATION:

Ph.D.,	University of Wisconsin - Madison,	1980,	(Biochemistry).
A.B.,	University of California - Berkeley,	1975,	(Biochemistry).
A.S.,	Reedley College (Reedley, Calif.),	1973,	(Chemistry).

PROFESSIONAL EXPERIENCE:

2007-present: Director, Great Lakes Research Consortium, Syracuse, NY 13210
1998-present: Professor of BioChemistry, State University of New York, College of Environmental Science and Forestry, (SUNY-ESF) Syracuse NY 13210.
2011-2014: Chair, Department of Chemistry, SUNY-ESF.
2010 E.T.S. Walton Visiting Professor, Cork Institute of Technology, Cork, Ireland
1991-1998 Associate Professor of Chemistry, SUNY-ESF.
1994 Visiting Scientist, Biology Dept., Woods Hole Oceanographic Institute, Woods Hole, MA
1986-1991 Joint Academic Appointment in the Faculty of Environmental Sciences, SUNY-ESF.
1985-1990 Assistant Professor of Chemistry, SUNY-ESF.
1983-1984 Research Associate, Dept. of Oceanography, Univ. of British Columbia, Vancouver, BC.
1980-1982 Research Associate, Michigan State Univ. - DOE, Plant Research Labs. East Lansing, MI.
1975-1980 Research Assistant, Department of Biochemistry, University of Wisconsin, Madison, WI.

HONORS and Supporting Activities:

Life member; Phi Beta Kappa - Alpha (UC-Berkeley Honor Soc.) and Alpha Gamma Sigma (Reedley College Honor Soc.), International Expert for IAEA (International Atomic Energy Agency) on PSP toxins (1999), Participant in EPA's "Creating a Cyanotoxin Target List for the Unregulated Contaminant Monitoring Rule" taskforce (2001), Participant in NOAA workshop entitled "Developing a National Plan for Remediation of Harmful Algal Blooms", Steering committee for "National Plan for Marine Biotoxins-2004; Elected member of the US National Harmful Algal Bloom Advisory Committee (2005-2008), Invited External Reviewer for EPA Toxicological Reviews of Cyanobacteria Toxins (2007), Elected to the Governing Body of the Northeast Algal Society 1999-2017; Recipient; State University of New York Research Foundations 2003 Award for Excellence in the Pursuit on Knowledge. Recipient; 2005 ESF award for integrating outreach activities with Teaching and Research. Recipient; ESF 2009 Exemplary Researcher Award ; Recipient; 2010 Science Foundation of Ireland E.T.S. Walton Visiting Professorship, Chief Scientist, Centers for Excellent in Ocean Science Education (COSEE) research cruises 2005, 2008 and 2009, Participate in more than 25 International Research Cruises (eight times as Senior or lead Scientist), Member of International Joint Commission's Council of Research Managers and New York's Great Lakes Basin Advisory Committee. Recipient; 2015 Lake Tear of the Clouds Award for outstanding service to the New York State Federal of Lake Associations. Recipient: 2016 Chancellors Award for Excellence in Faculty Service.

SELECTED PROFESSIONAL SOCIETIES: American Society of Limnology and Oceanography, North East Algal Society, International Association for Great Lakes Research, International, Society for the Study of Harmful Algal Blooms, North American Lake Management Society.

PUBLICATIONS OF GREGORY L. BOYER:

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6. Zeevaart, J.A.D. and G.L. Boyer (1984) Accumulation and transport of abscisic acid and its metabolites in *Ricinus* and *Xanthium*. Plant Physiol. 74: 934-939.
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11. Zeevaart, J.A.D., G.L. Boyer, K. Cornish, and R. Creelman (1986) Metabolism of abscisic acid. In: Plant Growth Substances 1985, M. Bopp, ed., Springer-Verlag, pp. 101-107.
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Exhibit B

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The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of *Microcystis* during cyanobacteria blooms

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ABSTRACT

In temperate latitudes, toxic cyanobacteria blooms often occur in eutrophied ecosystems during warm months. Many common bloom-forming cyanobacteria have toxic and non-toxic strains which co-occur and are visually indistinguishable but can be quantified molecularly. Toxic *Microcystis* cells possess a suite of microcystin synthesis genes (*mcyA–mcyJ*), while non-toxic strains do not. For this study, we assessed the temporal dynamics of toxic and non-toxic strains of *Microcystis* by quantifying the microcystin synthetase gene (*mcyD*) and the small subunit ribosomal RNA gene, 16S (an indicator of total *Microcystis*), from samples collected from four lakes across the Northeast US over a two-year period. Nutrient concentrations and water quality were measured and experiments were conducted which examined the effects of elevated levels of temperatures (+4 °C), nitrogen, and phosphorus on the growth rates of toxic and non-toxic strains of *Microcystis*. During the study, toxic *Microcystis* cells comprised between 12% and 100% of the total *Microcystis* population in Lake Ronkonkoma, NY, and between 0.01% and 6% in three other systems. In all lakes, molecular quantification of toxic (*mcyD*-possessing) *Microcystis* was a better predictor of *in situ* microcystin levels than total cyanobacteria, total *Microcystis*, chlorophyll *a*, or other factors, being significantly correlated with the toxin in every lake studied. Experimentally enhanced temperatures yielded significantly increased growth rates of toxic *Microcystis* in 83% of experiments conducted, but did so for non-toxic *Microcystis* in only 33% of experiments, suggesting that elevated temperatures yield more toxic *Microcystis* cells and/or cells with more *mcyD* copies per cell, with either scenario potentially yielding more toxic blooms. Furthermore, concurrent increases in temperature and P concentrations yielded the highest growth rates of toxic *Microcystis* cells in most experiments suggesting that future eutrophication and climatic warming may additively promote the growth of toxic, rather than non-toxic, populations of *Microcystis*, leading to blooms with higher microcystin content.

1. Introduction

Blooms of toxic cyanobacteria are a global public health and environmental concern. Toxic blooms are most commonly formed by *Microcystis*, a well-known producer of the hepatotoxin, microcystin (Carmichael, 1992, 1994; Fleming et al., 2002; Chorus and Bartram, 1999; Pearl, 2008). Long term exposure to microcystin has been associated with severe human health effects, including liver and colorectal cancers (Falconer et al., 1988; Carmichael and Falconer, 1993; Bell and Codd, 1994; Carmichael, 1994; Ito et al., 1997; Chorus and Bartram, 1999; Zegura and Sedmak, 2003). One complexity in field studies of harmful cyanobacteria blooms has been the existence and often co-existence of toxic and non-toxic strains of the same species within a genus that are morphologically and taxonomically indistinguishable (Otsuka et al., 1999; Fastner et al., 2001; Kurmayer et al., 2002). While it is clear that the occurrence of toxic cyanobacteria blooms around the world have increased during recent decades (Chorus and Bartram, 1999; Hudnell and Dortch, 2008), the underlying causes of such blooms and the factors influencing the dynamics of toxic and non-toxic strains within them are poorly understood.

Cyanobacteria blooms are typically associated with eutrophic and poorly flushed waters (Paerl, 1988; Paerl et al., 2001; Philipp et al., 1991; Carmichael, 1994; Rapala et al., 1997; Oliver and Ganf, 2000). Due to increases in human population density, agriculture, and industrial activities, nutrient loading rates into many freshwater ecosystems has increased (Carpenter et al., 1998). As surface waters become enriched in nutrients, particularly phosphorus (P), there is often a shift in the phytoplankton community towards dominance by cyanobacteria (Smith, 1986; Trimbee and Prepas, 1987; Watson et al., 1997; Paerl and Huisman, 2008). Higher

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phosphorus levels have been shown to yield higher microcystin content per cell in some cyanobacteria (Utkilen and Gjølme, 1995; Rapala et al., 1997). However, nitrogen (N) may be equally important in the occurrence of toxic, non-N fixing cyanobacteria blooms, such as *Microcystis* sp. Laboratory studies have indicated that increasing N loads increases the growth and toxicity of this species (Watanabe and Oishi, 1985; Orr and Jones, 1998; Codd and Poon, 1988). Moreover, previous laboratory research suggests toxic strains of *Microcystis* are able to outgrow non-toxic strains at high nitrogen levels (Vézie et al., 2002). However, no field study has examined how nutrients directly affect the growth of toxic and non-toxic strains of cyanobacteria within wild populations.

The burning of fossil fuels and subsequent rise in atmospheric carbon dioxide has caused the earth's surface temperature to increase by approximately 1 °C during the 20th century, with most of the increase having occurred during the last 40 years (IPCC, 2001). In the current century, global temperatures are expected to increase an additional 2–5 °C (Houghton et al., 2001). Frequently, cyanobacteria dominate phytoplankton assemblages in temperate freshwater environments during the warmest periods of the year, particularly in eutrophic systems (Paerl, 1988; Paerl et al., 2001; Paerl and Huisman, 2008; Paul, 2008). Harmful cyanobacteria such as *Microcystis* have been found to have an optimal temperature for growth and photosynthesis at, or above, 25 °C (Konopka and Brock, 1978; Takamura et al., 1985; Robarts and Zohary, 1987; Reynolds, 2006; Jöhnk et al., 2008; Paerl and Huisman, 2008). Furthermore, the cellular toxin content of multiple genera of cyanobacteria increases with increasing temperature to a maximum above 25 °C (Van der Westhuizen and Eloff, 1985; Codd and Poon, 1988; Sivonen, 1990; Rapala et al., 1997). However, the growth response of toxic and non-toxic strains of cyanobacteria to increasing water temperature in an ecosystem setting has yet to be explored.

The purpose of this study was to elucidate the conditions that favor the growth and proliferation of the toxic and non-toxic strains of *Microcystis*. A two-year field study was established in four diverse lake systems across the Northeast US to determine the dynamics of toxic and non-toxic strains of *Microcystis* using molecular quantification of total *Microcystis* cells and *Microcystis* cells possessing the microcystin synthetase gene. Levels of microcystin, nutrients, and other environmental parameters were assessed concurrently. Experiments were conducted to examine the impacts of elevated nutrient concentrations (N and P) and increased temperature on the growth rates of toxic and non-toxic *Microcystis* populations.

2. Materials and methods

2.1. Study sites

During this study, four sites within the Northeast US were investigated. Lake Champlain lies between New York and Vermont, and is connected to the Richelieu River to the north and the Hudson River to the south. It is the largest lake in the Northeast United States other than the Great Lakes. Lake Champlain serves as a drinking water supply to millions of individuals, has been subjected to eutrophication since the 1970s, and is dominated by cyanobacteria in some regions during warm months (Myer and Gruendling, 1979; Shambaugh et al., 1999; Boyer et al., 2004). Our sampling site was Missisquoi Bay (Latitude: 44.62°N; Longitude: 73.37°W), a basin in the northeast extent of Lake Champlain that experiences annual toxic cyanobacteria blooms (Boyer et al., 2004).

Our other sampling sites were located on Long Island, NY, USA, which has recently seen its population expand beyond seven million people. Lake Agawam (Latitude: 40.88°N; Longitude: 72.39°W) is a small (0.5 km^2), shallow (4 m maximum depth) system which experiences annual toxic cyanobacteria blooms dominated by *Microcystis* (Gobler et al., 2007). Mill Pond (Latitude: 40.91°N; Longitude: 72.36°W) is a deeper (8m maximum depth), hypereutrophic system (mean chlorophyll a = 200 μg L^{-1}) which also experiences dense cyanobacteria blooms dominated by *Microcystis* cells during summer months. Finally, Lake Ronkonkoma (Latitude: 40.83°N; Lon: 73.12°W), is the largest body of freshwater on Long Island (area = 1.5 km^2, maximum depth = 27 m), and experiences summer cyanobacteria blooms comprised of *Microcystis*. In all systems sampled on Long Island, lake transects indicated that blooms were spatially similar with regard to chlorophyll *a*, phycocyanin, and toxin concentrations (data not shown). As such, our sampling sites were representative of each system.

2.2. Water quality sampling

Field sampling was conducted bi-weekly before, during, and after cyanobacteria blooms (May–November). In 2005, Lake Ronkonkoma and Lake Agawam were sampled whereas in 2006, Lake Champlain, Lake Agawam, and Mill Pond were studied. At each site, general water quality was evaluated using a handheld YSI 556 sonde to determine surface and bottom temperatures and dissolved oxygen. Twenty liters of surface water was collected and taken to the lab where triplicate 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 and Baker, 1988; Lee et al., 1994). For microcystin analysis, whole water was filtered onto triplicate 47 mm GFF glass fiber filters and placed in 5 mL cryovials which were stored at −80 °C until analysis. Water samples were filtered through 0.2 μm capsule filters to obtain samples for dissolved nutrient analysis. Nitrate was analyzed by reducing the nitrate to nitrite using spongy cadmium as per Jones (1984). Nitrite, ammonium, phosphate, and silicate were analyzed using techniques modified from Parsons et al. (1984). Duplicate whole water samples were preserved with Lugol's iodine solution (5% final concentration) to quantify phytoplankton assemblages. For molecular analysis of cyanobacteria, bloom water was filtered onto triplicate 0.22 μm polycarbonate filters, immediately placed in CTAB lysis buffer, and stored at −80 °C.

2.3. Impacts of nutrients and temperature on toxic and non-toxic *Microcystis*

On selected dates (Table 3), experiments were conducted to assess the impact of increased nutrient concentrations and temperature on toxic and non-toxic *Microcystis* populations. For each of the six experiments, two sets of 12 triplicate, 1 L bottles (n = 24) were filled with surface water from each experimental site and were either left unamended to serve as a control, or amended with nitrogen (20 μM NO_3^-), phosphorus (1.25 μM orthophosphate), or both N and P in order to determine which nutrient may favor the proliferation of toxic and non-toxic *Microcystis*. One set of bottles (n = 12) was placed in an outdoor incubator receiving ambient light and circulating *in situ* water. In order to maintain *in situ* water temperatures, ambient lake water was continuously pumped through the incubation chamber throughout the duration of the experiment (48–72 h). The other set of bottles (n = 12) was incubated at natural light levels and circulating water maintained at elevated temperatures (+4 °C above ambient water temperature) achieved with a coupled heater/chiller design using commercially available heaters and chillers (Aquatic Eco-systems, Inc., FL, USA), approximating levels projected for the coming century (+4 °C; Houghton et al., 2001). Exact water temperatures administered during experiments were monitored every minute with *in situ* loggers (Onset Computer Corporation, MA, USA). At the end of the incubation period, aliquots from experimental bottles were filtered for levels of chlorophyll *a* and analyzed for *in vivo*

Table 1
A list of primers (Integrated DNA Technologies, IA, USA) and probes (Applied Biosystems, Foster City, CA, USA) used in the qPCR analysis.

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

F: forward primer R: reverse primer.
[a] 6-Carboxyfluorescein.
[b] Black Hole Quencher-1 (quenching range 480–580 nm).

phycocyanin. Samples were also filtered at the end of the incubation period as described above to preserve samples for determination of densities of toxic and non-toxic *Microcystis* using molecular methods. Net growth rates of each population were determined as follows: $\mu = \ln[N_t/N_0]/t$ where μ is the rate of population growth (d^{-1}), N_0 and N_t are initial and final cell densities, and t is the duration of incubation in days.

2.4. Sample analysis

2.4.1. Microscopic analysis

Densities of *Microcystis* and other co-occurring cyanobacteria were quantified using gridded Sedgewick-Rafter and Utermohl counting chambers. Utermohl chambers were used to quantify populations with cell densities that were low. For dense algal populations, a gridded Sedgewick-Rafter chamber allowed for accurate assessment of cell densities without the layering of cells which can occur when high biomass samples are concentrated within an Utermohl chamber. For all samples, at least 200 cells were enumerated. To quantify *Microcystis* as well as *Anabaena*, the number of colonies per chamber, as well as the number of cells in 20 colonies, was determined. Such a counting approach provided good reproducibility (<15% relative standard deviation) on live and preserved samples, as well as precise comparability between live and preserved samples. However, as individual trichomes of *Aphanizomenon* colonies are difficult to visually resolve, only colonies per chamber were counted.

2.4.2. Microcystin analysis

Filters for microcystin analyses were extracted in 50% methanol containing 1% acetic acid using ultrasound (four, 20 second bursts with a 20 second pause between bursts). Previous work has shown that this extraction protocol gives 90% recovery of microcystin-LR (Boyer et al., 2004). Following extraction, the methanolic extract was stored at −80 °C until analysis. Microcystin concentration was measured using the protein phosphatase inhibition assay (PPIA; Carmichael and An, 1999). This analytical protocol does not allow for specific congeners of microcystin to be distinguished but rather provides an indication of the potential biological impact of the microcystins, specifically their ability to inhibit protein phosphatases (Carmichael and An, 1999). A certified microcystin-LR standard (Alexis Biochemicals, San Diego, USA) was used to create the standards for this analysis. This assay yielded a 99.5 ± 8.2% recovery of samples spiked with known amounts of microcystin, a methodological relative standard deviation of 9.4%, and a detection limit between 0.05 and 0.50 μg L^{-1}.

2.4.3. Molecular analyses

Total cellular nucleic acids were extracted from field and experimental samples using methods described in Coyne and Cary (2005). Filtered environmental or experimental samples were submersed in CTAB buffer (Dempster et al., 1999), supplemented with 20 μg L^{-1} pGEM-3z(f+) plasmid (Promega; Table 1) which served as an internal control for extraction efficiency and PCR inhibition (Coyne et al., 2005). The filters were then flash frozen and stored at −80 °C until extraction. Nucleic acids were extracted after an initial heating step at 65 °C, followed by a double chloroform extraction, and an isopropanol precipitation. Extracted nucleic acids were resuspended in 20 μL of LoTE buffer. The quantity and quality of nucleic acids was assessed with a NanoDrop 1000 UV spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Two *Microcystis*-specific genetic targets were used during this study, the 16S rRNA gene (16S rDNA) and *mcyD* gene. The *Microcystis* 16S rRNA gene is specific to the *Microcystis* genus which allowed us to quantify 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) allowing for the quantification of the toxic *Microcystis* population (Rinta-Kanto et al., 2005). QPCR was carried out using an ABI 7300 Real Time PCR instrument using TaqMan® labeled probes (Applied Biosystems) and *Microcystis*-specific *mcyD* and 16 s rDNA primers (Table 1). Each 10 uL reaction included 5 uL of 2× TaqMan® Master Mix (Applied Biosystems), 10uM each primer (Integrated DNA Technologies), 10 uM 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. In order to amplify 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 *Microcystis aeruginosa*, clone LE-3 (Rinta-Kanto et al., 2005), was enumerated by standard microscopy and collected on polycarbonate filters which were prepared and extracted as outlined above. 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 toxic *Microcystis* cells. Since some *Microcystis* cells may carry multiple copies of the 16S rDNA gene and *mcyD* gene, data was generally expressed as "cell equivalents" (Rinta-Kanto et al., 2005). The numbers of toxic and total *Microcystis* cells were determined using the ΔΔCT method (Livak and Schmittgen, 2001; Coyne et al., 2005). The difference between the number of *mcyD* cell equivalents (toxic cells) and 16S rDNA cell equivalents (total cells) indicated the number of non-toxic cell equivalents (Rinta-Kanto et al., 2005). Toxic *Microcystis* cell abundances were determined for all locations and on all dates. Total *Microcystis* cells (16S rDNA) were quantified for all systems except Lake Agawam 2006 and Mill Pond 2006 when total *Microcystis* cells equivalents were only quantified on the days of experiments.

2.5. Statistical analysis

All time series data sets from each location (chlorophyll *a*, phycocyanin, toxic *Microcystis* cell densities, non-toxic *Microcystis* densities, total *Microcystis* densities, total cyanobacteria cells, microcystin concentration, dissolved inorganic phosphorus (DIP), dissolved inorganic nitrogen (nitrate + ammonium = DIN), and temperature) were statistically analyzed using a Pearson's correlation matrix to establish the degree to which individual variables were correlated. Student's *t*-tests were used to determine if the means of different populations (toxic and non-toxic) were significantly different from each other. The effects of nitrogen, phosphorus, and temperature on the net growth rate of toxic and non-toxic *Microcystis* populations were analyzed with three-way ANOVAs with nitrogen, phosphorus, and temperature considered treatment effects ($\alpha = 0.05$). Post-hoc comparisons of significant impacts were elucidated with Tukey's multiple comparison tests. For all results the standard variance presented is ± one standard deviation (SD).

3. Results

3.1. Dynamics of toxic Microcystis blooms

3.1.1. Lake Agawam, Long Island, NY

During 2005, Lake Agawam hosted mixed cyanobacteria blooms numerically dominated by *Microcystis* from June through September (Table 2). Blooms contained mean chlorophyll *a* levels of 97 ± 28 μg L^{-1} (mean ± SD) and coincided with peaks of *in vivo* phycocyanin (Fig. 1). Densities of non-toxic *Microcystis* ranged from 3.9×10^6 to 1.0×10^8 cell equivalents L^{-1} during blooms with peak densities achieved on 20 October (Fig. 1). Concurrently, toxic *Microcystis* cell densities (possessing the *Microcystis*-specific *mcyD* gene) ranged from 5.0×10^2 to 5.0×10^5 cell equivalents L^{-1} (Fig. 1), peaking on 21 June and representing between 0.01% and 1.56% of total *Microcystis* cells. Microcystin was detectable in the water column from May through October ranging from 0.86 to 11.8 μg L^{-1}, with the highest levels occurring at the peak in toxic *Microcystis* cell equivalents (Fig. 1). Concentrations of microcystin were significantly correlated with levels of toxic *Microcystis* cell equivalents ($p < 0.05$). DIN levels during the summer months (July–August) ranged from 2.67 to 6.52 μM, while DIP concentrations never exceeded 0.38 ± 0.08 μM (mean = 0.25 ± 0.06 μM; Fig. 1). During September when algal biomass levels declined, DIN and DIP levels increased (Fig. 1). Temperatures in Lake Agawam during 2005 rose from 13 °C in May to 29 °C in August, and then declined to 15 °C in late October.

During 2006, the temporal dynamics of cyanobacterial blooms in Lake Agawam differed from 2005. Lake Agawam hosted cyanobacteria blooms dominated by *Anabaena* during June and early July with maximal concentrations reaching 2.79×10^8 cells L^{-1} (Table 2). This bloom was succeeded by a bloom of *Microcystis* during mid-July which remained dominant through October (Table 2). *Aphanizomenon* was present throughout the sampling period but was never dominant (Table 2). Cyanobacteria blooms contained mean chlorophyll *a* levels of 209 ± 17.0 μg L^{-1} and coincided with peaks of *in vivo* phycocyanin (Fig. 2). Densities of toxic *Microcystis* ranged from 5.3×10^3 to 3.5×10^6 cell equivalents L^{-1}, peaking on 10 October (Fig. 2). Particulate microcystin was detectable throughout the study period ranging from 0.69 to 81.1 μg L^{-1}, with the highest levels occurring concurrently with the peak in toxic *Microcystis* cell equivalents (Fig. 2). In a manner similar to 2005, microcystin concentrations were significantly correlated with toxic *Microcystis* cell equivalents ($p < 0.001$). DIN levels during the bloom months (June–October) ranged from 3.03 to 48.81 μM and were inversely related to phycocyanin concentrations, being higher before and after bloom events (Fig. 2). During June and early July DIP concentrations were 1.54 ± 0.44 μM but rose later in the sampling season (Fig. 2). Surface temperatures in 2006 ranged from 22 °C in June to 29 °C in July, declining to 13 °C by October.

3.1.2. Lake Ronkonkoma, Long Island, NY

Compared to the other systems examined, Lake Ronkonkoma was the only system which hosted cyanobacteria blooms that were first dominated by *Microcystis* and were succeeded by *Anabaena* (Table 2). *Aphanizomenon* was either absent, or at low densities in Lake Ronkonkoma (i.e. <50,000 colonies L^{-1}; Table 2). Blooms (June–August) contained mean chlorophyll *a* levels of 20.2 ± 8.0 μg L^{-1} and coincided with peaks of *in vivo* phycocyanin (Fig. 3). May through June *Microcystis*-dominated blooms (Table 2) were comprised almost exclusively of toxic cells (1.98×10^8 cell equivalents L^{-1} maximum on 27 May; Fig. 3), until late June bloom composition shifted toward dominance by non-toxic cells (peak densities 1.20×10^6 cell equivalents L^{-1} on 25 July) before declining during the late summer (Fig. 3). Through the sampling period, toxic *Microcystis* cell equivalents comprised between 12% and 100% of the total *Microcystis* population and microcystin levels ranged from 1.28 to 78.8 μg L^{-1} (Fig. 3). In a manner similar to Lake Agawam, there was a significant correlation between microcystin concentrations and toxic *Microcystis* cell equivalents ($p < 0.001$). Nutrient concentrations during the bloom months (June–September) ranged from 4.17 to 11.1 μM DIN and 0.19 to 0.71 μM DIP (Fig. 3). Finally, surface temperatures ranged from 16 °C in May to 30 °C in August and decreased to 25 °C by September.

3.1.3. Mill Pond, Long Island, NY

Cyanobacterial bloom dynamics within Mill Pond during the summer of 2006 were similar to that of Lake Agawam during the same year. Mill Pond hosted cyanobacteria blooms dominated by *Anabaena* and *Aphanizomenon* from June through early July (Table 2). This bloom subsided and a bloom of *Microcystis* occurred and remained dominant throughout the summer and early fall (Table 2). Blooms (June–October) contained mean chlorophyll *a* levels of 96.9 ± 57.3 μg L^{-1} and coincided with peaks of *in vivo* phycocyanin (Fig. 4). Densities of toxic *Microcystis* ranged from 3.3×10^3 to 4.1×10^6 cell equivalents L^{-1}, achieving the highest concentration on 12 October coinciding with the highest levels of microcystin (Fig. 4). Microcystin levels ranged from 5.50 to 154 μg L^{-1}, and were significantly correlated with toxic *Microcystis* cell equivalents during the sampling period ($p < 0.001$). DIN concentrations varied widely (6.82–80.9 μM), with the highest concentrations present during late spring and fall (Fig. 4). DIP concentrations in Mill Pond were also elevated with a mean concentration of 4.93 ± 0.06 μM (Fig. 4). Surface temperatures in this system ranged from 22 °C in June to 28 °C in July declining to 12 °C by October.

3.1.4. Lake Champlain

Lake Champlain's Missisquoi Bay was devoid of cyanobacteria until July and was never numerically dominated by *Microcystis* in 2006 (Table 2). A cyanobacteria bloom which occurred during late summer contained mean chlorophyll *a* levels of 52.0 ± 27.5 μg L^{-1}, coincided with peaks of *in vivo* phycocyanin (mid-July through mid-October; Fig. 5), and was dominated by *Anabaena* at a density of $4.91 \pm 0.21 \times 10^7$ cells L^{-1} on 1 August (Table 2). *Microcystis* was present in the water column from July through October (Table 2) with densities of non-toxic *Microcystis* ranging from 1.4×10^4 to 2.1×10^7 cell equivalents L^{-1} during blooms (peak densities on 1 August; Table 2). Toxic *Microcystis* cells were detectable from August through September ($0.24–9.79 \times 10^5$ cell equivalents L^{-1}; Fig. 5) representing approximately 6% of total *Microcystis* cells. Microcystin was present in the water column from May through October at levels that were lower than other systems (0.10–1.95 μg L^{-1}, Fig. 5) and

Table 2
Mean cyanobacterial densities (cells* or colonies** L^{-1}) (SD in parentheses) for all systems sampled. Counts were made using light microscopy. Dashed lines indicate samples were not available.

	Microcystis*	Anabaena*	Aphanizomenon**
Lake Agawam 2005			
26-May-05	68,666,000 (3,433,000)	1,100,000 (141,000)	0 (0)
7-Jun-05	–	14,667,000 (1,604,000)	0 (0)
21-Jun-05	28,300,000 (1,415,000)	700,000 (141,000)	0 (0)
5-Jul-05	53,300,000 (2,665,000)	8,733,000 (2,810,000)	30 (0)
18-Jul-05	58,700,000 (2,935,000)	17,400,000 (3,504,000)	0 (0)
1-Aug-05	60,600,000 (3,030,000)	0 (0)	0 (0)
12-Aug-05	201,300,000 (10,065,000)	3,467,000 (611,000)	140,000 (14,000)
23-Aug-05	52,000,000 (2,600,000)	3,067,000 (1,007,000)	260,000 (30,000)
20-Sep-05	2,000,000 (100,000)	1,700,000 (141,000)	0 (0)
13-Oct-05	–	–	–
20-Oct-05	–	–	–
8-Nov-05	–	–	–
Lake Agawam 2006			
2-Jun-06	76,945,000 (3,847,000)	279,000,000 (13,950,000)	520,000 (26,000)
16-Jun-06	1,200,000 (60,000)	45,500,000 (2,275,000)	30,000 (2,000)
22-Jun-06	55,491,000 (2,775,000)	201,500,000 (10,075,000)	0 (0)
27-Jun-06	3,755,000 (188,000)	265,000,000 (13,250,000)	370,000 (19,000)
6-Jul-06	14,850,000 (743,000)	60,000,000 (3,000,000)	30,000 (2,000)
11-Jul-06	122,960,000 (6,148,000)	28,500,000 (1,425,000)	90,000 (5,000)
19-Jul-06	693,000,000 (34,650,000)	50,000,000 (2,500,000)	390,000 (20,000)
25-Jul-06	429,418,000 (21,471,000)	8,500,000 (425,000)	340,000 (17,000)
10-Aug-06	1,483,227,000 (74,161,000)	15,000,000 (750,000)	570,000 (29,000)
12-Sep-06	76,691,000 (3,835,000)	35,000,000 (1,750,000)	140,000 (7,000)
28-Sep-06	625,527,000 (31,276,000)	12,500,000 (625,000)	150,000 (8,000)
10-Oct-06	436,582,000 (21,829,000)	15,000,000 (750,000)	0 (0)
24-Oct-06	332,000,000 (16,600,000)	16,500,000 (825,000)	40,000 (2,000)
Lake Ronkonkoma 2005			
27-May-05	1,000,000 (0)	0 (0)	0 (0)
8-Jun-05	113,000,000 (6,062,000)	0 (0)	0 (0)
23-Jun-05	–	–	–
5-Jul-05	67,667,000 (6,506,000)	8,000,000 (1,000,000)	0 (0)
25-Jul-05	7,000,000 (2,000,000)	9,500,000 (1,000,000)	25,000 (7,000)
5-Aug-05	2,000,000 (0)	2,500,000 (0)	50,000 (10,000)
18-Aug-05	4,000,000 (0)	5,167,000 (764,000)	20,000 (10,000)
15-Sep-05	–	–	–
Lake Champlain 2006			
22-May-06	0 (0)	0 (0)	0 (0)
19-Jun-06	293,000 (15,000)	0 (0)	0 (0)
3-Jul-06	0 (0)	0 (0)	0 (0)
17-Jul-06	953,000 (48,000)	1,067,000 (53,000)	0 (0)
1-Aug-06	11,480,000 (574,000)	41,867,000 (2,093,000)	213,000 (11,000)
19-Sep-06	1,027,000 (51,000)	2,400,000 (120,000)	5,893,000 (295,000)
3-Oct-06	200,000 (10,000)	0 (0)	80,000 (4,000)
17-Oct-06	60,000 (3,000)	0 (0)	0 (0)
Mill Pond 2006			
2-Jun-06	9,082,000 (454,000)	27,000,000 (1,350,000)	1,050,000 (53,000)
16-Jun-06	18,600,000 (930,000)	78,500,000 (3,925,000)	1,970,000 (99,000)
22-Jun-06	22,636,000 (1,132,000)	46,000,000 (2,300,000)	620,000 (31,000)
27-Jun-06	5,155,000 (258,000)	0 (0)	840,000 (42,000)
6-Jul-06	61,600,000 (3,080,000)	3,000,000 (150,000)	13,970,000 (699,000)
11-Jul-06	244,873,000 (12,244,000)	0 (0)	490,000 (25,000)
19-Jul-06	268,736,000 (13,437,000)	0 (0)	2,370,000 (119,000)
25-Jul-06	181,909,000 (9,095,000)	1500,000 (75,000)	4,410,000 (221,000)
10-Aug-06	264,955,000 (13,248,000)	5,000,000 (250,000)	60,000 (3,000)
12-Sep-06	158,782,000 (7,939,000)	69,000,000 (3,450,000)	1,630,000 (82,000)
28-Sep-06	75,164,000 (3,758,000)	2,000,000 (100,000)	2,020,000 (101,000)
10-Oct-06	529,200,000 (26,460,000)	0 (0)	390,000 (20,000)
24-Oct-06	17,338,000 (867,000)	0 (0)	690,000 (35,000)

peaked on 1 August during the maximum densities of toxic *Microcystis* cells (Fig. 5). As was found in other systems, microcystin concentrations in Lake Champlain were significantly correlated with toxic *Microcystis* cell equivalents L^{-1} ($p < 0.05$). Nutrient levels were elevated before the onset of *Microcystis* blooms (May early July DIN = 11.95 ± 4.97 μM; DIP = 0.32 ± 0.08 μM), but were lower during the peak of the bloom (mid July–September DIN = 8.12 ± 3.91 μM; DIP = 0.26 ± 0.19 μM; Fig. 5). Nutrient levels increased again in October. The bloom peak also corresponded with the highest temperatures of the sampling campaign (26 °C), with initial and final sampling temperatures close to 10 °C.

3.2. Effects of increased temperature and nutrients on toxic and non-toxic strains of Microcystis

3.2.1. Lake Agawam

During the July 2005 experiment in Lake Agawam, ambient water temperature was 26.9 ± 1.7 °C while the elevated temperature

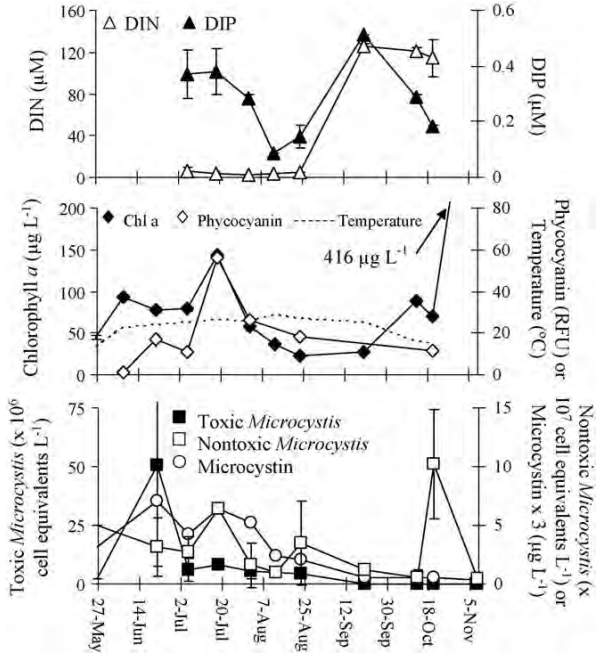

Fig. 1. Time series of parameters measured in the Lake Agawam, 2005. (A) Concentrations of dissolved inorganic nitrogen (DIN △) and orthophosphate (DIP ▲). (B) Levels of total chl *a* ◆, phycocyanin ◇, and temperature (- - -). (C) Densities of toxic ■ and non-toxic *Microcystis* □ as well as concentrations of microcystin ○. Error bars represent ±1SD of replicated samples.

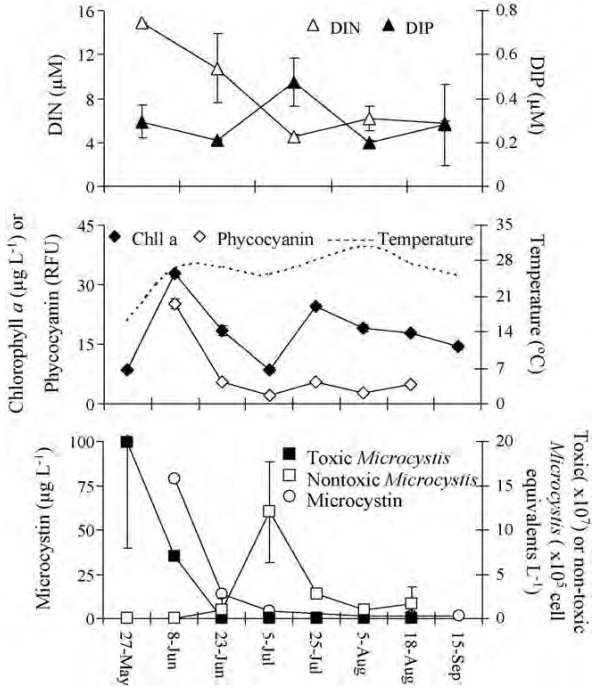

Fig. 3. Time series of parameters measured in Lake Ronkonkoma, 2005. Further details as in Fig. 1.

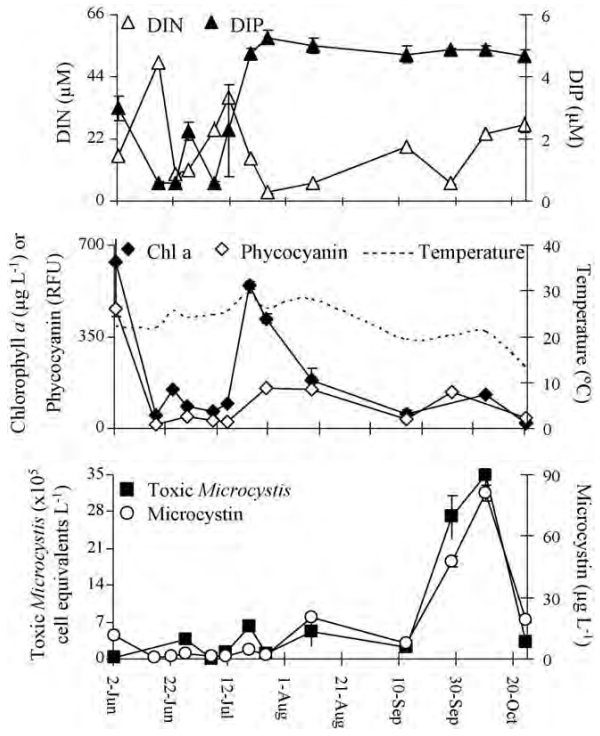

Fig. 2. Time series of parameters measured in Lake Agawam, 2006. Further details as in Fig. 1

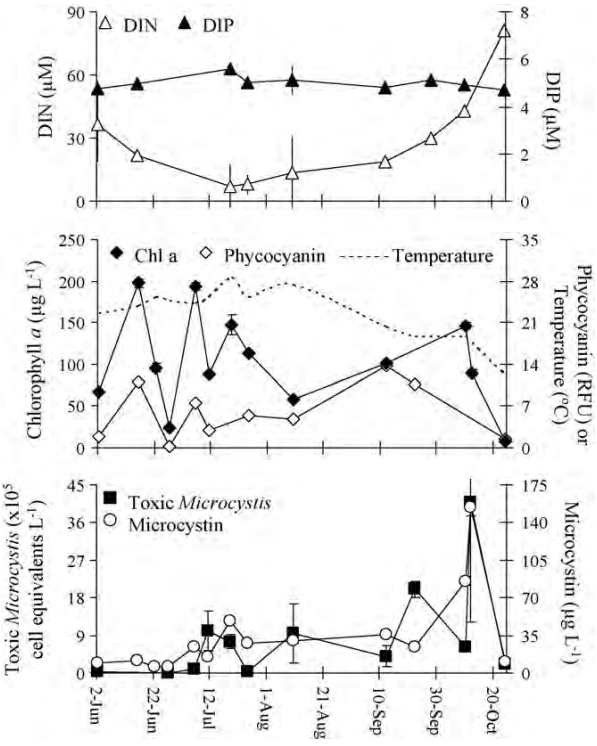

Fig. 4. Time series of parameters measured in Mill Pond, 2006. Further details as in Fig. 1.

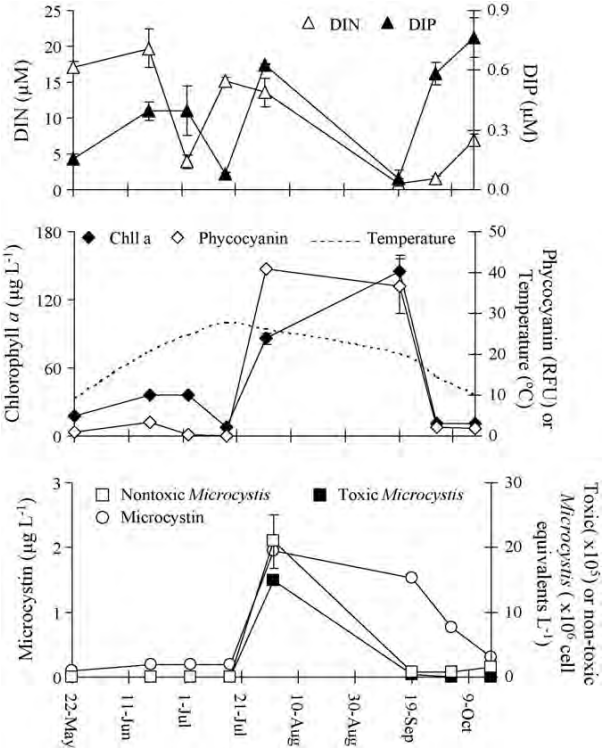

Fig. 5. Time series of parameters measured in Lake Champlain, 2006. Further details as in Fig. 1.

treatment was 30.6 ± 1.4 °C. Elevated temperature was a significant treatment effect which enhanced both toxic and non-toxic *Microcystis* growth rates by 2–3 fold ($p < 0.01$; Table 3; Fig. 6). Nutrients did not significantly alter growth rates and there were no significant interactions between treatments for either sub-population of *Microcystis* (Table 3; Fig. 6).

During the October 2006 experiment in Lake Agawam, temperature was a significant treatment effect which doubled toxic *Microcystis* growth rates compared to the control treatment ($p < 0.05$; Table 3; Fig. 6). Furthermore, although the interaction between temperature and phosphorus was not significant, this dual treatment yielded a growth rate that was 125% greater than the control and was the highest growth rate of either population in any treatment (1.87 ± 0.13 d^{-1}; Fig. 6). Interestingly, increased temperatures decreased the growth rate of the non-toxic population relative to the control ($p < 0.05$; Table 3; Fig. 6). Similarly, the interaction of temperature and phosphorus yielded a growth rate for non-toxic *Microcystis* that was significantly lower than the control ($p < 0.05$; Table 3; Fig. 6). Ambient and elevated temperatures were 10.6 ± 0.98 and 14.9 ± 0.81 °C, respectively for this experiment.

3.2.2. Lake Ronkonkoma

During the Lake Ronkonkoma experiment, ambient water temperature was 20.9 ± 0.97 °C. Experimentally enhanced temperatures (26.7 ± 0.7 °C) significantly increased growth rates of toxic *Microcystis* strains by 89% ($p < 0.05$; Table 3; Fig. 6). N enrichment lead to a significant decrease in the growth rate of the non-toxic subpopulation of *Microcystis* ($p < 0.05$; Table 3; Fig. 6), but did not alter the growth rate of the toxic strains (Table 3; Fig. 6). In addition, P enrichment yielded a significant, ~2-fold increase in the growth rates of both toxic and non-toxic strains of *Microcystis* ($p < 0.001$; Table 3; Fig. 6). There was a significant interaction between N and P on toxic *Microcystis* growth rates ($p < 0.01$; Table 3; Fig. 6), likely due to the slightly lower growth response in the N and P treatments compared to P only. There was a significant interaction between temperature and P on the non-toxic strains of *Microcystis* ($p < 0.01$; Table 3; Fig. 6). There was also a significant interaction between N, P, and temperature for the non-toxic population of *Microcystis* ($p < 0.05$) perhaps due to the growth rate being lower than that of the temperature/P enrichment (Fig. 6).

3.2.3. Mill Pond

During the June experiment in Mill Pond, ambient water temperature was 23.6 ± 2.7 °C and elevated temperature was 26.4 ± 1.9 °C. Temperature was a significant treatment effect which increased the growth rate of toxic *Microcysits* by 22% relative to the control ($p < 0.05$; Table 3; Fig. 6). Higher phosphorus concentrations also increased the growth rate of toxic *Microcystis* (by 33% relative to the control), but not significantly ($p > 0.05$; Fig. 6). However, the additive interaction between temperature and phosphorus yielded a growth rate for toxic *Microcystis* that was 63% higher then the control and was the highest growth rate of either population in any treatment (1.55 ± 0.36 d^{-1}, $p < 0.05$ for P/temperature interaction; Table 3; Fig. 6). The growth rate of the non-toxic *Microcystis* population was not significantly altered by any treatment or interaction (Fig. 6). Moreover, growth rates of this population were lower than the growth rates for toxic *Microcystis* in all treatments (Fig. 6).

During the July experiment, water temperatures were similar to that of the June experiment (23.8 ± 3.1 and 28.9 ± 1.6 °C for ambient and elevated, respectively). Although none of the individual treatments yielded a significantly increased growth rate for either population, the interaction between temperature and phosphorus was significant for toxic *Microcystis* cells as this dual treatment

Table 3
Experimentally significant treatment effects and interactions on the growth rates of toxic and non-toxic *Microcystis* as determined by a 3-way ANOVA.

	Treatment effects		Interactions	
	Toxic	Non-toxic	Toxic	Non-toxic
Lake Agawam				
18-Jul-05	T**	T**	–	–
24-Oct-06	T*	–T***	–T and N**, –T and P**	
Lake Ronkonkoma				
5-Jul-05	T*,P***	P***	N and P**	T and P**, T and N and P*
Lake Champlain				
1-Aug-06	T***, N**, P***	T***, P***	T and N**, T and P***, N and P***	–
Mill Pond				
27-Jun-06	T*	–	–	–
ll-Jul-06	–	–	T and P**	N and P**

A negative sign before a nutrient indicates that the treatment yielded a significant decrease in growth rates, whereas all others yielded significantly higher growth rates.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

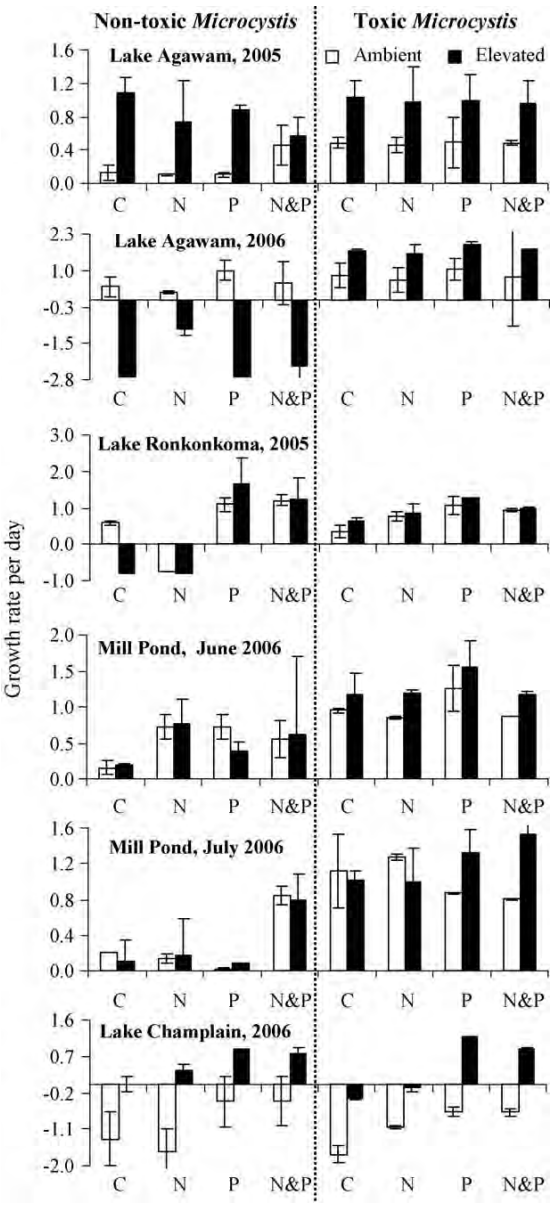

Fig. 6. Net growth rates of toxic *Microcystis* (right half of figure) and nontoxic *Microcystis* (left half of figure) during nutrient amendment experiments (t = 72 h) conducted in various systems during the 2005 and 2006 field seasons at ambient (white bars) and elevated (black bars) temperatures. C: control; N: nitrate; P: orthophosphate. Error bars represent ± 1 SD of triplicate experimental bottles.

yielded toxic population growth rates that were 20% higher than the control ($p < 0.01$; Table 3; Fig. 6). Furthermore, although not statistically significant, concurrent enrichment of temperature, N and P yielded a growth rate for toxic *Microcystis* that was 40% greater then the control and was the highest growth rate for either population among all the treatments (1.54 ± 0.11 d^{-1}; Fig. 6). On the other hand, the interaction between N and P was significant for the non-toxic population yielding growth rates which were enhanced 3-fold over unamended controls ($p < 0.01$; Table 3; Fig. 6).

3.2.4. Lake champlain

During the Lake Champlain experiment, ambient water temperatures were 24.9 ± 2.0 °C. Experimentally enhanced temperatures (29.1 ± 1.3 °C) significantly increased the growth rates of both toxic and non-toxic *Microcystis* populations by 80% and 101% respectively ($p < 0.001$; Table 3; Fig. 6). Increased nitrogen concentrations stimulated growth rates of the toxic *Microcystis* population, increasing them 40% over the unamended controls ($p < 0.01$; Table 3; Fig. 6). Furthermore, phosphorus enrichment significantly increased the growth rates of both toxic and non-toxic populations by 60% and 68%, respectively ($p < 0.001$; Table 3; Fig. 6). Finally, nutrients (N or P) interacted with temperature, to enhance toxic *Microcystis* growth rates ($p < 0.01$; Table 3; Fig. 6) with the enhanced P and temperature treatment yielding the highest growth rates of any population among all treatments (1.17 ± 0.03 d^{-1}).

4. Discussion

Harmful cyanobacterial blooms have increased globally in frequency and intensity in recent decades. Eutrophication and warmer temperatures are often cited as key factors which promote these events (Paerl, 1988; Chorus and Bartram, 1999; Hudnell and Dortch, 2008; Paerl and Huisman, 2008). Previous studies have investigated the effects of singular environmental factors on the growth and/or abundance and/or photosynthesis of total *Microcystis* populations such as light (Codd and Poon, 1988; Wiedner et al., 2003; Kim et al., 2005), nutrient enrichment (Watanabe and Oishi, 1985; Codd and Poon, 1988; Fujimoto et al., 1997; Orr and Jones, 1998; Lee et al., 2000; Oh et al., 2000; Paerl et al., 2001; Vézie et al., 2002; Downing et al., 2005; Gobler et al., 2007), salinity (Tonk et al., 2007) and temperature (Konopka and Brock, 1978; Takamura et al., 1985; Robarts and Zohary, 1987). Other studies have found that *Microcystis* can out-compete other species of phytoplankton at high temperatures (≥30 °C; Fujimoto et al., 1997). However, to our knowledge, this is the first field study to investigate the effects of increased temperature and nutrient concentrations on the growth rates of toxic and non-toxic subpopulations of *Microcystis*. Our ability to examine this phenomenon along with the *in situ* dynamics of these two populations within four distinct ecosystems during two years has generated data set which provides new insight regarding the ecology of toxic *Microcystis* blooms.

4.1. Seasonal dynamics of toxic and non-toxic Microcystis

Microcystin was detected in all four ecosystems studied on every date analyzed (Figs. 1–5). Toxic *Microcystis* cells comprised between 0.01% and 100% of the total *Microcystis* population among the four systems, a range larger than those found in prior studies (Kurmayer and Kutzenberger, 2003; Rinta-Kanto et al., 2005; Rinta-Kanto and Wilhelm, 2006; Yoshida et al., 2006; Hotto et al., 2008). However, there were notable differences, even among the systems presented here. In Lake Ronkonkoma, toxic *Microcystis* comprised between 12% and 100% of total cells, whereas the range was between 0.01% and 6% in other systems. The seasonal dynamics of toxic and non-toxic *Microcystis* observed in Lake Ronkonkoma, the only stratified lake studied, were consistent with the findings of Kardinaal et al. (2007) who also found toxic strains of *Microcystis* were succeeded by non-toxic strains in a deep stratified lake. However, in the well-mixed systems we studied (Lake Agawam, northeastern Lake Champlain, Mill Pond), toxic strains comprised a small portion of total cells where as Kardinaal et al. (2007) found the toxic strains dominated *Microcystis* populations in the two unstratified lakes. The seasonal dynamics of cyanobacterial blooms in general, and toxic and non-toxic strains of *Microcystis* in particular, likely vary based on system-specific physical and/or environmental conditions.

The World Health Organization (WHO) currently recommends monitoring chlorophyll *a* concentrations and total cyanobacterial

cell counts to protect against human exposure to high levels of microcystin (Chorus and Bartram, 1999). However, of the five field data sets generated by this study (Figs. 1–5), only two displayed mildly significant correlations between chlorophyll *a* concentrations and concentrations of microcystin ($p < 0.05$) while only three showed significant correlations between total cyanobacterial cell counts and microcystin concentrations ($p < 0.05$). This is not surprising as all phytoplankton contain chlorophyll *a* and nearly every major species of cyanobacteria has both toxic and non-toxic strains (Chorus and Bartram, 1999). However, in all five of the time series data sets generated by this study, densities of toxic *Microcystis* cells were significantly correlated with microcystin levels ($p < 0.05$ for all; Figs. 1–5), often at a very high level of significance (Lake Ronkonkoma, Lake Agawam, Mill Pond; $p < 0.001$). Obviously, the predominance of other microcystin producing genera will influence the relationship between toxic *Microcystis* cells and microcystin (Rantala et al., 2006; Rinta-Kanto and Wilhelm, 2006) and the presence of the *mcyD* gene does not necessarily translate into the synthesis of microcystin *in situ* (Gobler et al., 2007). Despite this, our data demonstrates that *mcyD*-containing *Microcystis* cells were a better predictor of pelagic microcystin concentrations than the total cell counts of cyanobacteria, total *Microcystis*, or chlorophyll *a* and thus may be a better predictor of microcystin in aquatic ecosystems than parameters currently recommended to be monitored by the WHO.

4.2. Effects of temperature on the growth rates of toxic and non-toxic strains of Microcystis

As seasonal temperatures increase from 10 to 30 °C in freshwater ecosystems, the phytoplankton group with the highest growth rates generally shifts from diatoms to green algae to cyanobacteria (Canale and Vogel, 1974; Reynolds, 1997). Furthermore, it has generally been accepted that cyanobacteria dominate phytoplankton assemblages in temperate freshwater environments during the warmest periods of the year, particularly in eutrophic systems (Canale and Vogel, 1974; Reynolds and Walsby, 1975; Konopka and Brock, 1978; Tilman and Kiesling, 1984; Paerl, 1988, 2008; Paerl and Huisman, 2008). Consistent with this idea, cyanobacteria in general, and *Microcystis* in particular, dominated all of our study sites as temperatures reached their annual pinnacle (Figs. 1–5). While toxic *Microcystis* cells densities also peaked during annual maximum temperatures in two of our study sites (Lake Agawam, Lake Champlain; Figs. 1 and 5) they were slightly off-set in two other sites (Mill Pond, Lake Ronkonkoma; Figs. 3 and 4) likely reflecting the additive role other factors, such as nutrients, can play in bloom dynamics (see Section 4.3). More importantly, during five of the six (83%) experiments conducted, a ~4 °C increase in experimental temperatures yielded significantly higher (22–115%) growth rates for toxic (*mcyD*-containing) *Microcystis* cells (Fig. 6). In contrast, the growth rates of non-toxic *Microcystis* were significantly increased by higher temperature in only a third of experiments conducted (Fig. 6). Furthermore, in five of six experiments, the growth rate of toxic *Microcystis* cells exceeded those of non-toxic cells within the enhanced temperature only treatment (Fig. 6). All experimentally elevated temperatures were between 15 and 30 °C the optimal range for *Microcystis* (Chorus and Bartram, 1999), and in all but one experiment (Lake Agawam, 2006; temperature = 15 °C), the elevated temperatures fell between 25 and 30 °C, a range which yields maximal cellular toxin content in *Microcystis* (Van der Westhuizen and Eloff, 1985; Codd and Poon, 1988). Kim et al. (2005) found that toxic *Microcystis* strains cultured at 25 °C had more *mcyB* transcripts than cultures reared at 20 or 30 °C. Our observed increase in the abundance of 'toxic' *Microcystis* during higher temperatures could represent more gene copies per cell, more toxic cells, or both. However, any of these scenarios could lead to higher levels of microcystin in aquatic ecosystems (Tillett et al., 2000; Rinta-Kanto and Wilhelm, 2006). Hence, as surface water temperatures continue to rise (Houghton et al., 2001), toxic *Microcystis* may out-grow non-toxic *Microcystis* or may synthesize more microcystin synthetase, yielding blooms that are comprised of a larger proportion of toxic cells and/or have higher microcystin concentrations. Moreover, as predicted by Paerl and Huisman (2008), our data also suggests future warming of temperate aquatic systems could lead to toxic *Microcystis* dominating for longer time periods than they presently do.

4.3. The effects of nutrient enrichment on the growth rates of toxic and non-toxic Microcystis

During two of the six experiments conducted (Lake Ronkonkoma, Lake Champlain), phosphorus loading yielded significantly higher growth rates for both toxic and non-toxic populations of *Microcystis* relative to control treatments, although growth rates were not significantly different between these populations (Table 3; Fig. 6). These findings are contrary to those of Vézie et al. (2002) who found that at higher P concentrations the growth rates of toxic *Microcystis* exceeded non-toxic strains. However, our results were reasonable in light of the ambient nutrient levels as DIP was low (0.2 and 0.6 μM, respectively) and DIN concentrations were greater than 11 μM during these experiments (Figs. 3 and 5). DIP levels were substantially higher within the two shallower and more eutrophic systems we studied (Mill Pond, Lake Agawam; Figs. 1, 2 and 4) and P never affected *Microcystis* growth in these systems. Nitrogen loading significantly increased the growth rate of toxic *Microcystis* in Lake Champlain (Table 3; Fig. 6), a result consistent with Vézie et al. (2002) who found that increasing N concentrations in N-limited cultures significantly increased the growth rates of both toxic and non-toxic subpopulations of *Microcystis*. This result is also consistent with Gobler et al. (2007) who found that nitrogen can promote the growth and microcystin production of *Microcystis* during bloom events.

During many experiments we conducted, nutrients (N or P) and temperature interacted to promote the growth of toxic *Microcystis*. In Lake Champlain, Mill Pond (June 2006), and Lake Agawam (2006), the concurrent enhancement of temperature and P yielded growth rates for toxic *Microcystis* that not only increased 170%, 125%, and 20%, respectively, relative to the controls but also yielded the highest growth rate of either population in any treatment (Fig. 6). Similarly, during the Mill Pond experiment conducted during July 2006, the enrichment of N, P, and temperature yielded a growth rate of toxic *Microcystis* that was 40% greater than the control and was the highest growth rate of either population in all treatments (Fig. 6). These results are somewhat consistent with the findings of Jiang et al. (2008), who documented that higher temperature and P yielded greater abundance of total *Microcystis* cells. More importantly, these results suggest that future nutrient loading coupled with climatic warming may promote toxic, rather than non-toxic, populations of *Microcystis* and thus may lead to more toxic blooms.

This study has demonstrated the manner in which temperature and nutrients can interact to strongly influence the abundance and relative dominance of toxic and non-toxic strains of *Microcystis* within bloom events across the Northeast US. However, our previous research has demonstrated that even toxic populations of *Microcystis* show seasonal changes in expression of the microcystin synthetase gene (Gobler et al., 2007). As such, an important open question is the degree to which environmental factors such as nutrient loading and temperature changes influence microcystin synthetase gene expression and cellular microcystin synthesis within an ecosystem setting. Moreover, whether other microcystin synthesizing strains of other toxic cyanobacteria genera are promoted by warming and eutrophication remains unknown.

In conclusion, we found that the portion of wild *Microcystis* populations that were comprised of toxic cells varied seasonally and by location from 0.01% to 100%. Molecularly quantifying toxic (*mcyD*-containing) *Microcystis* was a better predictor of *in situ* microcystin levels than proxies currently recommended by the WHO to protect against human exposure to microcystin (total cyanobacteria cell densities or chlorophyll *a*; Chorus and Bartram, 1999). Warmer temperatures frequently (83% of experiments) shifted *Microcystis* toward populations comprised of a larger percentage of toxic *Microcystis* and/or cells with more *mcyD* copies per cell, scenarios which could yield more toxic blooms in an ecosystem setting. Finally, this study demonstrated that higher temperatures coupled with elevated P concentrations frequently yielded growth rates of toxic *Microcystis* cells which exceeded all other treatments and populations. Therefore, continued climatic warming and eutrophication could lead to a shift in cyanobacterial dominance toward blooms that contain a greater percentage of toxic *Microcystis* cells and/or more microcystin synthetase gene copies and hence greater concentrations of microcystin.

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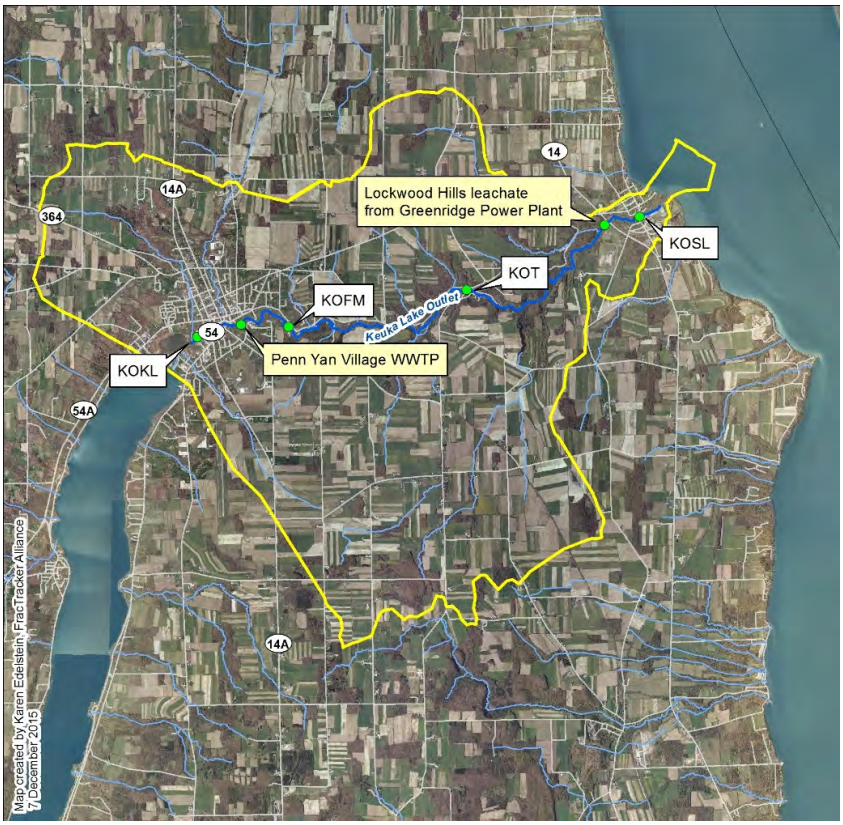
Exhibit C

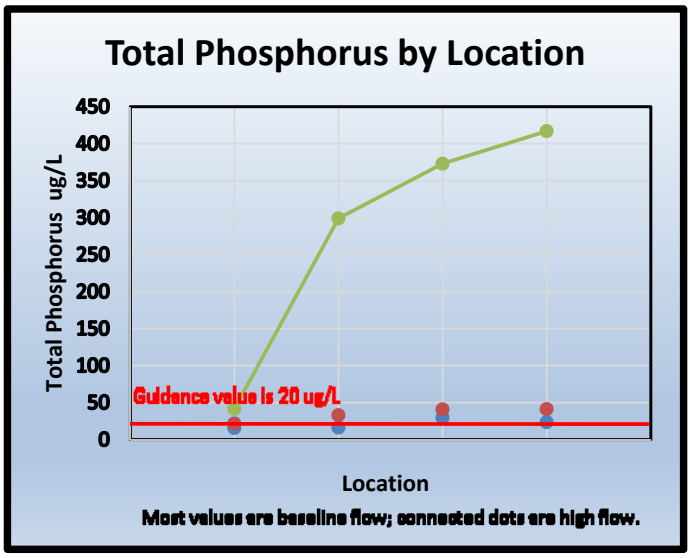
Keuka Outlet Fact Sheet

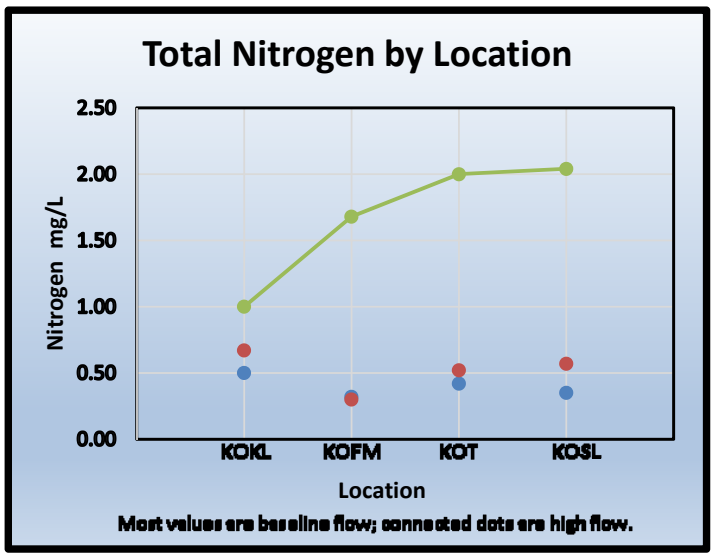
Keuka Outlet is the largest tributary to Seneca Lake, its watershed area representing 35% of total. It is the sole outlet of Keuka Lake, and flows northeast from Penn Yan to Dresden, mid-way on the western shore of Seneca Lake. The flow from Keuka Lake is controlled by gates in a dam at Penn Yan. The drainage area is largely made up of agriculture and forested land, though the outlet does receive effluent from the Penn Yan WWTP. The stream has a long history of mills and industry on its shores, and historically provided water for a canal connecting the two lakes. The Keuka Outlet stream is a DEC Class C fishing stream. The maps shows the sampling locations on the outlet stream, though the watershed encompasses all that flows into Keuka Lake.



SLPWA (and its partner CSI) has sampled and tested the outlet throughout 2015 at the four locations shown on the map (KOKL, KOFM, KOT, KOSL). Results for phosphorus and nitrogen (nutrients that encourage plant and algae growth), and E. coli bacteria (indicator of human and animal wastes) are the most concerning of the attributes tested.

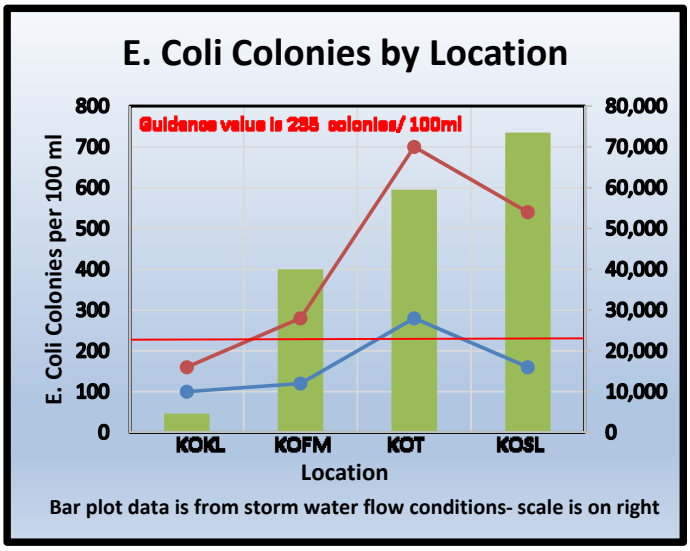
Results





The graph showing phosphorus levels, at the locations sampled, shows that at normal stream flow conditions the levels are reasonable at the 1-2X the DEC guidance level of 20ug/l. During the one storm event tested the phosphorus levels increased dramatically to ~20X the guidance level. The graph for nitrogen is similar, with normal flow conditions showing levels at or below the guidance level. Again the high flow of the storm event showed nitrogen levels 4X the guidance level. High flow levels combined with high levels of nutrients results in much added nutrients flowing into Seneca Lake, adding to the issue of weed and algae growth. 2015 saw Seneca Lakes first confirmed toxic blue-green algae blooms.

The graph to the right shows the levels of E.coli during normal flow and the one high flow event captured. At normal flow, the E.coli levels increase as the stream flows from Keuka to Seneca Lake, starting below the guidance level and ending at or above that level, The real concern is the dramatically high levels seen during the high flow event, with levels increasing from 20X to 310X . E. coli is not regulated in Class C streams. The very high levels that are seen in the highest of flow conditions pollute Seneca Lake (a "Class A" drinking water supply) with this dangerous bacteria. Investigative sampling will be required to determine if the Penn Yan waste water treatment facility is a significant contributor to the high bacteria levels.



Actions to address these issues can occur at the community and state level. Upgrades or replacement of the WWTP can address the high phosphorus output. Improvement and controls on agricultural practices can have impact on the dramatic run-off effects seen for both phosphorus and E.coli levels

Keuka Outlet Data

Location Code GPS Coordinates	Site Name	Team Contact	Sampling Date	Type of Event	Water Temperature (degrees C)	pH	Dissolved Oxygen mg/L	Chloride mg/L	Soluble Reactive Phosphorus ug/L	Total Phosphorus (ug/L)	Nitrate-Nitrite Nitrogen (mg/L)	Kjeldahl Total Nitrogen (mg/L)	Specific Conduct (Ms/cm)	Coliform (Col/100 ml)	E.coli (Col/100 ml)	Turbidity (NTU)	Hardness Mg/L as CaCo3	Total Suspended Solids (ml/L)
Guidance Values						6.5-8.5 (Classes A- C) 6.0-9.0 (Class D)		50.0		20.0	1.00		150-500	2400	235			
KOKL	Penn Yan Boat Launch	Fred Geyer	7/13/2015	BF	23.5	8.00	7.8	35.2	15.10	15.8	0.08	0.50	315.0	4000	100	1.71	117.0	0.63
42.6574			8/17/2015	BF	25.2	8.25	6.8	37.5	17.20	21.8	0.08	0.67	305.0	9000	60	3.89	110.0	0.63
-77.0589			9/30/2015	SW	21.5	7.25	5.1	31.7	23.20	41.7	0.07	1.00	287.0	20000	4620	80.00	141.0	53.00
KOFM	Fox Mills Rd.	Fred Geyer	7/13/2015	BF	23.5	8.25	7.6	35.9	9.14	16.3	0.09	0.32	323.0	7000	120	2.62	118.0	2.25
42.6596			8/17/2015	BF	24.8	8.25	7.4	38.6	20.00	32.9	0.05	0.30	331.0	5500	160	4.59	130.0	12.50
-77.0371			9/30/2015	SW	-	8.00	7.7	33.0	175.00	299.0	2.16	1.68	384.0	100000	40000	80.00	141.0	53.00
KOT	KO Tributary near Ridge Rd.	Larry Martin	7/13/2015	BF	23.5	8.25	7.9	37.3	16.40	29.6	0.30	0.42	333.0	5000	280	4.82	122.0	15.30
42.6669			8/17/2015	BF	23.2	8.25	---	42.6	32.50	40.8	0.72	0.52	366.0	12500	420	1.86	132.0	1.25
-76.9947			9/30/2015	SW	17.0	8.25	8.0	38.6	204.00	373.0	4.80	2.00	454.0	100000	59500	95.00	177.0	65.00
KOSL	Charles St. Bridge, Dresden	Larry Martin	7/13/2015	BF	23.2	8.00	7.8	39.3	12.60	23.5	0.27	0.35	339.0	20000	160	1.05	119.0	7.00
42.6805			8/17/2015	BF	22.7	8.25	7.4	47.1	30.10	41.3	1.04	0.57	402.0	11000	380	2.79	138.0	0.63
-76.9538			9/30/2015	SW	16.8	8.00	8.0	31.3	215.00	417.0	4.25	2.04	401.0	100000	73500	150.00	161.0	102.00

BF= Base Flow; SW= Storm Water