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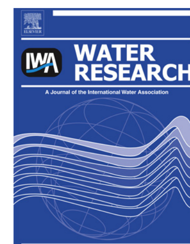

Nitrogen availability increases the toxin quota of a harmful cyanobacterium, *Microcystis aeruginosa*

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ABSTRACT

An important objective in understanding harmful phytoplankton blooms is determining how environmental factors influence the toxicity of bloom-forming species. We examined how nutrients and grazers (dreissenid mussels) affect the production of microcystin (a liver toxin) by the cyanobacterium *Microcystis aeruginosa*, via a combination of field and laboratory experiments, and field observations in Lake Erie. The field experiment revealed no effect of mussel density on microcystin quota (particulate microcystin per unit *Microcystis* biomass). In contrast, in both field and laboratory experiments, nitrogen-limited conditions led to substantially reduced microcystin quota relative to phosphorus-limited or nutrient-saturated conditions. In the field experiment, microcystin per unit of *mcyB* gene was strongly reduced under nitrogen-limited conditions, indicating a phenotypic response. Results from a seasonal survey in the western basin of Lake Erie revealed a similar negative influence of nitrogen limitation (as indexed by nitrate concentration) on microcystin quota. Our results are consistent with stoichiometric considerations in that the cell quota of a nitrogen-rich secondary metabolite, microcystin, was reduced disproportionately under nitrogen limitation.

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1. Introduction

Harmful phytoplankton blooms (HABs) are a major environmental concern in both marine and freshwaters throughout much of the world (Huisman et al., 2005), and the impacts of HABs on both ecosystems and human health are well documented (Carmichael, 2001; Chorus and Bartram, 1999; Smith and Schindler, 2009). A critical aspect of HAB ecology is the production of toxins by HAB species during blooms. As one noteworthy example, concentrations of microcystin, a potent liver toxin produced by the freshwater cyanobacterium *Microcystis aeruginosa* (hereafter *Microcystis*), can greatly exceed the World Health Organization (WHO) guideline for drinking water during the summer months in Lake Erie (Michalak et al., 2013), which is a source of drinking water for four million people (Fuller et al., 2002).

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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 cell or per unit biomass). Toxin quota can be a highly variable trait within a species. For example, among genotypes within some species, toxin quota can range from essentially zero to over 5 μg toxin mg^{-1} dry mass (Sivonen and Jones, 1999). The implication of a highly variable toxin quota is that blooms with high biomass and low toxin quota may, in some cases, pose a smaller public-health 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 important to understanding the impacts of HABs.

Microcystis is a cosmopolitan cyanobacterium that develops toxic blooms in both temperate and tropical freshwaters. Within populations of *Microcystis*, microcystin concentrations per unit biomass can vary by either: a) changes in the relative abundance of more toxic vs. less toxic genotypes; or b) changes in toxin production by individual genotypes 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 microcystin-producing genotypes of *Microcystis* in a large reservoir over the course of the growing season. Kardinaal et al. (2007) demonstrated in laboratory experiments that non-toxic strains of *Microcystis* outcompeted toxic strains under light-limited conditions, and suggested that light limitation induced by self-shading might explain a commonly observed temporal trend in some lakes for blooms to become less toxic over time.

Alternatively, many laboratory studies indicate that microcystin quota is a plastic trait and can be a function of growth rate (Orr and Jones, 1998; Watanabe et al., 1989), nutrient concentration (Downing et al., 2005; Oh et al., 2001; Van de Waal et al., 2009), temperature (van der Westhuizen and Eloff, 1985), light levels (Utkilen and Gjolme, 1992; Wiedner et al., 2003) and grazers (Jang et al., 2003). With respect to *Microcystis*, several laboratory studies have shown a strong positive correlation between nitrogen concentration (or N:P ratio) and microcystin quota (Long et al., 2001; Orr and Jones, 1998). More recent studies have parsed out the effects of growth rate and confirmed that microcystin quota is positively affected by increased nitrogen levels (or N:P ratio) independent of growth rate (Downing et al., 2005; Lee et al., 2000). Jähnichen et al. (2011) additionally reported limiting effects of low sulfate concentrations on microcystin quota, as well as an inverse relationship between quota and phosphorus concentrations. One study has shown that selective grazing by zooplankton can increase the microcystin quota of *Microcystis* by up to a factor of five (Jang et al., 2003). We are aware of no direct tests of the latter phenomenon for a benthic grazer such as dreissenid mussels, which are now a major source of phytoplankton mortality in the Laurentian Great Lakes (Vanderploeg et al., 2001, 2002).

Demonstrating statistically-significant effects of environmental factors in tightly-controlled laboratory experiments with clonal cultures is relatively straightforward given the high precision of the laboratory. However, it remains to be seen which of many potentially-important factors (as listed above) has major effects on toxin quotas in natural populations, since the latter are genetically diverse (Wilson et al., 2005) and embedded within complex communities of interacting species and genotypes.

Within natural phytoplankton communities where *Microcystis* is a dominant species, there is evidence that ambient microcystin concentrations can be correlated with nitrogen and/or phosphorus concentrations (Graham et al., 2004; Orihel et al., 2012; Rinta-Kanto et al., 2009). 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 microcystin concentrations.

Only a handful of studies have specifically focused on microcystin 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 few details about the nutrient environment. A survey of lakes in Quebec found ~10 fold variation in microcystin quota among lakes, but reported no environmental correlates with quotas (Giani et al., 2005). A multi-year survey of eutrophic lakes in Alberta showed that microcystin quota was significantly positively correlated with total phosphorus concentrations, but not with N availability (Kotak et al., 2000). These field data do not provide much consistent support for any particular nutrient driver. In many cases however, field data can be problematic for inferring the microcystin quota of any particular species since, in many lakes, microcystin can be produced by certain co-occurring cyanobacterial taxa other than *Microcystis* (Giani et al., 2005; Kotak et al., 2000). Clearly there is a need for more research on the controls of microcystin production by cyanobacteria in natural systems, and research that specifically brings together laboratory and field experimentation/observation.

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 field experiment, a single-genotype laboratory experiment, and an extensive field survey of the western basin of Lake Erie. Results from all three approaches were congruent in pointing to a positive effect of N availability on microcystin quota. Furthermore, genetic analyses of the microcystin gene (*mcyB*) suggested that phenotypic plasticity (rather than changes in gene density) can be largely responsible for this effect.

2. Materials and methods

2.1. *Gull Lake enclosure experiment*

Gull Lake is a large, hardwater lake (surface area = 8.2 km^2, 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 low (less than 15 μg L^{-1}), but NO_3^- levels are usually above 300 μg L^{-1} due to significant external contributions from the watershed (Brusewitz et al., 2012). Consequently, phytoplankton growth is P-limited (Hamilton et al., 2009; Sarnelle et al., 2012). Sulfate is readily

available and concentrations show little seasonal variability (mean, 23 mg/L: S. Hamilton, unpublished data). Zebra mussels (*Dreissena polymorpha*) invaded the lake in 1994, and *Microcystis* biomass has been markedly higher after 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 Michigan lake of comparable TP 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 (Sarnelle et al., 2012). *Microcystis* biomass in Gull Lake generally first reaches detectable levels in early July (J. White, unpublished data), so we timed the start of our field experiment to coincide approximately with this initial "appearance".

During the summer of 2007, we conducted an enclosure experiment in the lake to test for the effects of P addition and *D. polymorpha* density on the abundance and toxin quota of *Microcystis*. The enclosures were deployed in the same manner as described in Sarnelle et al. (2012). 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 and passed through a 150-μm mesh net to remove zooplankton that may have been killed by the pump. Natural densities of zooplankton (dominant species: *Daphnia mendota*, *Bosmina longirostris*, *Diaphanosoma brachyurum*, *Diaptomus sp.*, unidentified cyclopoids; Sarnelle et al., 2012) were then re-established on 29 June by pooling repeated zooplankton tows (1 m diameter, 150 μm mesh) from the mixed layer (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 ~8–10 μg L^{-1}, referred to as "low P"), mesotrophic (TP amended to ~15 μg L^{-1}, "medium P") and mildly eutrophic (TP amended to ~25 μg L^{-1}, "high P") conditions. We added P alone to simulate a eutrophication gradient since the N:P ratio in lakes typically declines with increases in P (Downing and McCauley, 1992) 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 target TP levels. We subsequently maintained target TP levels via weekly TP monitoring and addition of phosphate solution as needed.

Within each set of 10 enclosures for each P treatment, we established a gradient of *Dreissena* ranging from 0 to 4 g m^{-2} (dry tissue mass) as follows. Mussels were collected in late June from the littoral zone of Gull Lake. After sorting by size (range used: 16–20 mm, shell length), mussels were counted out individually into open-top baskets (16 cm diameter, 13 cm high) made of stiff plastic mesh (mesh size: 3.5 mm) until each basket contained the targeted number of mussels (e.g., for the 4 g m^{-2} treatment, baskets were stocked with ~90 individuals). Baskets of mussels were suspended in Gull Lake until we were ready to stock them into the enclosures. Eight baskets of mussels were hung on a PVC frame at a depth of 2.5 m in each of 24 enclosures to initiate the *Dreissena* manipulation on 5 July (day 0 of the experiment). The 6 mussel-free enclosures were stocked with the same number of empty cages.

We sampled the enclosures weekly by pooling duplicate depth-integrated tube samples (2.5 cm inside diameter) collected from the surface to 7 m depth. Sampled water was stored in clean, polyethylene containers and transported in coolers to a lakeside laboratory, where samples were initially processed within 1 h into four sub-samples: 1) raw water stored at 4 °C; 2) filtered water passed through a Pall A/E glass fiber filter (1-μm nominal pore size) 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 h of collection. Soluble reactive P (SRP) and ammonium (NH_4^+) were measured on filtered water samples using long-pathlength spectrophotometry (Wetzel and Likens, 2000). Nitrate (NO_3^-) was measured on filtered water samples by Dionex membrane-suppression ion chromatography. Total phosphorus was measured on persulfate-digested raw water samples and then analyzed spectrophotometrically as SRP. Chlorophyll *a* was extracted from frozen filters in 90% ethanol and measured fluorometrically (Welschmeyer, 1994). Particulate microcystins were extracted from frozen filters with 75% methanol (Chorus and Bartram, 1999) and then diluted 1:5 with deionized water before analysis by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Envirologix, Inc.). The detection limit for microcystin 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 100× 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 a surface-area to cell-count relationship established for *Microcystis* samples from 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 mass (mg) to biovolume (mm^3) ratio of 0.4 (White et al., 2011). Microcystin toxin quota (μg mg^{-1} dry mass) was calculated by dividing particulate microcystin concentration (μg L^{-1}) by *Microcystis* dry biomass concentration (mg L^{-1}).

Alkaline phosphatase activity (APA), a measure of P limitation in the planktonic community, was measured fluorometrically in 10 ml aliquots of freshly-collected raw water samples from the enclosures (Pettersson, 1980). Enzyme activity was divided by chlorophyll *a* concentration (SpAPA). *Microcystis*-specific alkaline phosphatase activity was also assessed using enzyme-labeled fluorescence (ELF) microscopy (Gonzalez-Gil et al., 1998). This microscopy-based method is

analogous to bulk alkaline phosphatase analysis, whereby P-limited organisms produce phosphatase enzymes, which are then detected visually for cells of a particular species. Fluorescence 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 h, 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 phosphatase 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.

2.2. *mcyB* gene copy density

We processed seston samples collected from the enclosures on day 31 of the experiment 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 elsewhere (Dyble et al., 2008). Briefly, water samples were filtered onto 0.8-μm membrane filters and stored at −80 °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 (qPCR) in 20 μL volumes using an Applied Biosystems® 7500 Fast Real-Time PCR system. Amplification used fast conditions with an initial denaturing step of 95 °C for 10 min followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °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.

2.3. 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 toxin-producing *Microcystis* strain isolated from the lake (strain 2006-B) was exposed to varying N versus P availability. In contrast to most culture-collection strains, our strain had retained the typical floating colony-forming phenotype when the laboratory experiment was conducted.

Before the experiment, *Microcystis* strain 2006-B was grown in four 2-L culture vessels containing sterilized half-strength WC-S media (1/2 WC-S, Stemberger, 1981), with an N:P ratio by mass of 9:1 (3500 μg N L^{-1} as KNO_3, 388 μg P L^{-1} as K_2HPO_4). 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 1/2 WC-S: 1) P-limited (3500 μg N L^{-1}: 78 μg P L^{-1} [45:1], $N = 4$); 2) N-limited (438 μg N L^{-1}: 388 μg P L^{-1} [1.1:1], $N = 8$); and 3) unaltered 1/2 WC-S ($N = 4$). The treatment flasks were filled to 450 ml with their respective media and set under the same fluorescent lighting on a 12 h:12 h light:dark cycle at 19 °C.

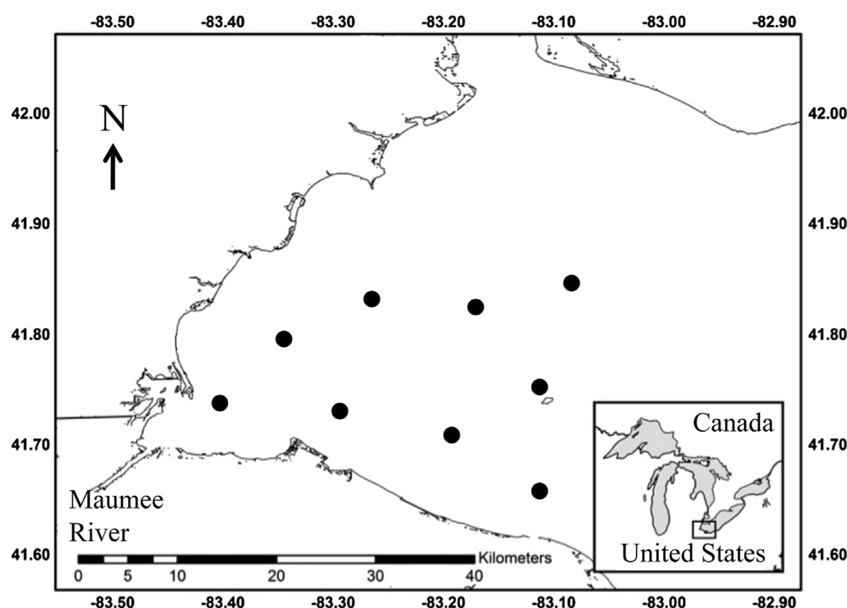

Fig. 1 – Map of the western basin of Lake Erie (modified from Chaffin et al., 2011) showing sampling stations in relation to a major nutrient source, the Maumee River.

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 that with the respective fresh medium for each treatment. To check for any effects of inorganic carbon availability, we gently aerated half the flasks from the N-limited treatment (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 or not, so the results for all eight flasks were pooled into one treatment.

Microcystin and SRP were analyzed in the same fashion as the enclosure experiment. Concentrations of NO_3^- 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 NaOH digestion (Reynolds and Jaworski, 1978) to break the colonies into individual cells for easier counting.

2.4. *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 μg L^{-1}) that enters from the southwest (Fig. 1) and contributes more than half of the P loading in the southern and western portions of the basin where *Microcystis* blooms are a recurring water quality problem (Bridgeman et al., 2012; Chaffin et al., 2011). Nine stations were sampled to provide a broad representation across the basin, with stations spaced approximately 6–10 km apart (Fig. 1). Depth varied from 4–8 m for all stations except the one nearest the Maumee River (Fig. 1), which was 1 m deep. Sampling trips were conducted at intervals of approximately two weeks, from July through September, 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 depths with a van Dorn bottle and pooled into clean, 10-L polyethylene containers. Temperature profiles confirmed that the lake was generally isothermal (i.e., <1 °C difference from top to bottom) and therefore subsurface samples should be representative of the water column. Secchi disk depth was recorded at each station and temperature, light, pH, conductivity, and dissolved oxygen profiles were taken from at least four stations on each sampling trip.

Water samples were kept at ~4 °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 h of collection and analyzed in the same manner (with NO_3^- via spectroscopy) as described for the enclosure and laboratory experiments. *Microcystis* biomass was measured via microscopic analysis of Lugol's-preserved samples as described for the enclosure experiment.

Microcystin cell quota was 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 μg L^{-1} dry mass), and thus readily measurable. This criterion excluded five pre-bloom samples from 22 July.

2.5. *Statistical analyses*

For variables measured over time in the enclosure experiment (Fig. 2), we employed a repeated-measures analysis of variance (R-M ANOVA) with P treatment and *Dreissena* density as independent variables. Variables measured on day 31 of the

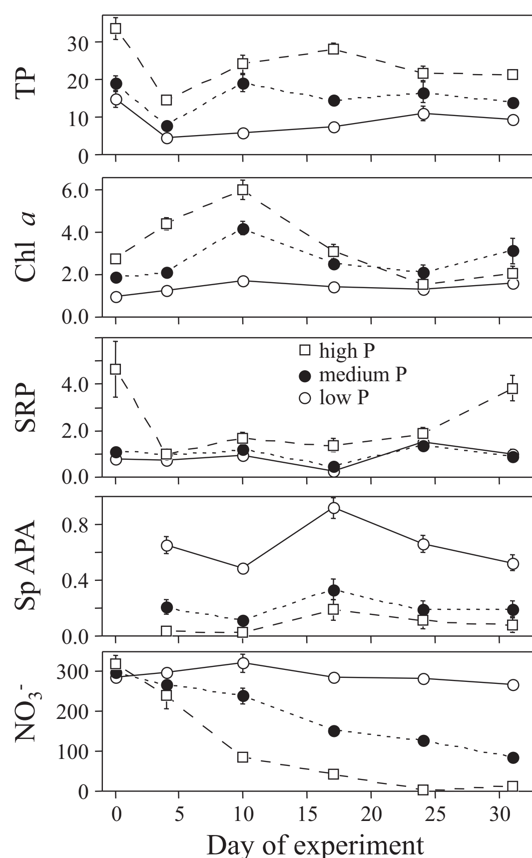

Fig. 2 – Dynamics of total phosphorus (TP, μg P L^{-1}), chlorophyll *a* (Chl *a*, μg L^{-1}), soluble reactive phosphorus (SRP, μg P L^{-1}), chlorophyll-specific alkaline phosphatase activity (SpAPA, nmol $μg^{-1}$ min^{-1}) and nitrate (NO_3^-, μg N L^{-1}) during the field experiment in Gull Lake. Open circles = low-phosphorus treatment (no P addition), solid circles = medium-phosphorus treatment, open squares = high-phosphorus treatment. Data points represent means (±1 standard error) averaged across a gradient of 10 *Dreissena* densities. In many cases, the error bar is contained within the symbol. Note that the phosphorus-addition treatments were initiated 7 days before day 0, whereas the *Dreissena* treatments were initiated on day 0.

enclosure experiment were first analyzed with an analysis of covariance (ANCOVA) having three levels of P and *Dreissena* density as a continuous independent variable. There were no effects of *Dreissena* on the parameters of primary interest in this paper (see Results), so we followed the ANCOVA with a one-way ANOVA of the effect of P treatment. Differences among treatment levels were assessed with Tukey's HSD multiple comparison test. The laboratory results were analyzed with a one-way ANOVA followed by Tukey's HSD. Relationships between continuous variables (all three data sets) were analyzed by linear regression. Data were log transformed as needed to correct for heterogeneity of variances.

3. Results

3.1. *Gull Lake enclosure experiment*

We were reasonably successful in establishing and maintaining TP near target levels in the enclosures (Fig. 2, R-M ANOVA for TP: $p < 0.0001$). Soluble reactive phosphorus was less than 2 μg L^{-1} in the low and medium P treatments, while concentrations were slightly higher and reached 4 μg L^{-1} in the high P treatment by day 31 (Fig. 2, R-M ANOVA for SRP: $p < 0.0001$). As expected, phytoplankton biomass (as chlorophyll *a*) increased in response to P addition (Fig. 2, R-M ANOVA for chlorophyll *a*: $p < 0.0001$). After the initial response to P addition, chlorophyll *a* declined in the P-addition treatments, with the decline being most pronounced in the high-P treatment (Fig. 2).

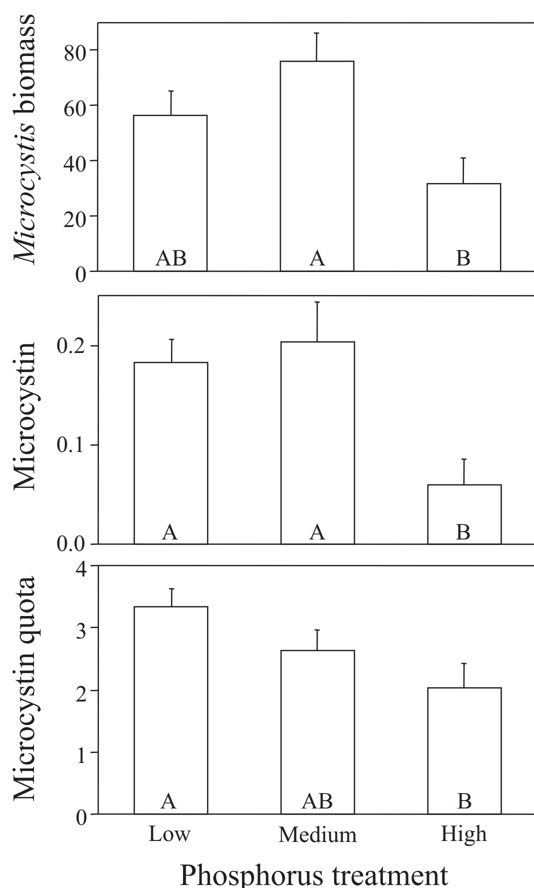

Fig. 3 – *Microcystis* dry biomass (μg L^{-1}), particulate microcystin (μg L^{-1}) and microcystin quota (μg mg dry $biomass^{-1}$) on day 31 of the field experiment in Gull Lake. Columns represent means (±1 standard error) averaged across a gradient of 10 *Dreissena* densities. Columns with different letters denote treatment means that were different via Tukey's HSD multiple-comparison tests ($p < 0.05$). Error bars denote 1 standard error.

At the beginning of the experiment, NO_3^- was ~300 μg N L^{-1} and remained relatively unchanged throughout the experiment in the low-P treatment that received no P addition (Fig. 2). Nitrate 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 μg L^{-1} in the medium-P treatment (Fig. 2, R-M ANOVA for NO_3^-: $p < 0.0001$). Ammonium levels remained less than 20 μg L^{-1} in all treatments throughout the experiment and did not contribute significantly to variation in dissolved inorganic N.

Alkaline phosphatase activity of the planktonic community was inversely related to ambient P throughout the experiment, with enzyme activity in the low-P treatment typically 3–4 times higher than in the medium- and high-P treatments (Fig. 2, R-M ANOVA for SpAPA: $p < 0.001$). At the taxon-specific level, the relative alkaline phosphatase activity for *Microcystis* measured with ELF 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, $p < 0.03$). These data indicate that P addition alleviated P limitation in the phytoplankton generally, and in *Microcystis* specifically. None of the variables listed above were affected by *Dreissena* density (R-M ANOVA $p > 0.05$).

Microcystis biomass on day 31 was affected by both *Dreissena* density (Appendix Fig. A-1, ANOVA, $p < 0.01$) and P addition (Fig. 3, ANOVA $p < 0.03$), but with no significant interaction. The response of *Microcystis* biomass to P addition was similar to that observed for chlorophyll *a* on day 31, with highest abundance in the medium-P treatment (Figs. 2 and 3). 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. 3; ANOVA, $p < 0.005$), but not affected by *Dreissena* density (Appendix Fig. A-2, ANOVA, $p > 0.50$). The negative effect of P addition on microcystin quota resulted in a significant positive relationship between quota and nitrate concentration on day 31 of the experiment (linear regression, $R^2 = 0.22$, $N = 30$, $p < 0.002$).

Density of the *mcyB* gene was higher in enclosures with lower available N (P-addition treatments, Figs. 2 and 4, ANOVA, $p < 0.05$), providing no evidence of selection for strains with low copy numbers at reduced NO_3^-. More importantly, microcystin per *mcyB* gene copy was strongly reduced by P addition (Fig. 3, ANOVA, $p < 0.0001$), and hence was lowest at the lowest NO_3^- (Figs. 2 and 4).

3.2. *Monoculture laboratory experiment*

Microcystin quota of the Gull Lake strain of *Microcystis* in the laboratory experiment was lowest in the N-limited treatment

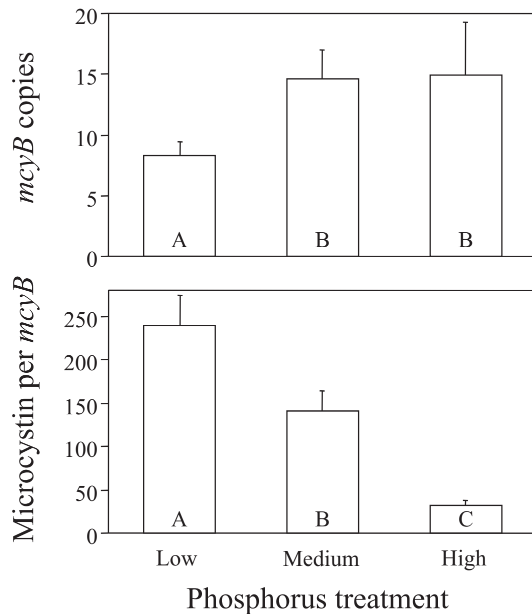

Fig. 4 – Copy density of the *mcyB* gene ($\times 10^9$ L^{-1}) and microcystin per gene copy (pg 10^6 copies^{-1}) on day 31 of the field experiment in Gull Lake. Columns represent means (±1 standard error) averaged across a gradient of 10 *Dreissena* densities. Columns with different letters denote treatment means that were different via Tukey's HSD multiple-comparison tests ($p < 0.05$). Error bars denote 1 standard error.

and highest in the P-limited treatment (Fig. 5; ANOVA, $p < 0.005$). *Microcystis* grown in media replete with N and P (1/2 WC-S) had about the same quota as the P-limited treatment. Thus N availability, not P availability, affected toxin quota in the laboratory experiment. Nitrate concentrations averaged 14 μg N L^{-1} in the N-limited treatment when quota was measured.

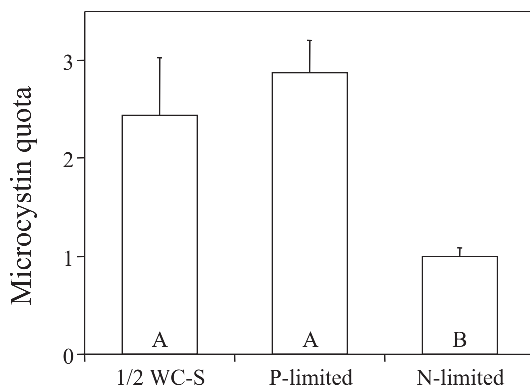

Fig. 5 – Microcystin quota (μg mg dry biomass^{-1}) of Gull Lake *Microcystis* strain 2006-B on day 50 in the three media treatments of the laboratory experiment. 1/2 WC-S = N- and P-replete culture medium (N:P = 9:1). Columns with different letters denote treatment means that were different via Tukey's HSD multiple-comparison tests ($p < 0.05$). Error bars denote 1 standard error.

3.3. *Lake Erie surveys*

During the Lake Erie survey in 2008, *Microcystis* was the only significant (i.e., >1% of total phytoplankton biomass) microcystin-producing taxon present. *Anabaena* was present at some sites but always at levels less than 1% of total biomass. There was a strong trophic gradient (TP, chlorophyll *a*, *Microcystis* biomass, microcystin, etc.) with distance from the mouth of the Maumee River, but we did not find consistent spatial correlates of microcystin quota across sampling dates. Given the latter, and the fact that the spatial gradient has been described well by others (Chaffin et al., 2011), we focused our attention on temporal rather than spatial patterns in microcystin quota.

Averaging across the western basin, *Microcystis* biomass increased by over two orders of magnitude during August, reaching a peak of 3500 μg L^{-1} on 3 September before declining in mid-late September (Fig. 6). Microcystin concentrations mirrored these trends (Fig. 6). Basin-wide microcystin quota declined by more than two fold from July to September in concert with a ten-fold decline in NO_3^- (Fig. 6). Total phosphorus more than doubled during this same period (Fig. 6). Quota was both high and low during the exponential growth phase of the bloom (4 August, 18 August), and also low during peak biomass (3 September) and decline (22 September). The latter observations do not support the hypothesis that quota was driven by net population growth rate. Rather, results over time from the western basin of Lake Erie are consistent with results from the field and laboratory experiments in showing a positive influence of available N on microcystin quota (Fig. 7).

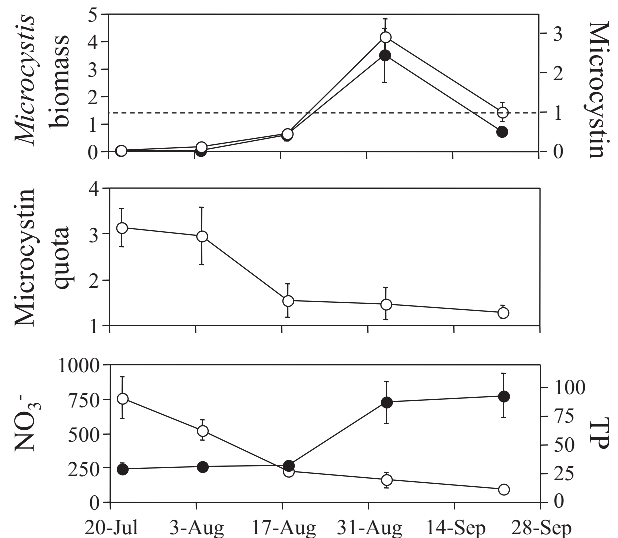

Fig. 6 – *Microcystis* dry biomass (mg L^{-1}, solid circles), particulate microcystin (μg L^{-1}, open circles), microcystin quota (μg mg dry biomass^{-1}), nitrate (NO_3^-, μg N L^{-1}, open circles) and total phosphorus (TP, μg P L^{-1}, solid circles) for the western basin of Lake Erie from July–September 2008. Dotted line on upper panel is WHO guideline for microcystin (1 μg L^{-1}) in drinking water. Error bars denote ±1 standard error and represent spatial variation across nine sampling stations.

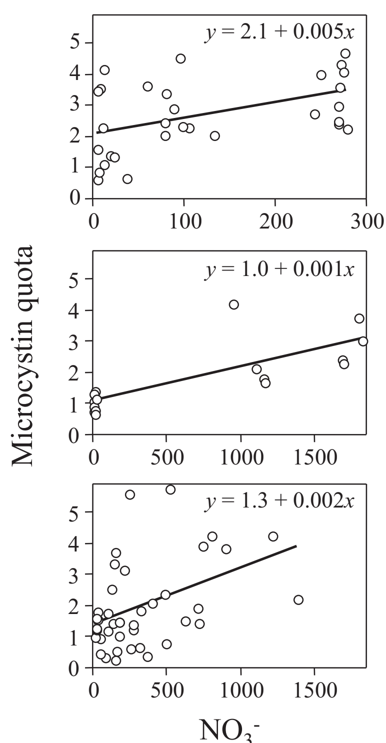

Fig. 7 – Relationships between microcystin quota (μg mg *Microcystis* dry biomass^{-1}) and nitrate (NO_3^-, μg N L^{-1}) for the three data sets. Top panel: results on day 31 of the enclosure experiment in Gull Lake ($R^2 = 0.22$, $p < 0.01$, N = 30), middle panel: results on day 50 of the laboratory experiment with Gull Lake *Microcystis* strain 2006–B ($R^2 = 0.61$, $p < 0.001$, N = 12), lower panel: results from nine stations in the western basin of Lake Erie, sampled from July–September, 2008 ($R^2 = 0.18$, $p < 0.01$, N = 40).

4. Discussion

In this paper we have presented multiple lines of evidence supportive of a major role for N availability in mediating the microcystin quota of *Microcystis aeruginosa*, one of the most important phytoplankton species responsible for toxic phytoplankton blooms in freshwaters (Boyer, 2008). Results from a field experiment in large enclosures showed a strong association between reduced NO_3^- concentration (generated by P addition) and reduced microcystin quota (Figs. 3 and 7). This relationship was shown to be consistent with observations from a seasonal survey of the western basin of Lake Erie showing concomitant declines in NO_3^- and microcystin quota (Figs. 6 and 7). Genetic evidence from the enclosure experiment suggests 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. 4). 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 confirm cause-and-effect and phenotypic plasticity in *Microcystis*, we followed up our field experiment 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 microcystin quota compared to N-replete conditions, whereas P limitation had no detectable effect on microcystin quota (Fig. 5). Combining the results from all three approaches we employed, we conclude that N availability is likely an important causative driver of microcystin quota in natural populations of *Microcystis*, and that this effect may be largely mediated by the phenotypic responses of individual cells. The latter conclusion is supported by other recent work with laboratory cultured *Microcystis* (Harke and Gobler, 2013).

We observed a qualitatively consistent pattern of increased microcystin quota with increased available N across the three study systems (Fig. 7). Not surprisingly, the laboratory experiment was the most precise, but all three relationships suggest a major influence of NO_3^- concentration on microcystin quota (Fig. 7). The magnitude of the N availability effect (i.e., slope of quota versus NO_3^-) was fairly similar across study systems, although most similar between the laboratory experiment and the Lake Erie survey where the range of NO_3^- concentrations was more similar. In both the laboratory and Lake Erie, microcystin quota approximately tripled from the lowest to the highest N availability, a result that is remarkably consistent given the large methodological differences between these two data sets.

The positive effect of N availability on microcystin quota that we observed is largely consistent with previous laboratory research (Downing et al., 2005; Lee et al., 2000; Van de Waal et al., 2009). More recently, Jähnichen et al. (2011) found that microcystin quota decreased with increasing P, as we observed in the enclosure experiment (Figs. 3 and 4). Our laboratory experiment showed, however, that the P "effect" in the enclosures was more likely driven directly 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 Jähnichen et al. (2011). Other work in Lake Erie also shows a shift to N limitation of *Microcystis* growth late in the summer (Chaffin et al., 2013).

The proportion of a *Microcystis* population harboring toxin-producing genes can vary in space and time (Briand et al., 2009; Rinta-Kanto et al., 2009). If selection for strains of *Microcystis* with lower capacity for microcystin production was causing the decrease in toxin quota at reduced NO_3^- (high P) in our field enclosures (Fig. 3), we would have expected a lower relative *mcyB* gene frequency (*mcyB* genes per *Microcystis* biomass) at low NO_3^-. In fact, relative *mcyB* gene frequency was actually higher in enclosures with lower available N (respective frequencies: low-P mean = 1.5×10^8, SE = 1.6×10^7, medium-P mean = 2.5×10^8, SE = 6.1×10^7, high-P mean = 6.6×10^8, SE = 1.3×10^8 gene copies μg dry *Microcystis* biomass^{-1}).

An alternative explanation is that toxin quota is a function of phenotypic plasticity caused by a change in the abiotic or biotic environment. In the enclosures, toxin produced per *mcyB* gene was more than 8 fold higher in the low-P (high NO_3^-)

vs. high-P (low NO_3^-) enclosures (Fig. 4). This evidence suggests a high plasticity in microcystin production physiology mediated by N availability and is consistent with stoichiometric theory in that production of N-rich secondary metabolites, such as microcystins, should be reduced disproportionately under N limitation (Sterner and Elser, 2002; Van de Waal et al., 2009).

In contrast to the marked effect of N availability on microcystin quota, we found no evidence that zebra-mussel grazing or infochemicals affect the quota under field conditions. This result is congruent with lake-survey results (Knoll et al., 2008), and suggests that mussels do not consistently select against *Microcystis* with high microcystin quotas when feeding. There is precedent for this conclusion from feeding studies (Vanderploeg et al., 2001; Dionisio Pires et al., 2007; White et al., 2011). Thus, our results reinforce the conclusion that compounds other than microcystin may be more responsible for *Dreissena*'s selective feeding on *Microcystis* colonies within the edible size range (White et al., 2011).

Clearly, both *Microcystis* biomass and microcystin quota contribute to overall toxin concentrations in nature. In our survey of the western basin of Lake Erie, seasonal variation in microcystin concentrations was strongly related to the dynamics of *Microcystis* biomass ($R^2 = 0.98$, $p < 0.002$, $N = 5$) despite declining microcystin quota. This indicates that biomass was the primary driver of microcystin concentrations (Fig. 6). However, the role of seasonally-variable microcystin quota (and thus presumably N availability) is also important, as illustrated by a simple calculation. During peak *Microcystis* biomass in early September, microcystin levels in the western basin peaked at $\sim 3\ \mu g\ L^{-1}$, or three times the WHO limit for drinking water (Fig. 6) despite a relatively low microcystin quota for *Microcystis* ($\sim 1.5\ \mu g\ mg^{-1}$). If the quota had remained at levels observed in July ($\sim 3\ \mu g\ mg^{-1}$), perhaps as a result of higher N availability, microcystin levels might have reached over $6\ \mu g\ L^{-1}$, or six times the WHO limit. Of course, we do not fully 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 (Orihel et al., 2012; Scott et al. 2012).

The results of this work have potential implications for understanding controls on the toxicity of *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) and Lewis et al. (2011) present arguments in favor of both N and P control in some freshwaters. In this paper, we present evidence for an important role of N availability in determining toxin quota via phenotypic plasticity in nature. Increasing N pollution of watersheds and recipient freshwaters (Carpenter et al., 1998) could therefore lead to increased toxicity of harmful phytoplankton blooms. Even when N is not the proximate limiting factor for biomass production in a bloom (although there is recent evidence of N limitation of *Microcystis* growth in late summer in Lake Erie- Chaffin et al., 2013), enhanced N availability could make a bloom more toxic, which is another reason to perhaps consider measures to limit further increases in N pollution of watersheds, in addition to controlling P. This conjecture merits further examination.

5. Conclusions

1. Results of a field experiment in oligotrophic Gull Lake showed a significant negative effect of P addition on the microcystin quota of *Microcystis aeruginosa*, but no significant effect of zebra mussel (*Dreissena*) density.
2. In the field experiment, we found a significant positive relationship between microcystin quota and nitrate concentration, suggesting that low N availability was driving a decrease in quota of this N-rich compound.
3. Results of a laboratory experiment with a strain of *M. aeruginosa* isolated from Gull Lake showed a negative effect of N limitation on microcystin quota.
4. A sampling survey in the western basin of Lake Erie showed a seasonal decrease in the microcystin quota of *M. aeruginosa* that was significantly associated with a decline in N availability, but not associated with variation in net population growth rate.
5. Taken together, the three data sets suggest that N limitation is an important driver of microcystin quota in *M. aeruginosa* in natural systems.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.01.063.

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