

ECOPHYSIOLOGY OF THE TOXIC CYANOBACTERIUM *MICROCYSTIS AERUGINOSA*
ACROSS NUTRIENT GRADIENTS

By

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ABSTRACT

ECOPHYSIOLOGY OF THE TOXIC CYANOBACTERIUM *MICROCYSTIS AERUGINOSA* ACROSS NUTRIENT GRADIENTS

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Harmful algal blooms (HABs) are a significant and growing problem worldwide in both freshwater and marine ecosystems. In many freshwater systems, cyanobacteria blooms can cause significant risks to public health due to the production of potent toxins. An important objective in managing harmful phytoplankton blooms is determining what factors influence the toxicity of bloom-forming species. In this dissertation, I combined lab and field experiments along with field measurements in Lake Erie to determine whether the production of microcystin (a potent liver toxin) by the cyanobacterium *Microcystis* is strongly affected by nutrient limitation. The field and lab experiments demonstrated that nitrogen-limited conditions led to significantly reduced microcystin quota relative to phosphorus-limited or nitrogen and phosphorus saturated conditions. Results an extensive seasonal survey in the western basin of Lake Erie revealed a similar influence of nitrogen availability on microcystin quota, indicating that nitrogen limitation appears to have a robust influence on the toxin quota of *Microcystis*. These results are consistent with stoichiometric considerations in that production of nitrogen-rich secondary metabolites, such as microcystins, should be reduced disproportionately under nitrogen limitation. The results of this work may be used to inform public decision making about managing nitrogen and phosphorus inputs into freshwater lakes.

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CHAPTER 1

Nitrogen availability increases the toxicity of the harmful cyanobacterium *Microcystis aeruginosa*

INTRODUCTION

Harmful phytoplankton blooms (HABs) are a major environmental concern in both marine and freshwaters throughout much of the globe (Huisman et al. 2005) and the impacts of HABs on both ecosystems and human health (Chorus & Bartram 1999; Carmichael 2001; Smith & Schindler 2009) are well documented. One of the most critical aspects of HAB ecology is the production of toxins by HAB species during blooms. In many cases, the impact of HABs on ecosystem and human health is directly related to the concentration of phytoplankton-produced toxins in the water. As one noteworthy example, concentrations of microcystin, a potent liver toxin produced by *Microcystis aeruginosa* (hereafter *Microcystis*), can exceed the World Health Organization guideline for drinking water during the summer months in Lake Erie which is the drinking-water source for four million people (Fuller et al. 2002).

The concentration of toxins in the water is a function of both the biomass of the toxin producing species and the toxin quota (i.e. the amount of toxin per unit biomass). Toxin quota is a highly variable trait within a species. For example, among genotypes within some species, toxin quota can range from essentially zero to over 5 $\mu\text{g toxin mg}^{-1}$ dry weight (Lukač 1993; Sivonen & Jones 1999). The implication of a highly variable toxin quota is that blooms with very high biomass and low toxin quota may actually pose a smaller concern (with respect to toxin concentrations in the water) than a bloom with less biomass but higher toxin quota. Therefore, understanding what factors contribute to variation in toxin quota is fundamental to understanding the impacts of HABs.

Microcystis is a cosmopolitan cyanobacterium that develops toxin producing blooms in both temperate and tropical freshwaters. Within populations of *Microcystis*, toxin quota can vary by either: a) changes in the relative abundance of more toxic vs. less toxic genotypes (i.e. intraspecific competition); or b) due to changes in toxin production induced by biological and/or physical factors (i.e. phenotypic plasticity). For example, Briand et al. (2009) showed dramatic spatio-temporal changes in the relative abundance of toxin producing genotypes of *Microcystis* in a large reservoir over the course of the growing season. Kardinaal et al. (2007) demonstrated in lab experiments that non-toxic strains of *Microcystis* outcompeted toxic strains under light limited conditions and light limitation induced by self-shading might explain a commonly observed temporal trend in some lakes for blooms to become less toxic (toxin per unit biomass) over time.

Alternatively, many laboratory studies indicate that microcystin quota is a plastic trait and can be a function of growth rate (Watanabe et al. 1989; Orr & Jones 1998; Oh et al. 2000), nutrient concentration (Oh et al. 2001; Downing et al. 2005; Van de Waal et al. 2009a), temperature (Westhuizen 1985), light levels (Utkilen and Gjølme 1992; Wiedner et al. 2003) and grazers (Jang et al. 2003). With respect to *Microcystis*, several lab-scale studies have shown a strong positive correlation between nitrogen concentration (or N:P ratio) and toxin quota (Orr & Jones 1998; Long et al. 2001). However, these data are confounded by the fact that toxin quota was also positively correlated with growth rate as a result of increased nitrogen availability. More recent studies have parsed out the effects of growth rate and confirmed that toxin quota is positively affected by increased nitrogen levels (or N:P ratio) independent of growth rate (Lee et al. 2000; Oh et al. 2000; Downing et al. 2005; Van de Waal et al. 2009). Jahnichen et al. (2011)

additionally reports the limiting effects of sulphur concentrations on microcystin quota as well as an inverse relationship between quota and phosphorus concentrations.

Demonstrating statistically-significant effects of environmental factors in tightly-controlled lab experiments with clonal monocultures is relatively straightforward given the high precision possible in the laboratory. However, it remains to be demonstrated which of many potentially-important factors has major effects on toxin quotas in natural populations, since the latter are genetically diverse (Briand et al. 2009) and embedded within complex communities of interacting species and genotypes.

Within natural phytoplankton communities where *Microcystis* is a dominant species, there is evidence that microcystin concentrations can be correlated with nitrogen and/or phosphorus concentrations (Graham et al. 2004; Rinta-Kanto et al. 2009; Orihel et al. 2012). Furthermore, Gobler et al. (2007) demonstrated with nutrient amendment assays to lake water samples that supplemental nitrogen late in the growth season led to increased toxin concentrations.

Only a handful of studies have specifically focused on toxin quotas in natural systems. Welker et al. (2003) documented a general pattern where the highest quotas occurred early in the season but decreased with increasing *Microcystis* abundance later in the season, but provided only relatively minor details about the nutrient environment. A survey of lakes in Quebec with generally low toxin concentrations ($< 1 \mu\text{g/L}$ microcystin) revealed a positive correlation between total nitrogen and toxin quota (Graham et al. 2004). By contrast, a multi-year survey of hyper-eutrophic lakes in Alberta showed that microcystin toxin quota was significantly positively correlated with phosphorus concentrations but inversely correlated with nitrate (NO_3)

and ammonium concentrations. Clearly there is a need for more work on the controls of microcystin production by cyanobacteria in natural systems.

In this study, we examined the role of nutrient limitation in controlling the microcystin quota of *Microcystis* by combining the results of a large-scale enclosure lake enclosure experiment, a single-genotype lab experiment and an extensive field survey of the western basin of Lake Erie. Results from all three approaches were congruent in terms of the positive effect of nitrogen availability on microcystin quota. Furthermore, genetic analyses of the microcystin gene (*mcyB*) from the enclosure experiment suggests that phenotypic plasticity (rather than changes in gene copy density) was largely responsible for this effect. This observed positive effect of nitrogen availability on the toxicity of *Microcystis* is particularly notable because the high N:P ratios in most freshwater lakes indicate that excess available nitrogen is common (Downing & McCauley 1992), particularly in landscapes with intensive row-crop agriculture where anthropogenic nitrogen inputs considerably exceed biological demand (Arbuckle & Downing 2001).

METHODS

Gull Lake enclosure experiment

Gull Lake is a large, hardwater lake (surface area = 8.2 km², mean depth = 12 m, maximum depth = 31 m) in southwestern Michigan adjacent to Michigan State University's Kellogg Biological Station. The lake is oligotrophic with average summer total phosphorus (TP) of 8 µg L⁻¹ and chlorophyll a of 4 µg L⁻¹. Ammonium levels are typically very low (less than 15 µg L⁻¹) but NO₃ levels are usually above 300 µg L⁻¹ due to significant external contributions from the watershed (Bruesewitz et al. 2012), and phytoplankton growth is P-limited (Hamilton et al. 2009). Sulfate is readily available and concentrations show little seasonal variability (mean, 23 mg L⁻¹; Hamilton unpublished data). Zebra mussels invaded the lake in 1994, and since then *Microcystis* biomass levels have been significantly higher than pre-invasion (Sarnelle et al. 2005). Microcystin concentrations in the mixed layer range up to 0.5 µg L⁻¹, which is about 8 times higher than a lake of comparable total phosphorus without zebra mussels (Sarnelle et al. 2010). The lake is ideal for experimental investigations of microcystin quota since *Microcystis* is the only significant microcystin producer and its low P and high nitrate-N concentrations allow manipulation of N and P availability by simply adding additional P.

During the summer of 2007 we conducted an enclosure experiment in the lake to test for effects of P addition and *Dreissena polymorpha* (zebra mussel) density on the abundance and toxin-quota of *Microcystis*. The enclosures were deployed in the same manner as described in Hamilton et al. (2009). In brief, enclosures were constructed from transparent polyethylene tubing (2 m diameter, 10 m long, total volume ~ 31,000 L) that was heat-sealed along the bottom end and open at the top to allow exposure to the atmosphere. Thirty enclosures were suspended

from a floating dock that was anchored in ~15 m of water. Over the course of 2 days (27-28 June), the enclosures were filled with lake water pumped from 4 m depth (mixed layer = ask Jeff) and passed through a 150 μm mesh net to remove crustacean zooplankton that might have been killed by the pump. Natural densities of zooplankton were then re-established on 29 June by pooling repeated zooplankton tows (1 m diameter, 150 μm mesh) from the lake epilimnion (7-8 m depth) and allocating equal aliquots to the enclosures.

Enclosures were randomly assigned to three P treatments intended to simulate ambient oligotrophic (no P addition, TP $\sim 8\text{-}10\ \mu\text{g L}^{-1}$, referred to as “low P”), mesotrophic (TP amended to $\sim 15\ \mu\text{g L}^{-1}$, “medium P”) and mildly eutrophic (TP amended to $\sim 25\ \mu\text{g L}^{-1}$, “high P”) conditions. We added P alone to simulate a eutrophication gradient since the N:P ratio in lakes typically decreases with increases in P (Downing et al. 2005) and since Gull Lake already has high NO_3 . Over the course of 7 days (29 June – 5 July), we dripped a concentrated P solution (as NaH_2PO_4) into the enclosures to reach the target TP levels. We subsequently maintained target TP levels via weekly monitoring and addition of additional phosphate solution as needed.

Within each set of 10 enclosures for each P treatment, we also established a gradient of *Dreissena* ranging from 0 – 4 g m^{-2} (dry tissue mass) on 5 July (day 0 of the experiment). We found no effects of *Dreissena* density on microcystin quota (as we had found earlier: Knoll et al. 2008), total phytoplankton biomass, concentrations of available nutrients, or indices of phytoplankton nutrient limitation, so we focus on the effects of phosphorus treatment in this paper. *Dreissena* had a positive influence on *Microcystis* biomass in the enclosures, as reported previously (Vanderploeg et al. 2002; Sarnelle et al. 2005; Knoll et al. 2008). Response variables were averaged across the *Dreissena* densities for all analyses reported in this paper.

We sampled for phytoplankton and nutrients weekly in the enclosures by pooling duplicate depth-integrated tube samples (2.5 cm inside diameter) from the surface to 7 m depth (approximate depth of mixed layer). Sampled water was stored in cleaned cubitainers and transported in coolers to a lakeside laboratory, where samples were initially processed within one hour into four sub-samples: 1) raw water sample stored at 4 °C; 2) filtered water sample passed through an A/E glass fiber filter and stored at 4 °C; 3) seston collected on A/E filters and immediately frozen at -20 °C; and 4) water preserved in Lugol's solution for microscope counting of the phytoplankton.

All nutrient analyses were performed within 48 hours of collection. Soluble reactive P (SRP) and ammonium (NH₄) were measured on filtered water samples using long-pathlength spectrometry (Wetzel and Likens 2000). NO₃ was measured on filtered water samples by Dionex membrane-suppression ion chromatography. TP was measured on persulfate-digested raw water samples and then analyzed spectrophotometrically as SRP. Chlorophyll was extracted from frozen filters in 90% ethanol and measured fluorometrically (Welschmeyer 1994). Particulate microcystins were extracted from frozen filters with 75% methanol and then diluted 1:4 with deionized water before analysis by Enzyme-Linked ImmunoSorbent Assay (ELISA) using a commercial kit (Enviroligix, Inc.). The detection limit for this protocol was ~0.02 µg L⁻¹.

Microcystis biomass was measured via inverted-microscope analysis on Lugol's preserved samples taken on the last day of the experiment (day 31). Sub-samples (50 ml) were transferred into 25 mm-wide settling chambers with a cover-slip bottom. After a 5-day settling

period, the entire chamber surface was surveyed for *Microcystis* colonies and the surface area of each colony was measured at 100X magnification from digitized photographs (SPOT Software V 4.0.9, Diagnostic Instruments). The cumulative colony surface area for a chamber was converted into *Microcystis* cell concentration (cells L^{-1}) based on the surface area to cell count relationship established for *Microcystis* samples in Lake Erie ($R^2 = 0.95$, $n = 21$) after confirming that cell packing density within Gull Lake colonies was not significantly different than that within Lake Erie colonies. *Microcystis* dry biomass concentration was then calculated by converting cell concentration to biovolume (based on measurements of cell dimensions) and subsequently converting biovolume to dry biomass assuming a dry weight (mg) to biovolume (mm^3) ratio of 0.42. Microcystin toxin quota ($\mu\text{g mg}^{-1}$ dry weight) was calculated by dividing the particulate microcystin concentration ($\mu\text{g L}^{-1}$) by *Microcystis* dry biomass concentration (mg L^{-1}).

Bulk-alkaline phosphatase activity (APA), a measure of phosphorus limitation of the planktonic community, was measured fluorometrically in 10 ml aliquots of freshly-collected raw water samples from the enclosures (Pettersson 1980). *Microcystis*-specific alkaline phosphatase activity was assessed using enzyme-labeled fluorescence (ELF) microscopy (Gonzalez-Gil and Aguilera 1998). This microscopy-based method is analogous to bulk alkaline phosphatase analysis, whereby phosphorus-limited organisms produce the enzyme alkaline phosphatase which is then detected visually for cells of a particular species. ELF substrate (0.1 ml, 1:20 v:v in buffer solution; E6601 from Invitrogen, USA) was added to separate 10 ml subsamples of freshly-collected raw water samples from the enclosures. After 1 hr, the treated subsample was filtered onto black 1 μm membrane filters and rinsed with phosphate-buffered saline to inhibit

further enzyme activity. The filters were then permanently mounted onto microscope slides and stored at -20 °C for up to three months before analysis.

Using a Nikon Eclipse E600 epifluorescence microscope, the mounted membrane filters were visually scanned for *Microcystis* colonies and evidence of alkaline phosphate activity as indicated by bright green precipitates on the surface of colonies. Each *Microcystis* colony (up to 200 per slide) was counted and marked as having either positive or negative enzyme activity, with the percent of active colonies per sample serving as a relative index of the population's alkaline phosphatase activity.

Laboratory experiment

To clarify the mechanistic effect of N versus P limitation on the microcystin quota of *Microcystis* in Gull Lake, we conducted a laboratory experiment in which a *Microcystis* strain from the lake was exposed to varying nitrogen versus phosphorus availability. We used a toxin-producing strain of *Microcystis* (E1-6) that was isolated from Gull Lake in 2007. In contrast to most culture-collection strains, our strain had retained the typical colony-forming phenotype when the laboratory experiment was conducted.

Before the experiment, *Microcystis* strain E1-6 was grown in four 2-L culture vessels containing sterilized half-strength WC-S media (Stemberger 1981), with an initial N:P ratio of 20:1 (250 µM N as KNO₃, 12.5 µM P as K₂HPO₄). These culture vessels were grown under constant light (PAR of 100 µmol m⁻² s⁻¹) with no supplemental aeration at 19 °C. After two weeks the culture vessels were pooled together and 250 ml of this seed inoculum was distributed to each of 16 500-ml sterilized flasks. The flasks were randomly assigned to one of three

nutrient treatments where the relative concentrations of N and P were modified from the half strength WC media recipe: 1) P-limited (250 μM N: 2.5 μM P [100:1]); 2) N-limited (31.25 μM N: 12.5 μM P [2.5:1]); and 3) half-strength WC media. The treatment flasks were filled to 450 ml with their respective media and set under the same fluorescent lighting on a 12hr:12hr light:dark cycle at 19 °C.

After a six-day initial growth period, we initiated a semi-continuous dilution regime of 0.1 d⁻¹ to maintain desired nutrient availabilities in the treatments. Dilutions were achieved by removing (after thorough mixing) 90 ml (i.e. 20%) of culture every two days and aseptically replacing with the respective media for each treatment. To ensure that inorganic carbon availability was not limiting, we included two sets of four flasks for the N-limited treatment with the additional set receiving aeration (to alleviate potential C limitation) starting on day 34. On day 50 (from the start of the dilution regime), we ended the experiment and sampled the flasks for nutrients, microcystin and *Microcystis*. In the N-limited treatment, there was no significant difference in biomass or microcystin levels between flasks that were aerated and not, therefore the results for all eight flasks were pooled.

Chlorophyll a, microcystin toxin and SRP were analyzed in the same fashion as the enclosure experiment. Concentrations of NO₃⁻ were analyzed with 2nd derivative UV spectroscopy (Crumpton et al. 1992) on a Perkin Elmer Lambda 20 UV/Vis spectrophotometer. *Microcystis* biomass was estimated from Lugol's preserved samples following a NaOH digestion (Reynolds and Jaworski 1978) to break the colonies into individual cells for easier counting.

Lake Erie surveys

We conducted an extensive seasonal survey of the western basin of Lake Erie, the most eutrophic of the Laurentian Great Lakes, from July through September of 2008. Nutrient concentrations in the basin are heavily influenced by loading from the Maumee River (TP typically above $200 \mu\text{g L}^{-1}$) that enters from the southwest. The Maumee contributes more than half of the phosphorus in the southern and western portions of the basin where the *Microcystis* blooms are a recurring water quality problem (Schwab et al. 2009; Chaffin et al. 2011; Bridgeman et al. 2012). Ten sampling stations, including one in the mouth of the Maumee River, were selected to provide a broad representation across the basin, with stations spaced approximately 6-10 km apart. Sampling trips were conducted at intervals of approximately two weeks, starting on 9 May and continuing through 22 September in order to capture seasonal changes in the physiology of *Microcystis* before, during and after the annual bloom.

On each sampling trip, water samples from each station were collected at 0.5 and 1 m depth with a van Dorn bottle and pooled into clean 10 L cubitainers. The western basin of Lake Erie is considered to be completely mixed during the summer due to the generally shallow depths and large fetch for wind mixing. Temperature profiles confirmed that the lake was generally isothermal (i.e. $< 1^{\circ}\text{C}$ difference from top to bottom) and therefore subsurface samples were assumed to be representative of the water column. Secchi disk depth was recorded at each station and temperature, light, pH, conductivity, *in situ* phycocyanin and dissolved oxygen profiles were taken from at least four stations on each sampling trip.

Water samples were kept at $\sim 4^{\circ}\text{C}$ immediately following collection and during transport back to the Michigan State University campus in East Lansing, MI. Samples for chlorophyll a,

microcystin and nutrients were processed within 12 hr of collection and analyzed in the same manner (with NO_3^- via spectrophotometry) as described for the enclosure and lab experiments.

Microcystis biomass was measured via microscopic analysis of Lugol's preserved samples as described for the enclosure experiment. Microcystin quota was then calculated as particulate toxin divided by *Microcystis* biomass. Since toxin quota is highly sensitive to the biomass estimate in the denominator, we only included samples for which *Microcystis* was relatively common (biomass concentrations $> 1 \text{ mg L}^{-1}$ dry weight) in our statistical analyses. This criterion excluded five, before-bloom, samples from 22 July.

mcyB gene copy density

We processed *Microcystis* samples collected from the Gull Lake experimental enclosures to assess whether treatment effects on microcystin quota were associated with effects on the frequencies of the *mcyB* gene, which is required for microcystin production (Kurmayer et al. 2002). Protocols for quantifying *mcyB* gene copies are described in detail by Dyble et al. (2008).

Briefly, water samples from the enclosure were filtered on $0.8 \text{ }\mu\text{m}$ membrane filters to collect *Microcystis*. The filters were stored at $-80 \text{ }^\circ\text{C}$ until all the samples could be processed. Cells were then lysed using DNAzol and DNA was extracted using standard phenol-chloroform procedures. The *mcyB* gene was amplified for quantification in $20 \text{ }\mu\text{L}$ volumes using an Applied Biosystems® 7500 Fast Real-Time PCR system. Amplification used fast conditions with an initial denaturing step of $95 \text{ }^\circ\text{C}$ for 10 minutes followed by 40 cycles of 3 seconds at $95 \text{ }^\circ\text{C}$ and 30 seconds at $60 \text{ }^\circ\text{C}$. The standard curve for absolute quantification was derived from

Hudson Bay strain E4-13, but relative differences are sufficient for our objectives (Kurmayer and Kutzenberger 2003). The critical threshold values for the samples were compared to the standard curve spanning five orders of magnitude ($10^2 - 10^6$ genes/filer) using Applied Biosystems SDS software.

Statistics

All statistical analyses were performed using Systat 9 software. In some cases, values were log transformed to correct for homogeneity of variances. Comparisons among treatments in the experiments were tested following the Bonferonni correction for multiple comparisons.

RESULTS

Gull Lake enclosure experiment

We were successful in establishing and maintaining TP near target levels in the enclosures (i.e. low $\sim 8\text{-}10\ \mu\text{g L}^{-1}$, medium $\sim 15\text{-}20\ \mu\text{g L}^{-1}$ and high $\sim 25\ \mu\text{g L}^{-1}$) (Fig. 1.1).

Soluble reactive phosphorus (SRP) was less than $2\ \mu\text{g L}^{-1}$ for the low and medium P treatments, while concentrations were marginally higher and reached $4\ \mu\text{g L}^{-1}$ in the high P treatment by day 31. Phytoplankton biomass (measured as chlorophyll a) increased markedly in response to the P addition treatments (Fig. 1), as expected given that Gull Lake is strongly P limited (Hamilton et al. 2009). After the initial strong response to P addition, chlorophyll declined in the P-addition treatments, and this decline was most pronounced in the high-P treatment (Fig. 1.1).

At the beginning of the experiment, NO_3 was $\sim 300\ \mu\text{g N L}^{-1}$ and remained relatively unchanged throughout the experiment in the low-P treatment (i.e. no phosphorus addition, Fig. 1.1). NO_3 concentrations in P-amended treatments decreased and by day 24 had fallen to undetectable levels in the high P treatment and to less than $150\ \mu\text{g L}^{-1}$ in the medium-P treatment. Ammonium levels remained less than $20\ \mu\text{g L}^{-1}$ in all treatments throughout the experiment.

Bulk alkaline phosphatase activity for the planktonic community was inversely related to ambient phosphorus throughout the experiment, with enzyme activity in the low phosphorus treatment typically 3-4 times higher than in the medium and high phosphorus treatments (Fig. 1.1). At the taxon-specific level, the relative alkaline phosphatase activity for *Microcystis*

measured with enzyme-labeled fluorescence microscopy was also significantly higher with 34% of colonies expressing activity in the low P treatment vs. 8% in the high P treatment by day 31 (ANOVA $F_{2,27}$ 4.16, $p = 0.027$). These data indicate that phosphorus addition alleviated P limitation in the phytoplankton generally, and in *Microcystis* specifically.

Microcystis biomass on day 31 was affected by both *Dreissena* (ANOVA $F_{1,24}$ 7.99, $p < 0.01$) and P addition (Fig. 1.2a; ANOVA $F_{2,24}$ 4.22, $p = 0.027$) but with no significant interaction. The response of *Microcystis* biomass to P addition was similar to that observed for chlorophyll on day 31, with highest abundance in the medium-P treatment (Figs. 1.1, 1.2). More importantly, the microcystin quota of *Microcystis* (μg of toxin per mg of *Microcystis* biomass) was negatively affected by P addition by the end of the enclosure experiment (Fig. 1.2; ANOVA $F_{2,27}$ 3.73, $p = 0.037$), and thus varied positively with available NO_3 concentration (Figs. 1.1, 1.2)

Density of the *mcxB* gene was highest in the enclosures with the lowest available N (high P treatment), indicating no selection for strains with low copy numbers at lower NO_3 . Microcystin per *mcxB* gene copy was highest at high NO_3 (low P treatment) and lowest at low NO_3 (high P treatment, Fig. 1.3).

Laboratory experiment

Microcystin quota of the Gull Lake strain of *Microcystis* in the laboratory experiment was significantly affected by nutrient treatments. (Fig. 1.4; ANOVA $F_{3,12}$ 7.87, $p = 0.004$). Toxin quota was lowest in the N limited treatment and highest in the P limited treatment. *Microcystis*

grown in media replete with N and P (WC media) had the same quota as the high N/low P treatment. These results indicate that N availability, not P availability, was the primary nutrient-mediated factor influencing toxin quota.

Lake Erie Surveys

During the Lake Erie survey in 2008, *Microcystis* was the only significant microcystin producing taxon present. *Anabaena* was present at some sites but always at levels less than 1% of the total phytoplankton biomass. Averaging across the western basin, *Microcystis* biomass increased by over two orders of magnitude during August, reaching a peak of $3500 \mu\text{g L}^{-1}$ on 3 September before declining in late September (Fig. 1.5). Basin-wide microcystin quota declined more or less continuously, and by more than two fold, from July to September. The decline in quota coincided with a ten-fold decline in NO_3 from late July to late September (Fig. 1.5). Total phosphorus increased from about $33 \mu\text{g L}^{-1}$ to $82 \mu\text{g L}^{-1}$ during this same period (Fig. 1.5). Thus, results from the western basin of Lake Erie are congruent with results from the field and laboratory experiments in showing a negative relationship between microcystin quota and NO_3 availability.

DISCUSSION

In this paper we have presented multiple lines of evidence supportive of a major role for N availability in mediating the toxin quota *Microcystis*, one of the most important cyanobacterium species responsible for harmful algal blooms in freshwaters. Results from a field experiment in large enclosures showed a strong association between reduced NO₃ concentration (generated by our P additions) and reduced microcystin quota (Figs. 1.2, 1.6). This relationship was shown to be consistent with observations from a seasonal survey of the western basin of Lake Erie showing concomitant declines in NO₃ and microcystin quota (Figs. 1.5, 1.6). Genetic evidence from the enclosure experiment suggested that decreases in microcystin quota were not driven by decreases in copy density of the microcystin-producing gene, *mcyB*, but rather more likely the result of phenotypic changes presumably mediated by N availability (Fig. 1.3). Taken together, these observations suggest a strong influence of N availability on cellular production of microcystin in nature, but do not demonstrate that low N is the cause of low quota.

To demonstrate cause and effect and phenotypic plasticity in *Microcystis*, we followed up field results with a laboratory experiment in which we exposed a single genotype of *Microcystis* from Gull Lake to strong N and P limited conditions. This experiment demonstrated that cells grown under N limitation had about a 2.5 fold lower toxin quota compared to N replete conditions, whereas P limitation had no detectable effect on toxin quota (Fig. 1.4). Thus, we conclude that N availability is a likely a strong causative driver of microcystin quota in natural populations of *Microcystis*, and that this effect may largely be mediated by phenotypic responses by individuals cells..

We established a qualitatively consistent pattern of increased microcystin quota with increased available N across a spectrum of study systems (less than 1 liter to over 100 km²).

Quantitatively, we compared across these study scales by regressing toxin quota on available NO_3 . As expected, there was a significant positive linear relationship between quota and NO_3 for each of the study systems (Fig. 1.6). Despite large differences among study systems, the range of microcystin quota was fairly constrained across the three systems ($\sim 1 - 3 \mu\text{g}$ toxin per mg *Microcystis* dry biomass).

The positive effect of N availability on microcystin quota that we observed is largely consistent with previous laboratory research (Lee et al. 2000; Oh et al. 2000; Downing et al. 2005; Van de Waal et al 2009). More recently, Janichen et al. (2011) found that microcystin quota decreased with increasing P (as we observed in the enclosure experiment). Our laboratory experiment showed, however, that the P "effect" in our enclosures was more likely directly driven by N availability, not P availability. In lakes, increasing P concentrations typically shift systems towards N limitation (Downing and McCauley 1992), which may have driven the decrease in quota observed by Janichen et al. (2011).

A number of studies have shown that the proportion of the *Microcystis* population containing toxin producing genes can vary in space and time (Briand et al. 2009, Otten et al. 2012), including in the western basin of Lake Erie (Rinta-Kanto et al. 2009). Briand et al. (2012) suggest that the toxicity or toxin quota of a bloom can be explained by variation in the proportion of toxic vs. non-toxic strains of the *Microcystis* population. If selection for strains of *Microcystis* more capable of toxin production was causing the increase in toxin quota at higher N levels, then we would have expected to observe a higher relative *mcyB* gene frequency (i.e. *mcyB* genes per *Microcystis* biomass). In fact, the relative *mcyB* gene frequency was actually higher in the enclosures with the lowest available N (i.e. the high P treatment).

An alternative explanation is that toxin quota is a function of phenotypic plasticity caused by a change in the abiotic or biotic environment. In this case, the toxin produced per mycB gene was over 8 fold higher in the low-P (high NO₃) vs. high-P (low NO₃) enclosures (Fig. 1.3). This evidence suggests a high plasticity in toxin production physiology mediated by N availability and is consistent with the stoichiometric theory that production of nitrogen-rich secondary metabolites, such as microcystins, should be reduced disproportionately under N limitation (Sturner & Elser 2002; Van de Waal et al. 2009). Furthermore, Van de Waal et al. (2009) also found that the most nitrogen-rich variants of microcystin were disproportionately reduced under N limiting conditions.

Microcystis biomass levels and the microcystin quota both contribute to the overall toxin concentrations in the water at a given location and time. Low biomass levels combined with high toxin quotas may result in the same toxin concentrations as a large bloom with lower toxin quotas. In our survey of the western basin of Lake Erie, microcystin concentrations were strongly correlated with the dynamics of *Microcystis* biomass ($r^2 > 0.9$) despite declining microcystin quota. This indicates that biomass was the major driver of microcystin levels (Fig. 1.5). However, the role of seasonally-variable microcystin quota (and thus presumably N availability as a driver) is also important, as illustrated by a simple hypothetical example. During peak *Microcystis* biomass in early September, microcystin levels in the western basin peaked at $\sim 3 \mu\text{g L}^{-1}$, or three times the WHO limit for drinking water (Fig. 1.5) despite a relatively low microcystin quota for *Microcystis* ($\sim 1.5 \mu\text{g mg}^{-1}$). If the quota had remained at levels observed in July ($\sim 3 \mu\text{g mg}^{-1}$), perhaps as a result of higher N availability, microcystin levels might have reached over $6 \mu\text{g L}^{-1}$, or six times the WHO limit. Of course, we do not yet

understand how an increase in N availability in the basin would affect the ecological success of *Microcystis* relative to non-microcystin containing taxa, so further research on the competitive dynamics of *Microcystis* under varying N:P ratios is needed.

The results of this work have potential implications for the monitoring and management of toxic *Microcystis* blooms. Current nutrient control strategies in freshwaters focus almost exclusively on P, supported by decades of research demonstrating the tight coupling between P and eutrophication in lakes (Schindler 1974; Schindler et al. 2008). However, Conley et al. (2009) argue that N and P control is needed for many lakes since P flux with the sediments can support large blooms of non nitrogen-fixing cyanobacteria, like *Microcystis*, when nitrogen is readily available. We present the first conclusive experimental evidence for the role of N availability in determining toxin quota in a natural lake environment and that evidence is supported by lab culture experiments, genetic analyses and surveys in eutrophic Lake Erie. The widespread N pollution of watersheds and recipient freshwaters (Carpenter et al. 1998) could therefore be leading to the increased toxicity of harmful algal blooms, even under conditions of P limitation. Thus, the control of N as well as P may be necessary to ameliorate harmful phytoplankton blooms that produce toxins with ecological and public health impacts.

FIGURES

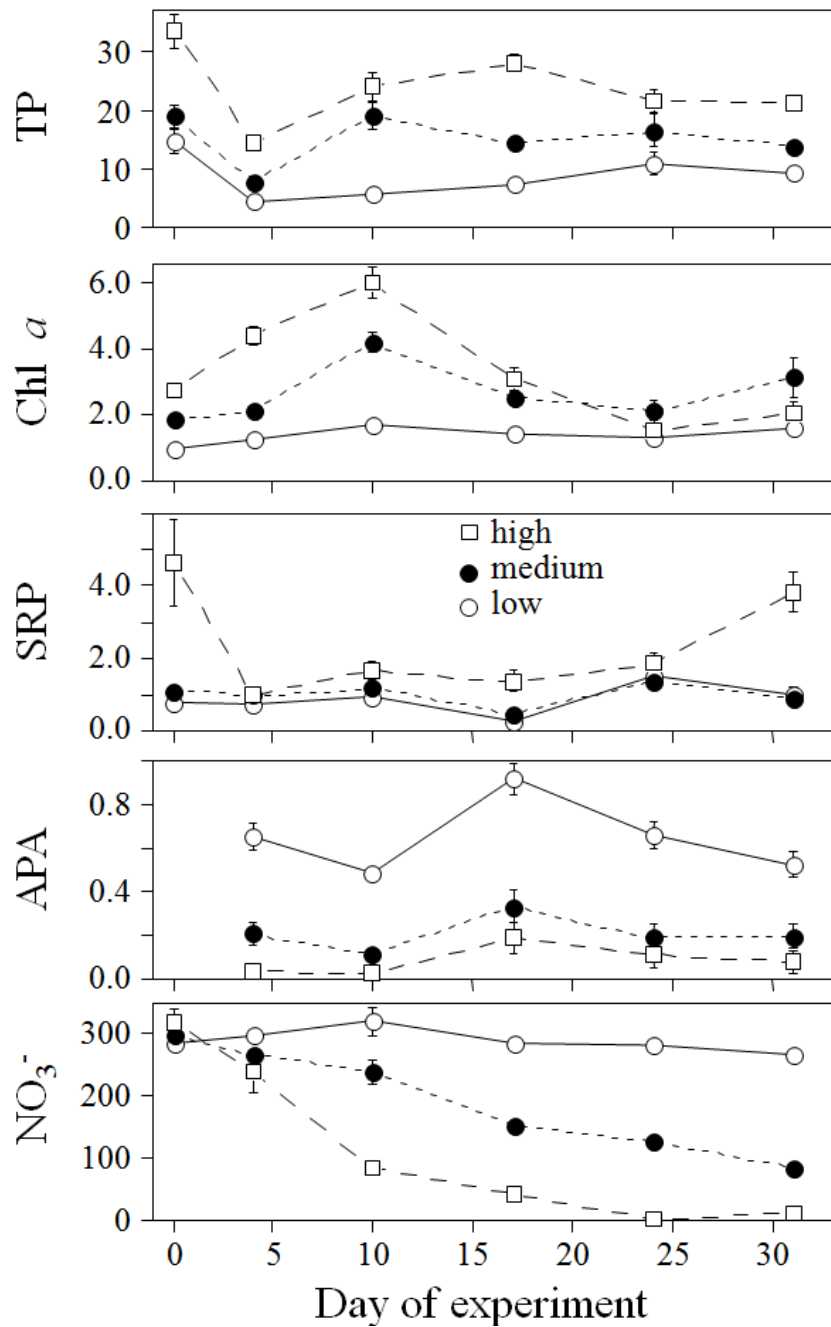


Figure 1.1 Total phosphorus, chlorophyll *a* (Chl *a*), soluble reactive phosphorus (SRP), bulk alkaline phosphatase activity (APA) and nitrate-nitrogen (NO₃) concentrations in the Gull Lake enclosure experiment (all units in ug/L). Values represent means $\pm$ SE ($n = 10$ enclosures per treatment).

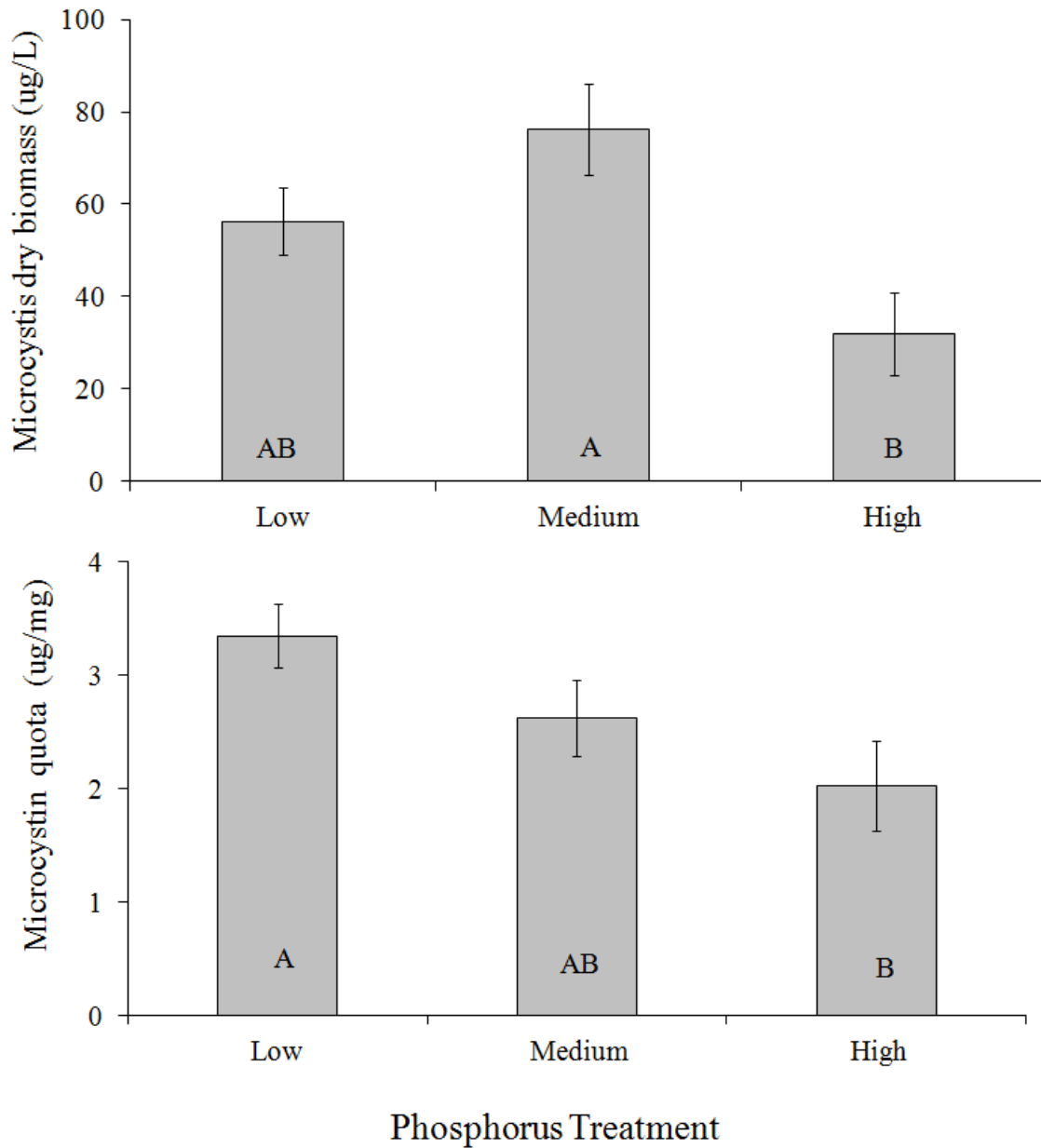


Figure 1.2 (A) *Microcystis* biomass (dry weight) and (B) microcystin toxin quota (ug toxin per mg *Microcystis* dry biomass) across nutrient treatments in the Gull Lake enclosure experiment on August. 6, 2007 (Day 31). Values represent means $\pm$ SE ($n = 10$). Bars sharing the same letter (A, B) were not significantly different following Bonferonni adjusted post-hoc analysis at $p = 0.05$.

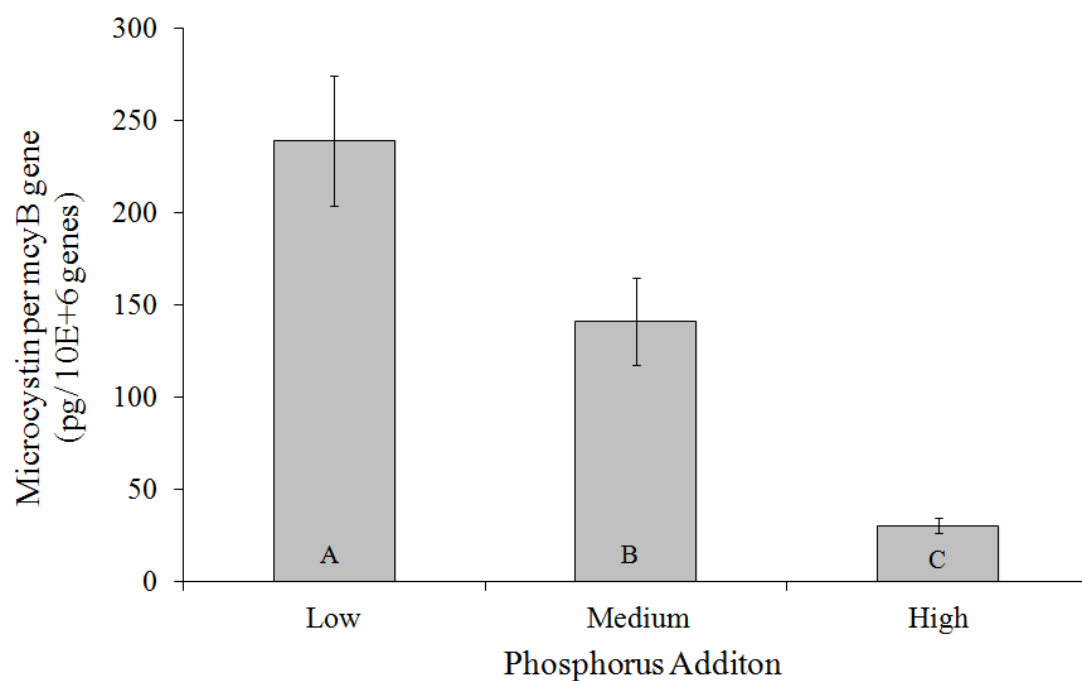


Figure 1.3 Microcystin per mcyB gene ($\text{pg}/10^6$ genes) across nutrient treatments in the Gull Lake enclosure experiment on August 6, 2007 (Day 31). Values represent means $\pm$ SE ($n = 10$). Bars sharing the same letter (A, B, C) were not significantly different following Bonferonni adjusted post-hoc analysis at $p = 0.05$.

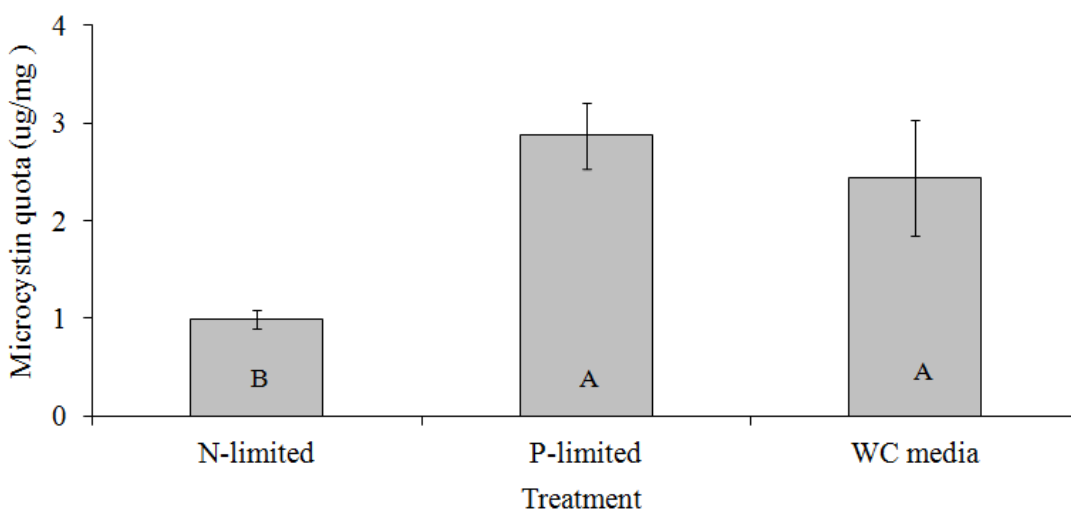


Figure 1.4 Microcystin toxin quota (ug toxin per mg *Microcystis* dry biomass) across nutrient treatments in the monoclonal lab experiment. Values represent means $\pm$ SE ($n = 8$ for N-limited, $n = 4$ for P-limited and WC media). Bars sharing the same letter (A, B) were not significantly different following Bonferonni adjusted post-hoc analysis at $p = 0.05$.

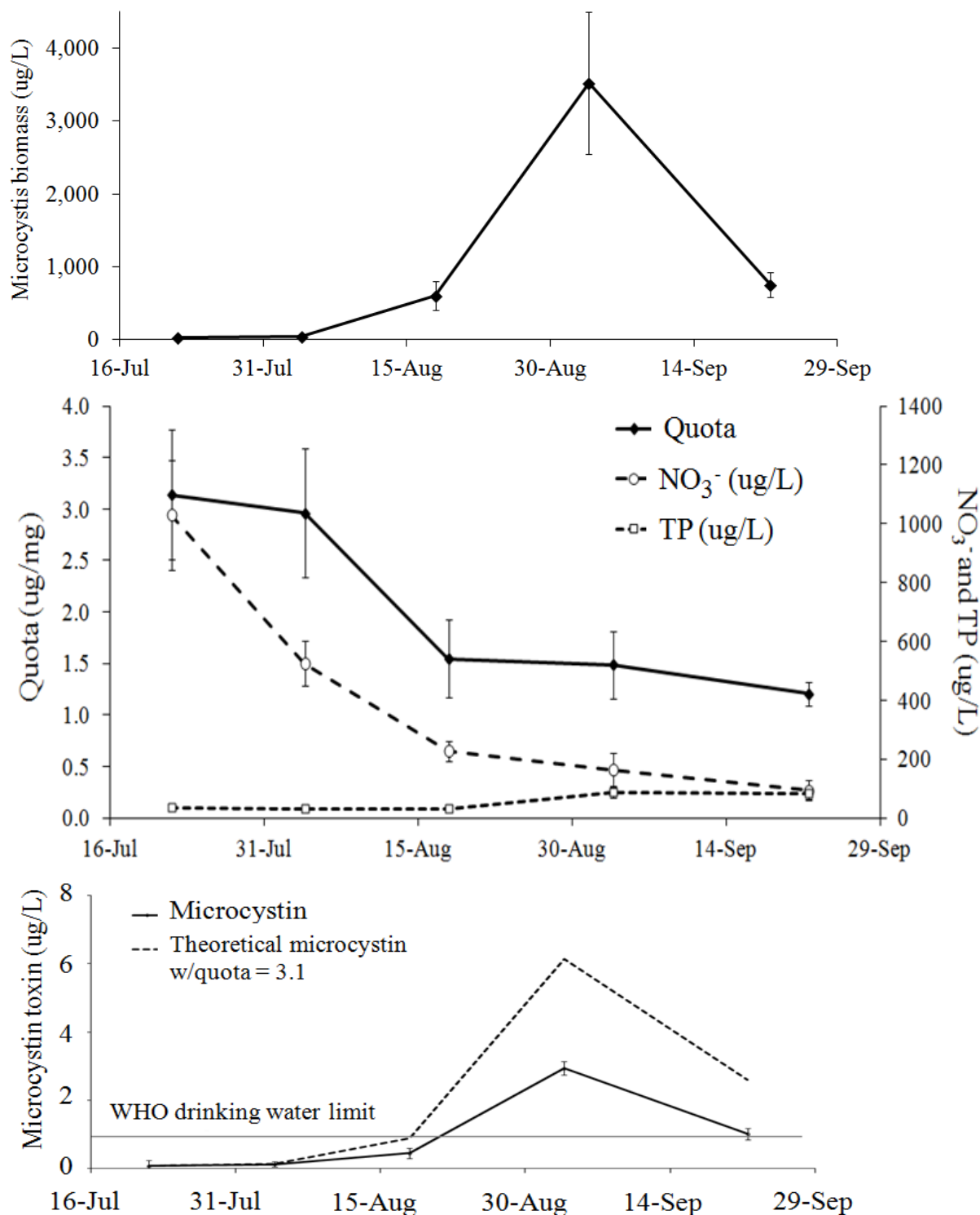


Figure 1.5 (A) Microcystis dry biomass; and (B) microcystin toxin quota, nitrate (NO₃) and total phosphorus (TP) concentrations throughout the summer of 2008 in the western basin of Lake Erie. (C) Measured microcystin levels (solid line) and theoretical toxin levels based on using a fixed toxin quota of 3.1 ug/mg (average quota from 22 July). Values are mean $\pm$ SE averaged across sites within the basin on each date.

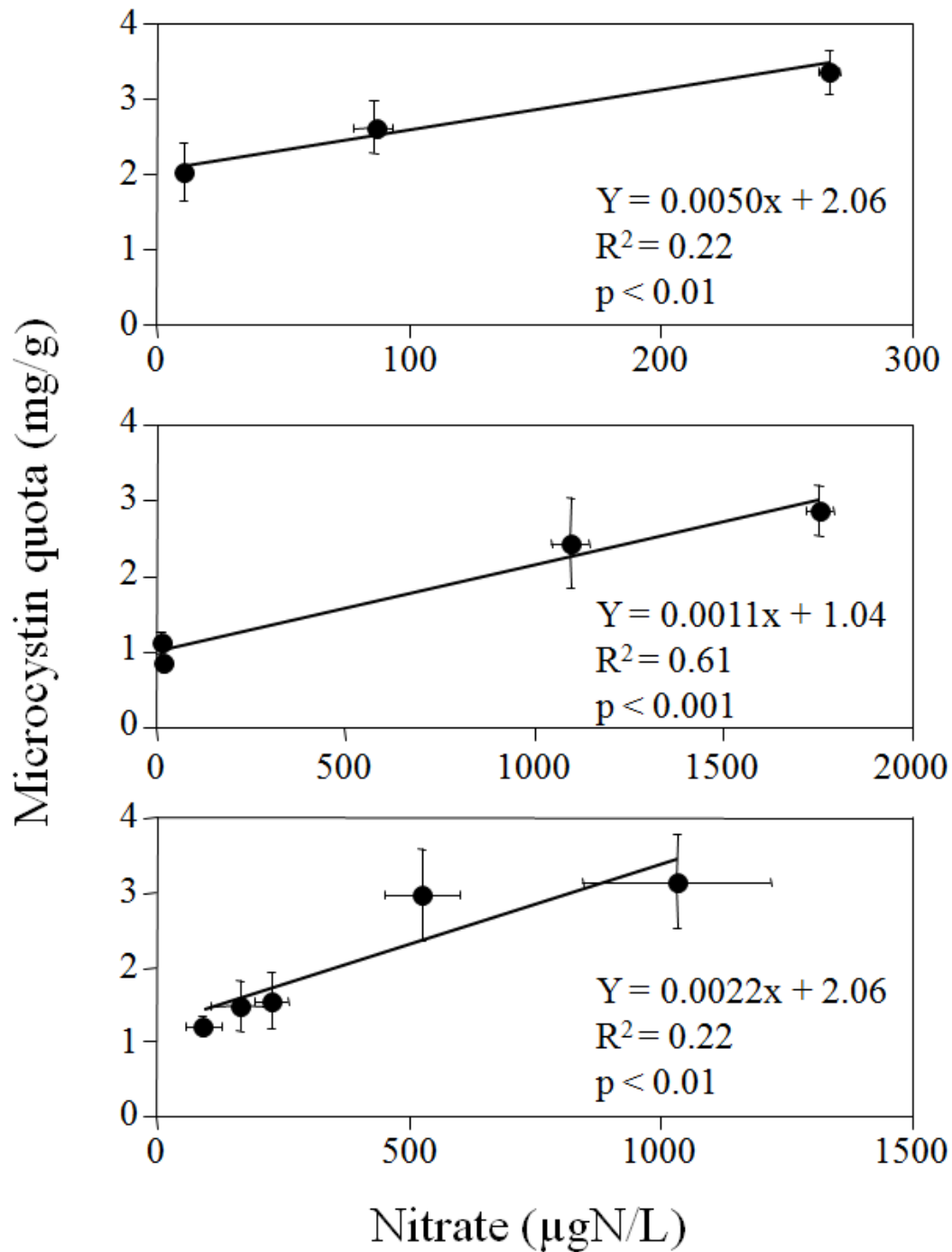


Figure 1.6 Microcystin toxin quota (ug toxin per mg *Microcystis* dry biomass) as a function of NO₃ (ug N/l) in: (A) the enclosure experiment; (B) the lab experiment with *Microcystis* strain; and (C) mean values on a given date from sites in the western basin of Lake Erie (points from left to right represent 22-Sep, 3-Sep, 18-Aug, 4-Aug, and 22-Jul).

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CHAPTER 2

Phosphorus limits *Microcystis aeruginosa* abundance in eutrophic western Lake Erie

INTRODUCTION

Harmful algal blooms (HABs) are a significant and growing problem worldwide in both freshwater and marine ecosystems (Chorus and Bartram 1999; Huisman et al. 2005; Paerl and Huisman 2008). In many freshwater systems, cyanobacteria blooms can cause significant risks to public health due to the production of potent toxins (Carmichael 1994, 2001; Chorus and Bartram 1999). The most cited causes for HABs relate to excessive nutrient loading, especially phosphorus (Carpenter et al. 1998; Paerl and Huisman 2008). In general, as phosphorus levels increase, phytoplankton biomass increases (Schindler 1974) and in particular, the proportion of cyanobacteria in the plankton community increases (Smith 1986; Watson et al. 1997; Downing et al. 2001). For this reason, substantial efforts and funding have been put in place to reduce phosphorus loading to freshwater ecosystems.

Lake Erie, in the Laurentian Great Lakes, historically experienced severe HAB events leading up to the 1970's, dominated by nitrogen-fixing cyanobacteria such as *Anabaena* and *Aphanizomenon* (Matisoff and Ciborowski 2005). Point source phosphorus control measures implemented by the Great Lakes Water Quality Agreement in 1972 dramatically reduced phosphorus loading (Richards et al. 2002). Subsequently, phytoplankton abundance in Lake Erie began to decrease through the mid 1990's, especially with respect to cyanobacteria abundance (DePinto et al. 1986; Makarewicz and Bertram 1991; Makarewicz 1993; Fahnenstiel et al. 1998). However, since the mid 1990's, phytoplankton abundance, especially the non-nitrogen fixing cyanobacterium, *Microcystis aeruginosa*, has increased significantly despite nutrient control measures (Vanderploeg et al. 2001; Conroy et al. 2005; Ouellette et al. 2006). Extensive

Microcystis blooms visible with satellite imagery, are now an annual occurrence in the western basin of the lake (Vincent et al. 2004; Millie et al. 2009; Chaffin et al. 2011a; Bridgeman et al. 2012a) and microcystin (a potent liver toxin) levels routinely exceed the World Health Organization's limit of $1 \mu\text{g L}^{-1}$ at sites throughout the western basin (Brittain et al. 2000; Dyble et al. 2008; Rinta-Kanto et al. 2009). Given that over 5 million people depend on western basin of Lake Erie as a source of drinking water, transportation, commercial fisheries and recreation (Fuller 2002), it's critical to understand the limiting factors controlling *Microcystis* abundance in order to mitigate its impact within the ecosystem.

The western basin of Lake Erie is functionally different from the central and eastern being relatively shallow (average depth $< 15 \text{ m}$), having a short hydraulic retention time and is heavily influenced by the Maumee River, which contributes only 10% of the total water flow but over 35% of the phosphorus loading to the basin (Schwab et al. 2009). As a result, there is typically a steep nutrient gradient extending outwards from the mouth of the Maumee River at the southwest corner of the basin with total phosphorus spanning a range from hypereutrophic to nearly oligotrophic conditions within a distance of less than 40 km (Moorhead et al. 2008). It is well established from field and lab studies that *Microcystis* grows faster with increasing phosphorus levels (Gobler et al. 2007; Jiang et al. 2008; Davis et al. 2009) and therefore it is not surprising that the highest abundance of *Microcystis* in the lake occurs closer to the mouth of the Maumee River where total phosphorus is highest (Dyble et al. 2008; Chaffin et al. 2011a). Temporally, *Microcystis* blooms usually occur from late August through September (Rinta-Kanto et al. 2009; Chaffin et al. 2011a; Bridgeman et al. 2012a).

Numerous studies have documented that *Microcystis* is prevalent in the western basin and that its abundance appears to be associated with the discharge plume of the Maumee River (e.g.

Vincent et al. 2004), however, survey studies that capture the spatial and temporal variation in bloom abundance and environmental factors that may affect *Microcystis* are rare. A survey by Chaffin et al. (2011) captured the temporal resolution of the 2008 *Microcystis* bloom at five sites that spanned much of the trophic gradient extending from the Maumee River and found higher *Microcystis* abundance at sites just outside the river mouth where phosphorus was higher than offshore sites. Additionally, Rinta-Kanto et al. (2009) surveyed between three and twelve sites in the western basin two to three times per year for three years and demonstrated a significant correlation between total phosphorus and microcystin and mcyD gene copies. Clearly, more extensive sampling is required in order to fully-characterize the seasonal bloom and determine to what extent phosphorus may be correlated with *Microcystis* abundance.

Since other environmental variables (e.g. water clarity) may co-vary with phosphorus along the trophic gradient extending from the Maumee River, it is important to identify additional independent measures of phosphorus-dependent growth of *Microcystis* before assigning a more causal link between phosphorus loading and the *Microcystis* blooms. One option is to measure phosphorus limitation in the phytoplankton community and specifically in *Microcystis* since it is established that phosphorus limited phytoplankton have lower growth rates (Litchman et al. 2003; Shen and Song 2007). A number of studies have independently confirmed that phosphorus can be limiting to at least some members of the phytoplankton community in the western basin using measures of biomass C:P ratios (Guildford and Hecky 2005; Rattan and Taylor 2012), nutrient addition assays (Wilhelm et al. 2003), and alkaline phosphatase activity (Guildford and Hecky 2005). More specifically, Chaffin et al. (2011) isolated *Microcystis* cells from the community and found that cells were typically phosphorus limited on a C:P ratio basis across most sampling sites in the basin and that the protein content of

cells at the most distant sites from the Maumee River were lower than cells collected closer to the river, perhaps as a result of higher ambient phosphorus levels.

The purpose of the present study was to: 1) capture spatio-temporal patterns of *Microcystis* abundance in the western basin at a short enough frequency and large enough spatial scale in order to fully characterize the extent of the annual *Microcystis* bloom and identify potentially important environmental factors associated with the bloom; and 2) determine whether the apparent association between *Microcystis* abundance and phosphorus is congruent with indices of phosphorus limitation and thus strengthening the causal link between phosphorus loading to the basin and *Microcystis* prevalence.

METHODS

We conducted an extensive seasonal survey of the western basin of Lake Erie, the most eutrophic of the Laurentian Great Lakes, from May through September of 2008. Nutrient concentrations in the basin are heavily influenced by loading from the Maumee River (TP typically above $200 \mu\text{g L}^{-1}$) that enters from the southwest. The Maumee contributes more than half of the phosphorus in the southern and western portions of the basin where the *Microcystis* blooms are a recurring water quality problem (Bridgeman et al., 2012; Chaffin et al. 2011; Schwab et al. 2009). Nine sampling stations were selected to provide a broad representation across the basin, with stations spaced approximately 6-10 km apart (Fig. 2.1). Sampling trips were conducted at intervals of approximately two weeks, starting on 9 May and continuing through 22 September in order to capture seasonal changes in the physiology of *Microcystis* before, during and after the annual bloom.

On each sampling trip, water samples from each station were collected at 0.5 and 1 m depth with a van Dorn bottle and pooled into clean 10 L cubitainers. The western basin of Lake Erie is considered to be completely mixed during the summer due to the generally shallow depths and large fetch for wind mixing. Temperature profiles confirmed that the lake was generally isothermal (i.e. $< 1^{\circ}\text{C}$ difference from top to bottom) and therefore subsurface samples were assumed to be representative of the water column. Secchi disk depth was recorded at each station and temperature, light, pH, conductivity, *in situ* phycocyanin and dissolved oxygen profiles were taken from at least four stations on each sampling trip.

The cubitainers were kept at $\sim 4^{\circ}\text{C}$ following collection and during transport back to the Michigan State University campus in East Lansing, MI, where the samples were processed into

four sub-samples: 1) raw water sample stored at 4 °C; 2) filtered water sample passed through an A/E glass fiber filter and stored at 4 °C; 3) seston collected on A/E filters and immediately frozen at -20 °C; and 4) water preserved in Lugol's solution for microscope counting of the phytoplankton.

All nutrient analyses were performed within 72 hours of collection. Soluble reactive P (SRP) was measured on filtered water samples using long-pathlength spectrometry (Wetzel and Likens 2000) and concentrations of NO_3^- were analyzed with 2nd derivative UV spectroscopy (Crumpton et al. 1992) on a Perkin Elmer Lambda 20 UV/Vis spectrophotometer. Total phosphorus (TP) was measured on persulfate-digested raw water samples and then analyzed spectrophotometrically as SRP. Chlorophyll was extracted from frozen filters in 90% ethanol and measured fluorometrically (Welschmeyer 1994).

Microcystis biomass was measured via inverted-microscope analysis on Lugol's preserved samples. Sub-samples (50 ml) were transferred into 25 mm-wide settling chambers with a cover-slip bottom. After a 5-day settling period, the entire chamber surface was surveyed for *Microcystis* colonies and the surface area of each colony was measured at 100X magnification from digitized photographs (SPOT Software V 4.0.9, Diagnostic Instruments). The cumulative colony surface area for a chamber was converted into *Microcystis* cell concentration (cells L^{-1}) based on the surface area to cell count relationship established for *Microcystis* samples in Lake Erie ($R^2 = 0.95$, $n = 21$). *Microcystis* dry biomass concentration was then calculated by converting cell concentration to biovolume (based on measurements of

cell dimensions) and subsequently converting biovolume to dry biomass assuming a dry weight (mg) to biovolume (mm^3) ratio of 0.42.

Bulk-alkaline phosphatase activity (APA), a measure of phosphorus limitation of the planktonic community, was measured fluorometrically in 10 ml aliquots of freshly-collected raw water samples from the enclosures (Pettersson 1980). *Microcystis*-specific alkaline phosphatase activity was assessed using enzyme-labeled fluorescence (ELF) microscopy (Gonzalez-Gil and Aguilera 1998). This microscopy-based method is analogous to bulk alkaline phosphatase analysis, whereby phosphorus-limited organisms produce the enzyme alkaline phosphatase which is then detected visually for cells of a particular species. ELF substrate (0.1 ml, 1:20 v:v in buffer solution; E6601 from Invitrogen, USA) was added to separate 10 ml subsamples of freshly-collected raw water samples from the enclosures. After 1 hr, the treated subsample was filtered onto black 1 μm membrane filters and rinsed with phosphate-buffered saline to inhibit further enzyme activity. The filters were then permanently mounted onto microscope slides and stored at -20°C for up to three months before analysis.

Using a Nikon Eclipse E600 epifluorescence microscope, the mounted membrane filters were visually scanned for *Microcystis* colonies and evidence of alkaline phosphate activity as indicated by bright green precipitates on the surface of colonies (Fig. 2.2). Each *Microcystis* colony (up to 200 per slide) was counted and marked as having either positive or negative enzyme activity, with the percent of active colonies per sample serving as a relative index of the population's alkaline phosphatase activity.

RESULTS

Early season survey sampling trips on 9 May, 23 May and 16 June spanned a period of rapidly increasing water temperatures from 12 to over 21 °C (data not shown). *Microcystis* was not observed via microscopy (detection limit of 1 ug L⁻¹) until 22 July (Fig. 2.3a) and then only increased from about 26 to 44 ug L⁻¹ between 22 July and 3 August when averaged across all sites within the western basin. Between 3 August and 3 September, *Microcystis* demonstrated strong exponential growth, increasing by nearly 100 fold to over 3500 ug L⁻¹. Following the peak abundance in early September, *Microcystis* decreased to less than 750 ug L⁻¹ by 22 September. Averaged across sites, chlorophyll a followed the same temporal growth pattern as *Microcystis*, but concentrations only increased by about 10 fold from 5 ug L⁻¹ in July to a peak of 57 ug L⁻¹ on 3 September before declining to about 30 ug L⁻¹ on 22 September.

Phosphorus concentrations in the western basin of Lake Erie are typically very high compared to the central and eastern basins of the lake due to the strong influence of high phosphorus loading from the Maumee River. Temporally, total phosphorus exhibited two phases during the sampling season of 2008, with an average concentration among all stations in the basin between 30 and 40 ug-P L⁻¹ during June, July and early August (Fig. 2.3b). Between 16 August and 3 September, total phosphorus more than doubled to over 85 g/L and remained above 90 ug L⁻¹ on 22 September. Nitrate, which is the dominant source of inorganic nitrogen in the basin, declined progressively over the course of the sampling period (Fig. 2.3b). Concentrations

of nitrate averaged around 800 ug-N L^{-1} through mid-July, then dropped to 200 ug-N L^{-1} by mid-August and reaching as low as 33 ug-N L^{-1} by 22 September.

Water temperatures in the western basin during the growing season rose quickly to over 20°C by mid-June and continued to climb to over 25°C by mid-July (Fig. 2.3c). Temperatures remained between 23 and 25°C through 3 September and then dropped to 20°C by 22 September. Water clarity was generally inversely proportional to chlorophyll a concentration and *Microcystis* abundance, with Secchi Disk depths averaging 2.5 to 3.5 meters among all sites in June, July and early August leading up to the bloom (Fig. 2.3c). By 3 September, the Secchi Disk depth had decreased to about 1 meter and remained low through 22 September.

Due to the strong influence of phosphorus inputs into the western basin of Lake Erie by the Maumee River (Schwab et al. 2009), we expected, and in fact found, a strong total phosphorus (TP) gradient extending outwards from the mouth of the Maumee River. On each sampling date there was a significant negative correlation between total phosphorus and distance from the mouth of the river (Fig. 2.4). As depicted in the temporal plots in Fig. 2.3, there was a dramatic increase in overall TP concentrations in the basin between 18 Aug and 3 Sep but Fig. 2.4, illustrates that this increase was also characterized by a dramatic increase in the steepness in the TP gradient, not simply an overall increase at all sites. For example, the sites closest to the mouth of the river typically averaged close to 50 ugP L^{-1} through 18 Aug, yet reached 150 to 200 ugP L^{-1} by 3 Sep and afterwards. The most offshore sites also increased but less dramatically from about 20 to 30 ugP L^{-1} through mid-August up to 25-50 ugP L^{-1} in September.

Microcystis biomass did not show a significant correlation with distance from the Maumee River during the early sampling dates through mid-August while concentrations were generally low (Fig. 2.4). However, as *Microcystis* reached peak abundance on 3 September, there was a strong and significant negative correlation between biomass concentration and distance from the Maumee River, paralleling the concentrations in total phosphorus and spatial pattern continued through 22 September.

Given the apparent co-variation in total phosphorus and *Microcystis* leading up to the peak bloom in early September, we regressed *Microcystis* against TP for all the sampling sites sampled during the growth period from 22 July to peak abundance on 3 September (Fig. 2.5). TP was, as expected, significantly correlated with *Microcystis* abundance over the growth period, explaining nearly half of the variation ($R^2 = 0.48$, $p < 0.0001$), despite a multitude of other potential abiotic and biotic variables that vary among sites within the $>500 \text{ km}^2$ sampling area over the span of six weeks. On 22 September, following the peak of the bloom, *Microcystis* was still positively correlated with TP, but at a lower slope than during the growth period (Fig. 2.5).

The strong correlation between TP and *Microcystis* abundance (Fig. 2.5) suggests that phosphorus was potentially limiting the growth of *Microcystis* and perhaps other phytoplankton species during the growth period in the western basin. In order to assess phosphorus limitation among sites and sampling dates, we measured bulk alkaline phosphatase activity on the entire plankton community. Despite this broad measure of phosphorus limitation in the entire community, *Microcystis* abundance demonstrated a very tight negative correlation with alkaline phosphatase activity (Fig. 2.5) during the growth period (22 July to 3 September) indicating that *Microcystis* abundance increased as phosphorus limitation decreased (i.e. lower alkaline phosphatase activity). Compared to total phosphorus, alkaline phosphatase activity for the entire

plankton community explained more than 70% of the variation in *Microcystis* abundance during the growth period ($R^2 = 0.71$, $p < 0.0001$). By 22 September, alkaline phosphatase activity was very low for all the sites, yet *Microcystis* abundance had already started to decline, suggesting that some other factor besides phosphorus limitation was likely limiting *Microcystis* growth after the bloom (Fig. 2.6).

In order to determine whether *Microcystis* specifically was experiencing phosphorus limitation, we employed a taxon-specific alkaline phosphatase activity using enzyme labeled fluorescence (ELF) and epi-fluorescence microscopy. Among sites and dates during the survey, *Microcystis* colonies displayed a range of ELF activity ranging from about 30% of colonies expressing ELF-based alkaline phosphatase activity to 0% of colonies expressing ELF (Fig. 2.7), indicating lower levels of alkaline phosphatase activity by *Microcystis*. For the period from 4 August till 22 September, the % of *Microcystis* colonies expressing ELF-based alkaline phosphatase activity was correlated with overall bulk alkaline phosphatase activity (APA) for the entire plankton community (Spearman rank correlation coefficient, $r_s = 0.34$, $t = 2.06$, $df = 33$, $p = 0.023$), indicating that phosphorus limitation for *Microcystis* was congruent with overall bulk alkaline phosphatase activity for the entire plankton community.

DISCUSSION

During the spring/summer of 2008, the western basin of Lake Erie experienced a pronounced bloom of *Microcystis* during late August and early September (Fig. 2.3), which resulted in microcystin toxin levels exceeding the World Health Organization drinking water limit of 1 ug/L at several sites throughout the basin. The *Microcystis* population was not even detectable via microscopy until mid-July when temperatures had warmed to 25 °C, but then grew exponentially through the first week September before dissipating. Noticeably, *Microcystis* grew exponentially during a period of increasing total phosphorus levels (Fig. 2.3) and *Microcystis* biomass was strongly correlated with total phosphorus leading up to the peak bloom (Fig. 2.5). More pertinently, *Microcystis* was inversely correlated with alkaline phosphatase activity (Fig. 2.6), a measure of phosphorus limitation in the phytoplankton community. In particular, *Microcystis* colonies expressed taxon-specific alkaline phosphatase activity in relative proportion to community-wide phosphorus limitation (Fig. 2.7). Together, these results indicate that phosphorus strongly regulates *Microcystis* abundance in the western basin.

It has been previously demonstrated that there is a pronounced phosphorus gradient extending from the mouth of the Maumee River (Moorhead et al. 2008), and this pattern was exhibited consistently throughout our survey (Fig. 2.4). Notably, the relative slope of this gradient increased dramatically between 18 August and 3 September, whereby total phosphorus at sites closest to the river mouth increased by nearly four-fold. This relative change in the gradient therefore explains the most of the apparent increase in phosphorus averaged over the whole basin sampling area during the same period. *Microcystis* did not exhibit a strong gradient until 3 September, perhaps because the slope of the phosphorus gradient in earlier dates was not as pronounced despite being statistically significant.

Despite the complexities of a dynamic ecosystem with multiple abiotic and biotic variables changing over the course of nine weeks and a 500 km² survey area, there was still a very strong positive correlation between total phosphorus concentrations and *Microcystis* abundance, explaining nearly 50% of the variation (Fig. 2.5). Several studies have found similar patterns across multiple lakes or within lakes (Oh et al. 2001; Otten et al. 2012), but we believe this is the first study to quantitatively demonstrate a correlation between phosphorus and *Microcystis* biomass during the course of developing bloom in Lake Erie. Additionally, this pattern is consistent with both field and lab studies that have experimentally demonstrated that increasing phosphorus increases *Microcystis* (Vézic et al. 2002; Strojsová et al. 2005; Gobler et al. 2007; Davis et al. 2009).

In order to further evaluate the role of phosphorus in driving the dynamics of *Microcystis* growth in the western basin, we quantified both the community-wide bulk alkaline phosphatase activity and taxon-specific alkaline phosphatase activity for *Microcystis*. In comparison to total phosphorus, bulk alkaline phosphatase activity was actually a better predictor of *Microcystis* abundance, explaining over 70% of the variation (Fig. 2.6). It was surprising to find such a strong relationship, but it has been well established that alkaline phosphatase activity is highly dynamic in phytoplankton (Jansson and Petterson 1988) especially in cyanobacteria (Sakshaug and Olsen 1986). Under phosphorus limitation, *Microcystis*, like other species should exhibit lower growth rates (Litchman et al. 2003; Shen and Song 2007) and therefore its consistent that *Microcystis* abundance should increase with decreasing levels of phosphorus limitation (i.e. lower alkaline phosphatase activity).

Despite the strong relationship between bulk alkaline phosphatase activity and *Microcystis* abundance, we also wanted to know whether we could specifically identify

phosphorus limitation in *Microcystis* without the laborious efforts of performing either nutrient amendment assays (e.g. Gobler et al. 2007, Davis et al. 2009) or isolating *Microcystis* cells from the community and analyzing C:P ratios. Fortunately, an enzyme-labeled fluorescence ELF assay (Gonzalez-Gil and Aguilera 1998; Rengefors et al. 2003) allows for the rapid detection of alkaline phosphatase activity via fluorescence microscopy (Fig. 2.2). *Microcystis* colonies did express a modest range of ELF phosphatase activity that correlated positively with bulk alkaline phosphatase activity (Fig. 2.7), demonstrating that *Microcystis* colonies were experiencing phosphorus limiting conditions in conjunction with bulk alkaline phosphatase activity for the entire plankton community. While the ELF method is relatively fast and simple to process, its limitation may be that in many samples, there was no indication of taxon-specific ELF phosphatase for *Microcystis* (i.e. 0% of colonies expressing), yet clearly there was some positive level of bulk alkaline phosphatase activity.

The majority of the focus for this study has been on the factors controlling the growth of *Microcystis* in the western basin of Lake Erie, however, we did collect samples during the period of declining *Microcystis* biomass (3 Sep to 22 Sep) which may offer insight into factors that lead to a bloom collapse. First, phosphorus levels remained high following the bloom (Figs. 2.3 and 2.4), alkaline phosphatase activity remained low, and no *Microcystis* colonies were expressing ELF phosphatase activity, all of which indicates that phosphorus limitation was not apparent and likely did not contribute to the population decline. One explanation for the decline may be nitrogen limitation as nitrate availability declined from over 800 ug L^{-1} in early summer to only 30 ug L^{-1} by 22 Sep. This would have resulted in a nitrate-N:TP ratio of less than 1, which is considered strongly nitrogen limited by many measures (Healey and Hendzel 1980). Second, the water temperature dropped relatively rapidly from 24 to 20 °C between 3 September and 22

September and it is well established that *Microcystis* growth rates are strongly temperature dependent in this range (Nalewajko and Murphy 2001; Davis et al. 2009). Third, water clarity was dropping quickly with the concomitant rise in both chlorophyll a and *Microcystis* abundance in late August and early September and it's possible that light levels in the water column could have limited *Microcystis* growth rates (Wiedner et al. 2003). It is plausible that a combination of these environmental factors could have contributed to the rapid decline in *Microcystis* abundance following the bloom.

Our study has provided multiple lines of evidence that *Microcystis* abundance is regulated by phosphorus availability in the western basin of Lake Erie at least during the 2008 season. The implications of this work are that phosphorus loading into the western basin via the Maumee River can have dramatic impacts on the scope of the *Microcystis* bloom in a given year. Clearly, an overall increase or decrease in phosphorus loading would translate into some direct impacts on *Microcystis* abundance, and unfortunately, the current trend indicates that phosphorus loading from the Maumee has been increasing since the mid 1990's (OEPA 2010). The timing of the phosphorus loading within the growing season may also have unknown variable impacts. For example, in 2008, there was a dramatic rise in the phosphorus gradient between 18 August and 3 September (Fig. 2.4). The rapid increase in available phosphorus then contributed to the rapid growth of phytoplankton, including *Microcystis*, within the basin (Figs. 3 and 4). If this pulse of phosphorus had arrived later in September, then it's possible that *Microcystis* would not have been phosphorus limited at that point and would have been more limited by temperature or nitrogen. Likewise, if the pulse had arrived earlier in the season when temperatures were lower, then perhaps a different taxa besides *Microcystis* would have bloomed. Given that the Maumee watershed is largely dominated by agriculture (Richards et al. 2002) there may be land

management practices that could reduce overall phosphorus loading or at least alter the timing of the phosphorus loading into the river.

FIGURES

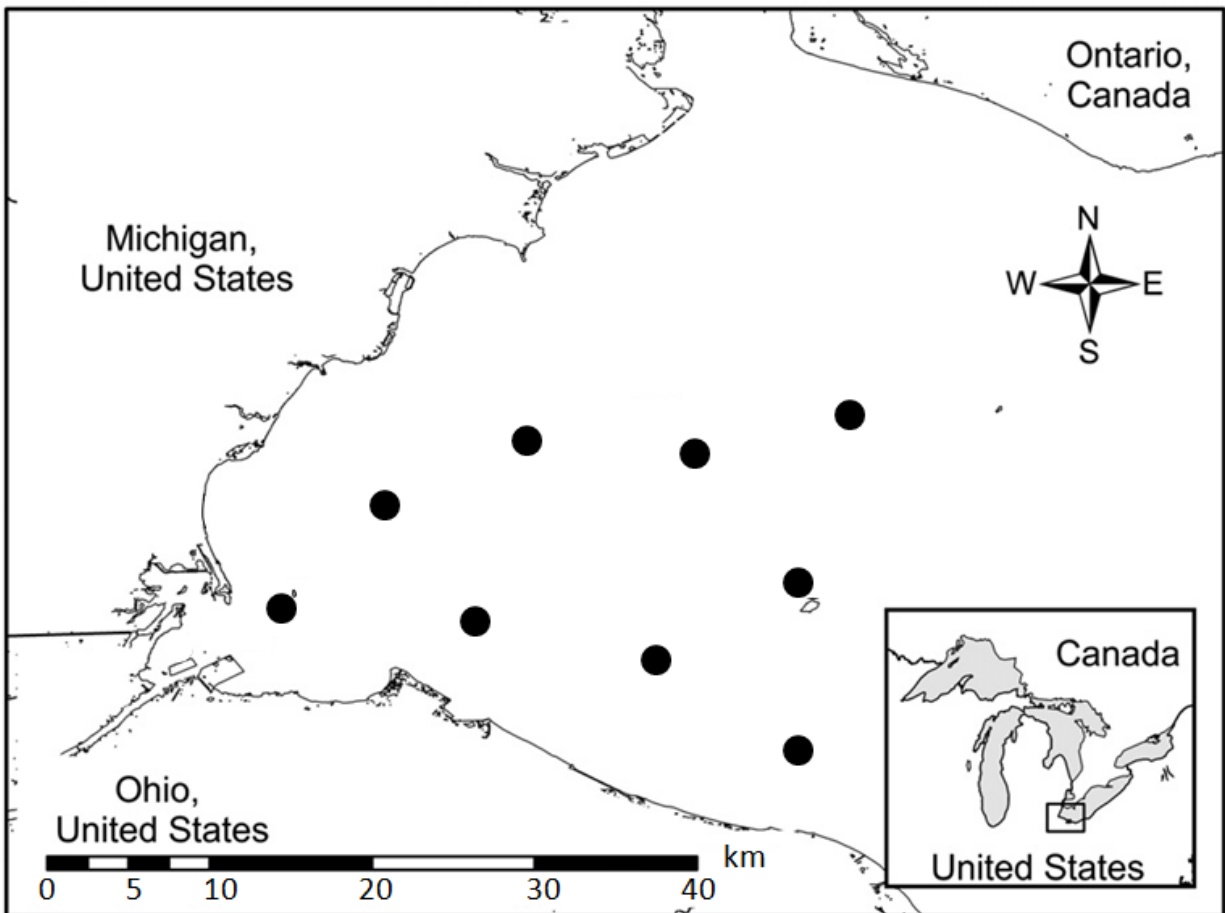


Figure 2.1 Map of the western basin of Lake Erie and locations of the survey sampling stations. Map derived from Chaffin et al. (2011).

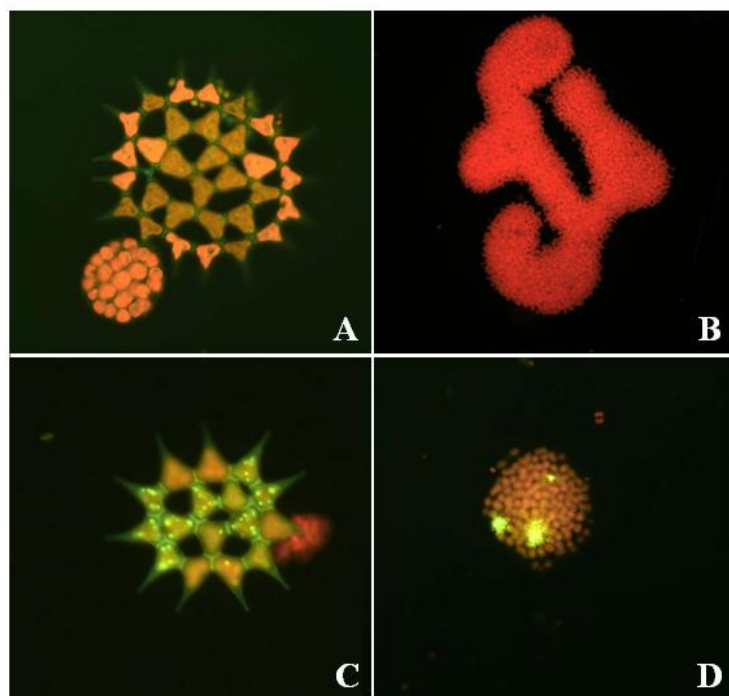


Figure 2.2 Taxon-specific alkaline phosphatase activity using enzyme-labeled fluorescence (ELF) and epi-fluorescence microscopy. Chlorophyll a in cells fluoresces orange-red, while expression of alkaline phosphatase activity is represented by bright-green precipitates formed by the alkaline phosphatase enzyme. Panel A and B illustrate colonies of *Pediastrum* and *Microcystis aeruginosa* that are not expressing ELF, while panels C and D illustrate colonies expressing ELF. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

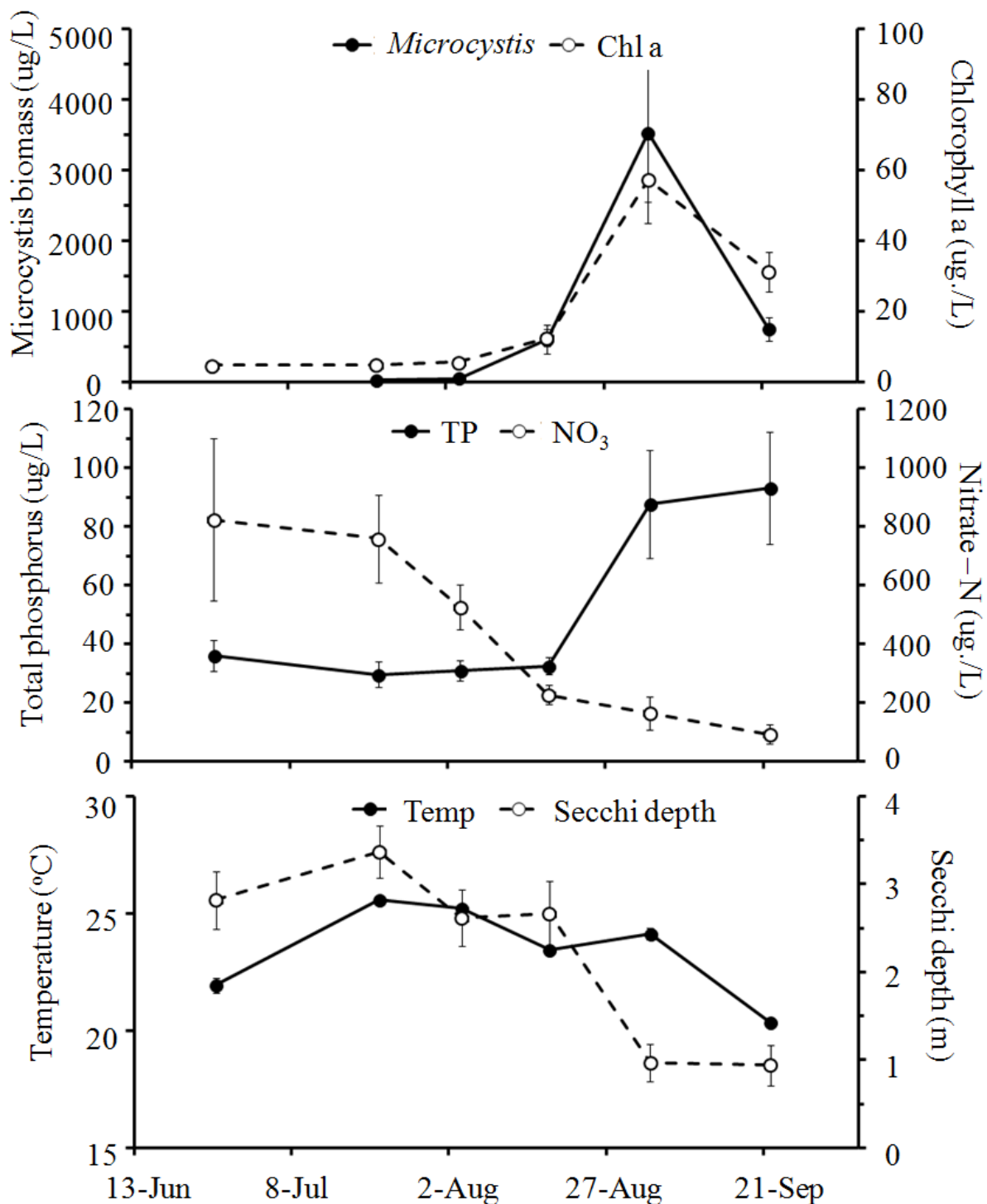


Figure 2.3 (Top) *Microcystis aeruginosa* (ug dry biomass/L) and chlorophyll a (ug/L); (middle) total phosphorus (ugP/L) and nitrate (ugN/L); and (bottom) water temperature (°C) and Secchi disk depth (m) throughout the summer of 2008 in the western basin of Lake Erie. Values are means $\pm$ SE averaged across sites within the basin on each date.

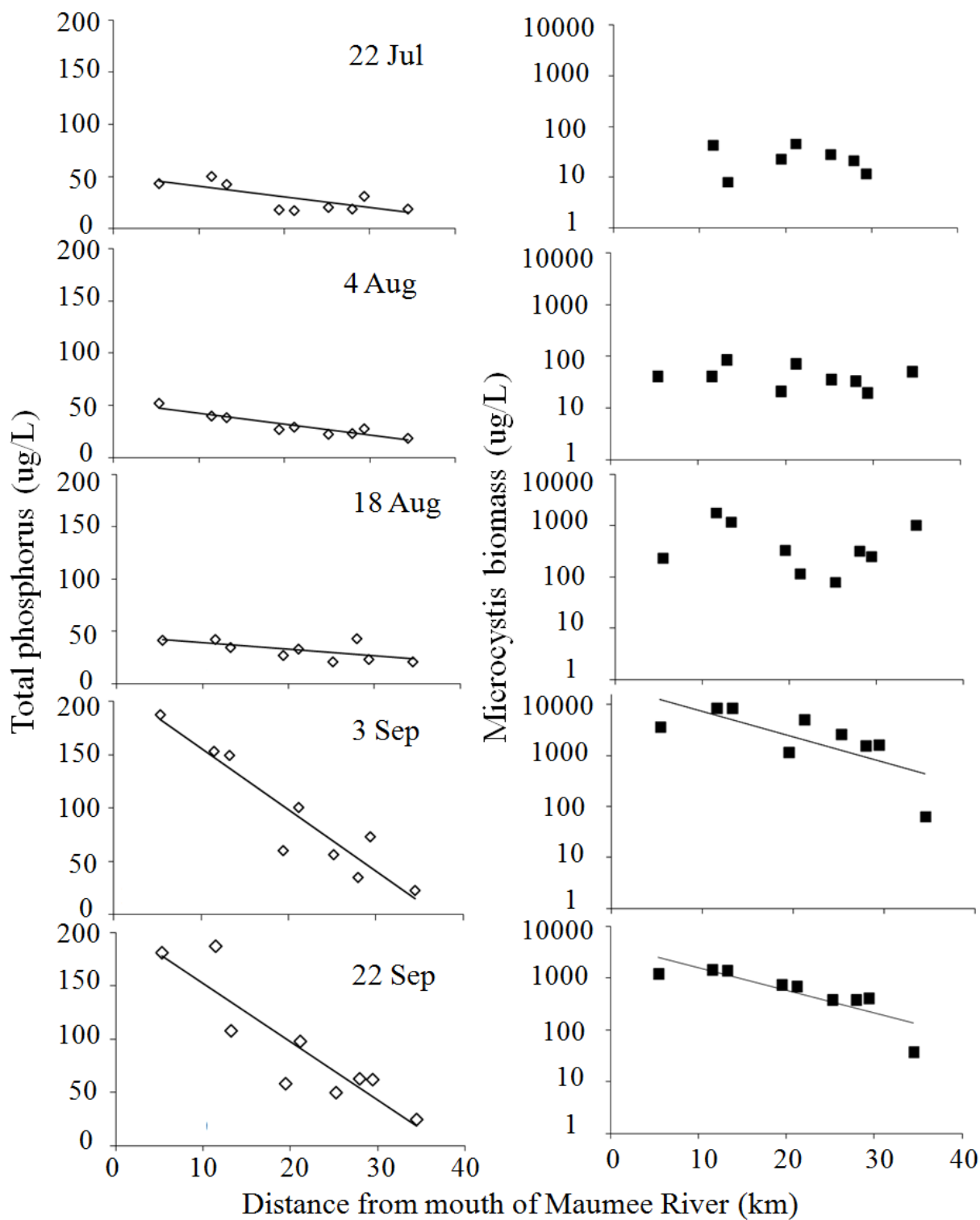


Figure 2.4 Total phosphorus (ug P/L) and *Microcystis aeruginosa* biomass (ug dry weight/L) in relation to distance from the mouth of the Maumee River in the western basin of Lake Erie. Trend lines are plotted for significant correlations ($p < 0.05$).

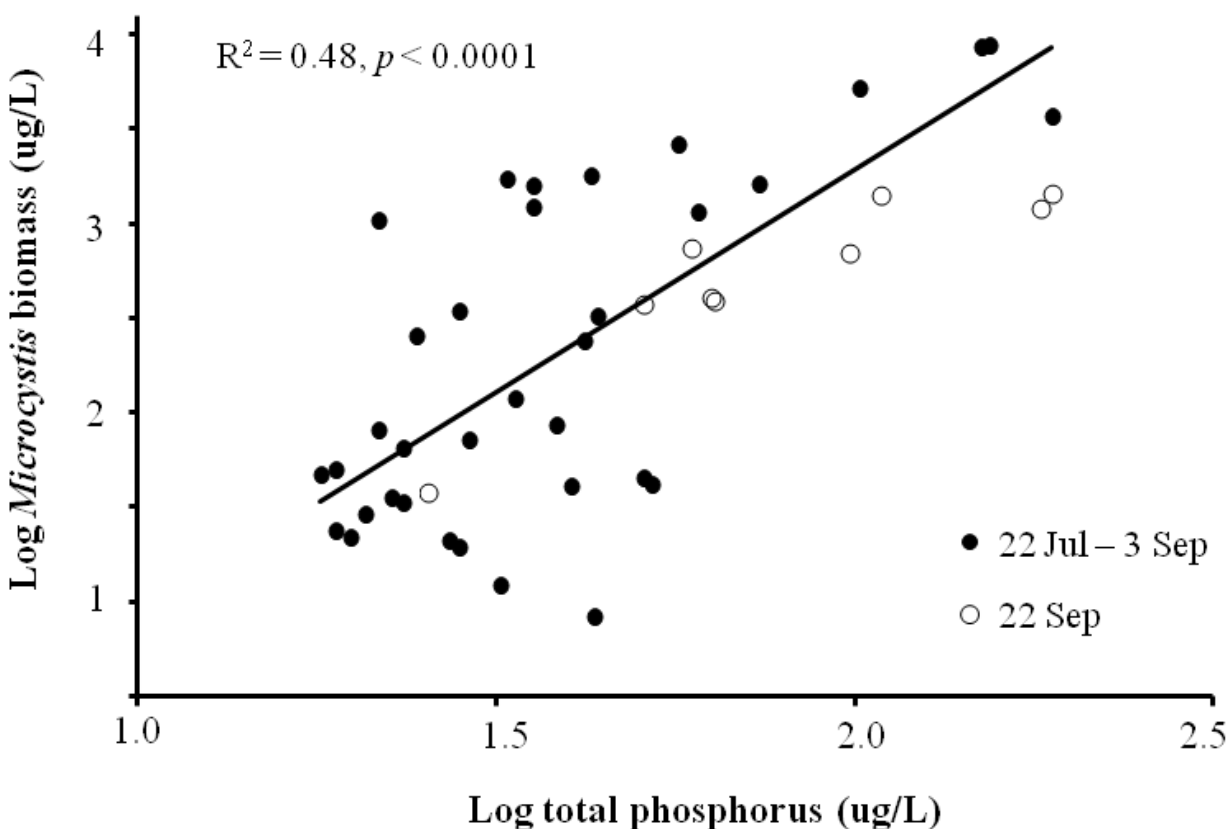


Figure 2.5 *Microcystis aeruginosa* biomass (ug dry weight/L) as a function of total phosphorus (ugP/L) during the sampling dates leading up to peak *Microcystis* abundance (Solid circles: 22 Jul, 4 Aug, 18Aug and 3 Sep) and after the bloom (Open circles: 22 Sep). Trend line represents the linear regression of log10 transformed biomass and log10 transformed phosphorus concentrations for the sampling period leading up the bloom (22 Jul – 3 Sep).

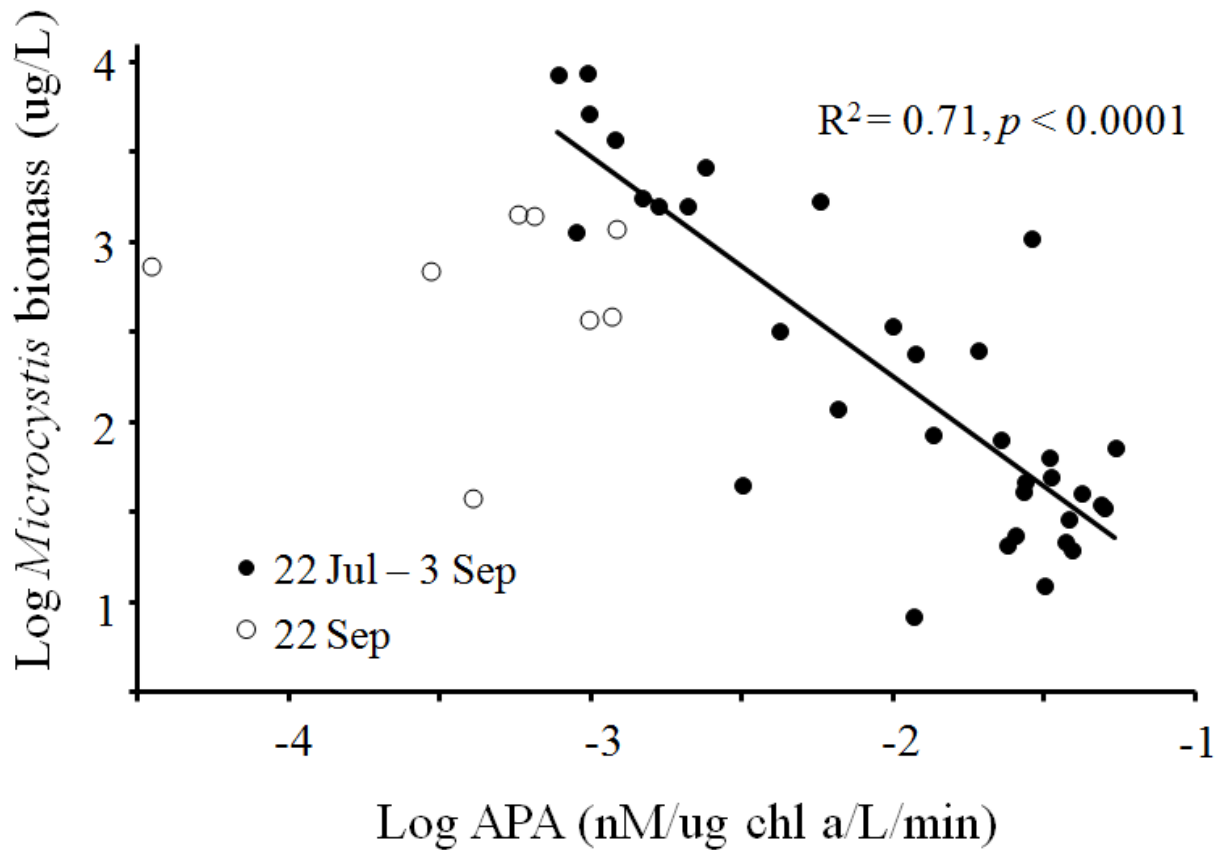


Figure 2.6 *Microcystis aeruginosa* biomass (ug dry weight/L) as a function of bulk alkaline phosphatase activity (APA) (nM/ug chlorophyll a/L/min) during the sampling dates leading up to peak *Microcystis* abundance (Solid circles: 22 Jul, 4 Aug, 18Aug and 3 Sep) and after the bloom (Open circles: 22 Sep). Trend line represents a linear regression of log10 transformed biomass and log10 transformed APA for the sampling period leading up to the bloom (22 Jul – 3 Sep).

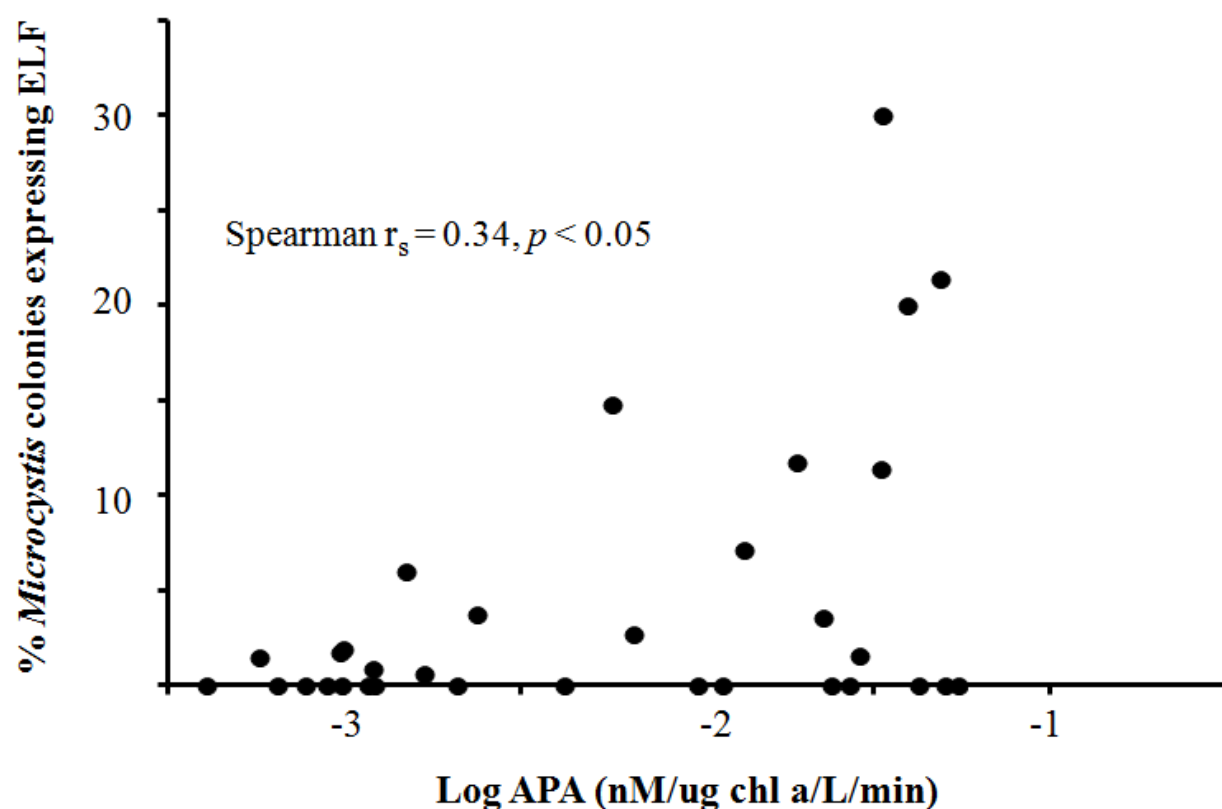


Figure 2.7 The fraction of *Microcystis aeruginosa* colonies expressing taxon-specific enzyme labeled fluorescence (ELF) of alkaline phosphatase as a function of bulk alkaline phosphatase activity (APA) (nM/ug chlorophyll a/L/min) for the entire seston community during on 4 Aug, 18 Aug, 3 Sep and 22 Sep. Spearman rank correlation coefficient, $r_s = 0.337$, $t = 2.06$, $df = 33$, $p = 0.023$.

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