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# The influence of nutrients limitation on phytoplankton growth and microcystins production in Spring Lake, USA

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## HIGHLIGHTS

- Nitrogen or phosphorus addition alone was not sufficient to induce algal blooms.
- N + P treatment resulted in high concentrations of chlorophyll-*a* and microcystins.
- Past management strategy solely on reducing P loads is insufficient in Spring Lake.
- Reduction of both N and P are necessary to control algal blooms and MC production.

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## ABSTRACT

Due to excessive loadings of nitrogen (N) and phosphorus (P), frequent blooms of harmful cyanobacteria and their associated cyanotoxins pose serious threats to recreational usage and human health. However, whether cyanobacteria growth and toxin production are limited by N, P, or both N + P is still not clear. Thus, we conducted a nutrient enrichment bioassay *in situ* in Spring Lake, a eutrophic lake in west Michigan, USA, to examine the influence of nutrient limitation on the proliferation of algal blooms and the production of microcystins (MC). N or P addition alone resulted in a slight increase in the concentration of chlorophyll-*a* (Chl-*a*), suggesting a positive effect on phytoplankton growth, but alone, neither were sufficient to induce algal blooms. In contrast, the combination of N and P had a significant and positive influence on phytoplankton growth and MC production. Compared to controls, the N + P treatment resulted in high concentrations of Chl-*a* and MC, as well as high pH and dissolved oxygen. In addition, significant increases were observed in different MC analogues for each treatment; the highest concentrations of intracellular MC-LR, -RR, -YR, and TMC (total MC) were found in the N + P treatment with values of 9.16, 6.10, 2.57, and 17.82 μg/L, respectively. This study suggests that at least in this temperate coastal lake, cyanobacterial blooms and associated MC are influenced more by combined N and P enrichment than by N or P alone, indicating that managing both nutrients is important for effectively reducing algal blooms and MC production.



## 1. Introduction

Excess enrichment of nutrients from both internal and external loading accelerates water eutrophication and promotes the growth of phytoplankton, leading to frequent cyanobacterial blooms and subsequent decline in water quality in freshwater ecosystems (Paerl et al., 2015; Smith et al., 2016; Xu et al., 2017). The role of nutrients, mainly nitrogen (N) and phosphorus (P), in controlling phytoplankton growth has received considerable attention but the results have been inconsistent (Conley et al., 2009; Paerl et al., 2016; Steinman et al., 2016). Some researchers consider P to be the primary nutrient limiting the occurrence of cyanobacterial blooms, and therefore they promote methods to reduce P input or inactivate P already in the lake, as the most effective method to restore freshwater ecosystems (Bormans et al., 2016; Lürling et al., 2016). However, according to the results of the nutrient enrichment bioassay by Xu et al. (2010) conducted in Lake Taihu, the third

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largest freshwater lake in China, it is more likely that bioavailable N is the key factor controlling the proliferation of cyanobacterial blooms, especially toxic *Microcystis* spp. blooms. Recently, Steinman et al. (2016) suggested that phytoplankton were P-limited and benthic algae were co-limited by N and P in hypereutrophic Lake Macatawa, USA. Therefore, limitation by N and/or P appears to vary depending on habitat (benthic vs. planktonic), local environmental conditions (e.g., ambient nutrient concentrations and light conditions), and the taxonomic composition of the algae ($N_2$-fixing and non-$N_2$-fixing cyanobacteria). Furthermore, the nutrients limiting phytoplankton growth can vary seasonally, likely due to temperature and meteorological conditions, with P limitation often occurring in spring and winter, and N limitation being observed in summer and fall when the environmental conditions are more conducive to phytoplankton growth (Xu et al., 2010; Paerl et al., 2016).

It is widely accepted that there is no N limitation or P limitation if hypereutrophic lakes are nutrient replete (Xie et al., 2003; Xu et al., 2010). For example, when N and P water column concentrations were greater than 0.8 mg/L and 0.2 mg/L, respectively, the growth of the dominant bloom-forming cyanobacterium *Microcystis* spp. was not nutrient-limited in Lake Taihu (Xu et al., 2010). Therefore, reducing cyanobacterial blooms by controlling both N and P simultaneously rather than N alone or P alone may be the most effective management strategy in seriously eutrophic freshwater ecosystems (Conley et al., 2009; Paerl et al., 2016).

The production of microcystins (MC), the dominant cyanotoxin produced by freshwater cyanobacteria, is mainly regulated by environmental parameters, including water temperature, light intensity, pH, N, and P (Graham et al., 2004; Boopathi and Ki, 2014; Lee et al., 2015). Traditional approaches to manage cyanobacterial blooms and decrease MC production have focused on controlling P levels in water (Levy, 2017). However, recent studies suggest that increased N loadings are contributing to *Microcystis* blooms and MC release (Horst et al., 2014). Nitrogen was the primary factor limiting MC production (Yan et al., 2015) and different N forms likely influenced the concentration and composition of MC via changes in the cyanobacterial community structure (Monchamp et al., 2014). Therefore, it is important to understand which nutrient controls cyanobacterial blooms and impacts MC production in order to reduce the potential environmental risks posed by MC in aquatic ecosystems. In addition, extensive studies have shown that *in situ* nutrient enrichment bioassays are useful methods to investigate the influence of nutrients on phytoplankton growth (Elser et al., 1990; Xu et al., 2010, 2013, 2015; Deng et al., 2014; Steinman et al., 2016). However, their utility to examine the effect of nutrients on toxin production has received less attention, even though the global distribution of harmful cyanobacterial blooms and associated hepatotoxins have been well documented in many countries and territories (Harke et al., 2016).

Spring Lake is a eutrophic lake located in west Michigan and has been characterized by very high total P (TP) concentrations. An alum (aluminum sulfate) treatment with a concentration of 10–20 mg Al/L was applied in the surface water of Spring Lake in autumn 2005 to reduce internal P loading from the sediments (Steinman and Ogdahl, 2008). The alum treatment remained extremely effective both 8 months and 5 years after the application, based on the reduction of both TP concentrations in the surface water and measured P release rates from the sediment (Steinman and Ogdahl, 2012). However, TP concentrations near the bottom of the water column showed an unexpected increase and reached as high as 1.005 mg/L in 2016 (Steinman et al., 2018), suggesting that internal loading may be increasing and alum treatment is possibly losing its efficacy, some 11 years following application. Thus, it is necessary to keep track of Spring Lake nutrient dynamics, especially in summer from June to August, when planktonic algae are abundant and dominant in the surface water.

Given the concerns over frequent cyanobacterial blooms and possible toxin production in this heavily used lake, an *in situ* nutrient addition bioassay was conducted in Spring Lake to evaluate the influence of N and P, both separately and in concert, on phytoplankton growth and MC production. In addition, a survey of Spring Lake was performed after the bioassay to examine the distribution and variation in nutrients and MC at two different sites during the summer. We hypothesized the following: (1) N is now the primary factor controlling phytoplankton growth in Spring Lake given the recent high P concentrations, and (2) the production of MC is mainly regulated by N, via its influence on the MC-producing cyanobactera.

## 2. Materials and methods

### 2.1. Study area

Spring Lake (43.0770° N, 86.1970° W) is located in western Michigan and connects to the Grand River, which flows east into Lake Michigan (Fig. 1). This drowned river-mouth lake has a surface area of 5.25 $km^2$, with a mean depth of 6 m and a maximum depth of 13 m. Hydraulic retention time in this lake is approximately 330 days in the summer and 105 days in the winter. Mean annual precipitation is 87.6 cm as rain and 200.1 cm as snow; temperatures vary from July mean high of 26.7 °C to January mean low of −6.9 °C. The Spring Lake watershed covers 134 $km^2$, with major land use/land cover of forest (41%), residential (28%), cropland/pasture (11%), and 6% wetland (Steinman et al., 2015). The lake's shoreline is densely populated with primary residences (Steinman and Ogdahl, 2008) and annually abundant cyanobacteria are observed during the summer (Xie et al., 2012). To evaluate the environmental conditions and investigate the distribution of MC in Spring Lake, we conducted a survey at two sites (Fig. 1), including surface and near-bottom water column samples every two weeks in July and August 2017. Our experimental bioassay station is located along the western shoreline of Spring Lake (Fig. 1).

### 2.2. Experimental design

A bioassay experiment was performed to determine the influence of nutrients on phytoplankton growth and MC production during the summer of 2017 in Spring Lake. Twelve carboys, each filled with 10-L (L) of unfiltered surface lake water collected near the incubation location, were connected to a square polyvinyl chloride (PVC) frame. The PVC frame was fixed to metal stakes, driven into the bottom of the lake and the carboys were suspended just below the water surface to mimic the ambient water temperature and natural light conditions (Fig. S1). Carboys were amended with 4 nutrient treatments, each replicated 3 times: N as $KNO_3$ (10 × ambient concentration); P as $KH_2PO_4$ (10 × ambient concentration), N + P (each 10 × ambient concentrations), and control (no nutrient amendments). Placement of carboys on the PVC frame was determined randomly. Incubation time lasted 7 days from 23 June to 30 June 2017, which is considered sufficient to detect a nutrient effect but short enough to avoid possible containment artifacts, such as nutrient limitation (Steinman et al., 2016). During the experimental period, we checked the carboys every 2–3 days to make sure they remained intact.

Initial water samples were collected prior to nutrient additions for the determination of chlorophyll-*a* (Chl-*a*) (500 mL volume) and MC (250–500 mL according to the amounts of algae). Carboys were gently inverted 30 times to ensure the water was well-mixed immediately after nutrients were added. Carboys were

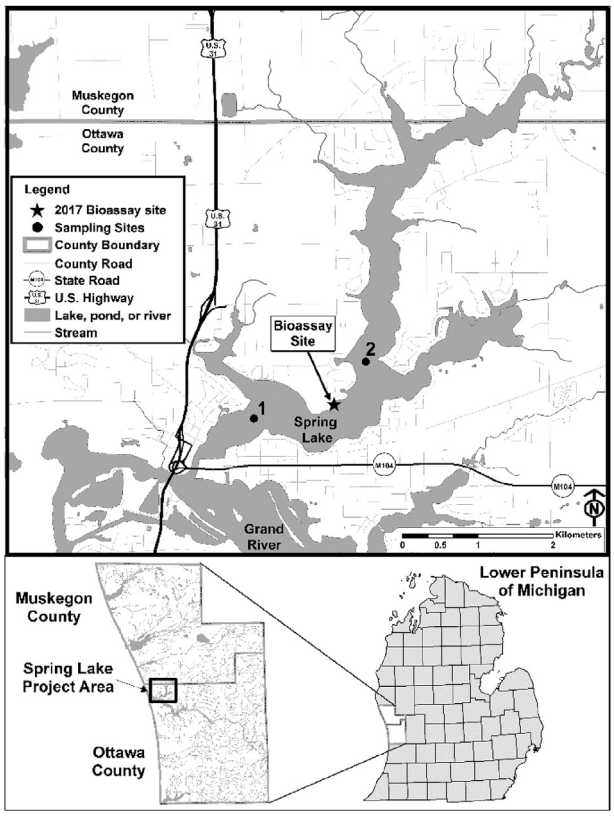


**Fig. 1.** Top: Map of Spring Lake showing the location of bioassay and sampling sites. Bottom: Location of Muskegon and Ottawa Counties in west Michigan's (USA) lower peninsula. Sites 1 and 2 represent MC monitoring stations during Spring Lake's cyanobacterial blooms in July and August 2017.

subsampled again after mixing to measure the initial concentrations of nitrate ($NO_3^-$) and soluble reactive P (SRP) by filtering 40 mL of water (0.45 μm cartridge filters) into two 20-mL scintillation vials, one for each nutrient. Vials were stored on ice until transported back to the lab. SRP was stored at 4 °C until analyzed, whereas $NO_3^-$ was frozen until analyzed. At the end of the experimental period, carboys were again gently inverted 30 times and subsampled for chemical and biological analysis as described above.

Physical parameters, including water temperature (WT), pH, dissolved oxygen (DO), turbidity, and electrical conductivity (EC) were measured in the water column at the center of the PVC square at the start, and for each carboy at the end of the bioassay period, using a Yellow Springs Instruments (YSI) 6600 multi-sensor sonde.

### 2.3. Field survey

One-meter integrated water samples were collected from 2 sites including the surface and bottom samples on July 17, August 1, August 16, and August 30, 2017. At each site, WT, pH, DO, turbidity, and EC were measured at the surface and near-bottom of the water column using a YSI 6600 sonde. Water samples were stored in a 5-L glass bottle and transported to the laboratory on ice. The environmental parameters analyzed in this study were the same as with the bioassay experiment.

### 2.4. Laboratory analysis

Chemical analyses of water samples included total Kjeldahl N (TKN), $NO_3^-$, ammonia ($NH_4^+$), TP, and SRP. TKN, $NH_4^+$, TP, and SRP were analyzed on a Bran + Luebbe Autoanalyzer (SEAL Analytical, Mequon, US). $NO_3^-$ was analyzed by ion chromatography on a Dionex ICS-2100 (APHA, 1998). Chl-*a* samples were filtered on glass-fiber filters (GF/F, Whatman, UK), frozen for 24 h and concentrations determined spectrophotometrically after extraction in 90% (v/v) acetone/water solution on a Shimadzu UV-1601 spectrophotometer (APHA, 1994).

### 2.5. MC analysis

Dissolved MC were measured using the enzyme-linked immunosorbent assay (ELISA) test kit according to the manufacturer's instructions. The limit of detection (LOD) of the EnviroLogix Microcystin Tube Kit (Portland, ME, USA) is 0.05 μg/L. Results below the detection limit were considered as one-half of the LOD for statistical analysis.

To further confirm the occurrence of intracellular MC and identify different MC analogues, we performed solid phase extraction to concentrate MC and identified them using high performance liquid chromatography (HPLC). The freeze-dried GF/F filter was extracted with 5% (*v*/*v*) acetic acid by ultra-sonication for 5 min, and the suspension was centrifuged at 10,000 r/min (15 min at 4 °C). This procedure was repeated three times, and the supernatants were collected for the next step. The HLB (Hydrophilic-Lipophilic-Balanced) cartridges (200 mg, Oasis®, Waters, MA, USA) were previously activated with 5 mL of methanol and balanced with 5 mL of distilled water. Afterward, the supernatant was applied at a flow rate of 1 mL/min, with a further washing step of 15 mL 5% (*v*/*v*) methanol, and a final elution with 10 mL 100% methanol. Finally, the eluent was dried under $N_2$ gas prior to reconstitution in 1.0 mL of methanol. A 500-μL subsample was prepared for HPLC analysis.

Quantification of MCs was performed on an Agilent 1200 series HPLC system with a DAD (Diode Array Detector) (Agilent, CA, USA) equipped with an ODS (octadecylsilyl) column (Agilent Eclipse XDB-C18, 5 μm, 4.6 mm × 150 mm) as described previously (Su et al., 2015). Standards for MCs were obtained from Sigma-Aldrich (München, Germany). The intracellular concentration of total MC (TMC) is the sum of three MC analogues, including MC-LR, MC-RR, and MC-YR (L, R, and Y are abbreviations of leucine, arginine, and tyrosine, respectively).

### 2.6. Statistical analysis

The differences in pH, DO, turbidity, and EC among the various bioassay treatments were analyzed by one-way analysis of variance (ANOVA). Post-hoc multiple comparisons of treatment means were performed by Tukey's least significant difference procedure. The effects of nutrients on phytoplankton biomass (Chl-*a*) and MC concentrations among treatments were carried out with 2-factor ANOVA (Tank and Dodds, 2003). We used Fisher's least significant difference (LSD) method to examine pairwise contrasts if the overall ANOVA model was statistically significant. The Kruskal-Wallis nonparametric test was conducted to determine the differences in environmental variables between the sampling stations. Statistical analyses were performed using the SPSS 22.0 and the level of significance was set at $P < 0.05$. Results are shown as mean ± standard deviation (SD).

## 3. Results

### 3.1. Bioassay pH, DO, turbidity, and EC results

Compared with the initial values, pH, DO, and turbidity values all increased significantly ($P < 0.05$), while EC decreased

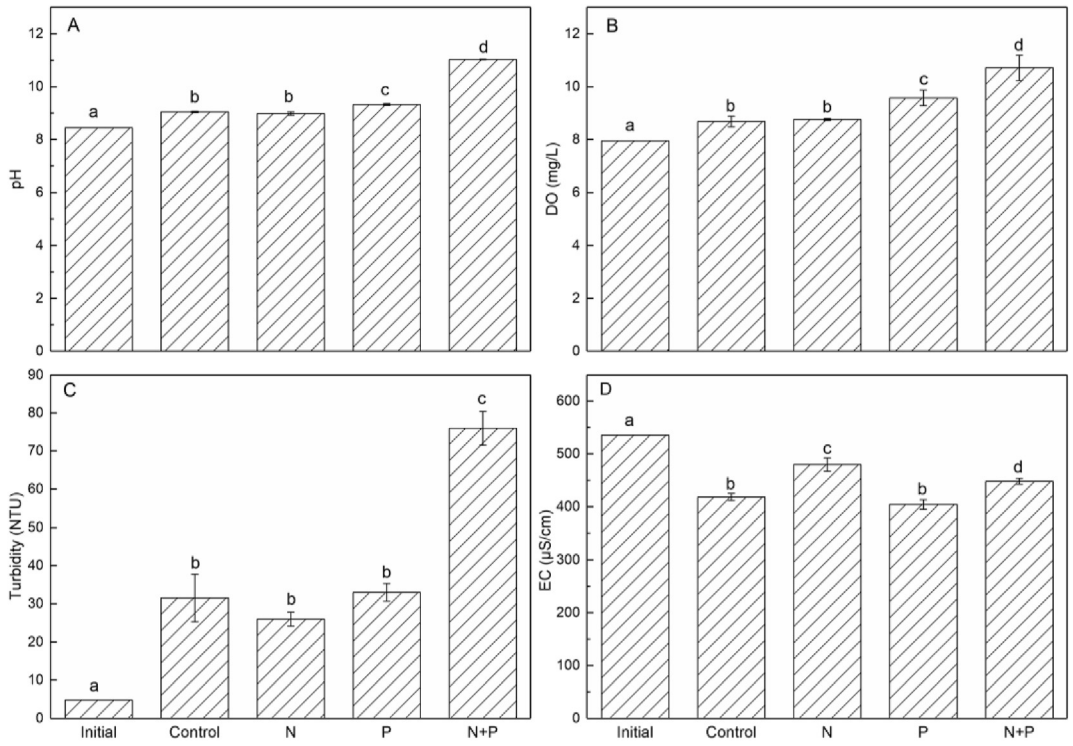


**Fig. 2.** Responses of (A) pH, (B) DO, (C) turbidity, and (D) EC after 7-day bioassay incubation. Initial water samples for bioassay were collected from the lake surface water at the incubation location. Mean values are shown and error bars represent standard deviation (SD) of triplicates. Differences among treatments are shown with different letters based on ANOVA post-hoc tests. Note different y-axis scales.

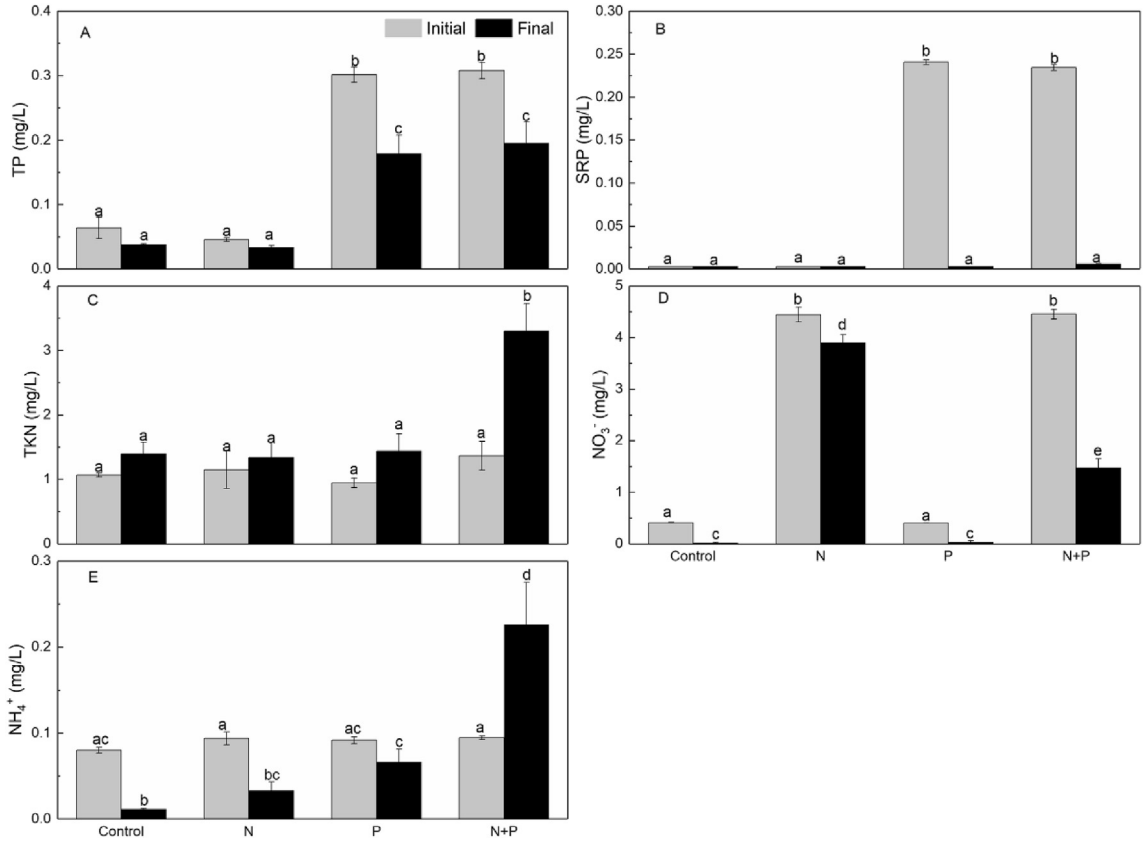


**Fig. 3.** The variations in (A) TP, (B) SRP, (C) TKN, (D) nitrate ($NO_3^-$), and (E) ammonium ($NH_4^+$) among various treatments at the start and the end of bioassays. Incubation time was 7 days. Mean values are shown and error bars represent standard deviation (SD) of triplicates. Differences between treatments are shown with different letters based on ANOVA post-hoc tests. Note different y-axis scales.

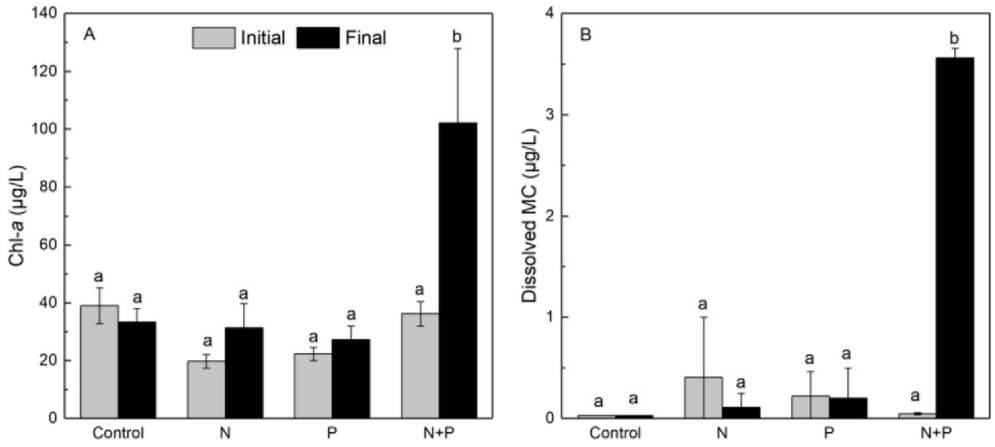


**Fig. 4.** (A) Chl-*a* and (B) dissolved MC responses among various treatments at the start and the end of bioassays. Incubation time was 7 days. Mean values are shown and error bars represent standard deviation (SD) of triplicate replicates. Differences among treatments are shown with different letters based on ANOVA post-hoc tests. Note different y-axis scales.

significantly ($P < 0.05$) at the end of the bioassay experiment (Fig. 2). In addition, pH increased significantly ($P < 0.05$) in the P and N + P treatments compared with the control and N treatment; mean ambient pH was 8.45 and increased to 11.04 in the N + P treatment. DO concentrations increased in a similar fashion to pH, reaching 10.72 mg/L in the N + P treatment (Fig. 2). In contrast, turbidity increased significantly ($P < 0.05$) only in the N + P treatment compared with the other treatments, more than doubling in concentration (Fig. 2). EC decreased significantly ($P < 0.05$) in the control and P treatment compared with the N and N + P treatments.

### 3.2. Bioassay nutrient concentration responses

Ambient concentrations of TP and SRP were 0.064 mg/L and below detection (<0.005 mg/L), respectively, in Spring Lake. The treatment spike increased TP and SRP concentrations to 0.30 mg/L and 0.24 mg/L, respectively (Fig. 3A and B). The final concentrations of both TP and SRP decreased in all treatments, but the declines were statistically significant ($P < 0.05$) only in the P and N + P treatments (Fig. 3A and B). The final TP concentrations in the P and N + P treatments were still greater than the final concentrations in the control and N treatments, while the final SRP concentrations were below detection in all treatments (Fig. 3A and B).

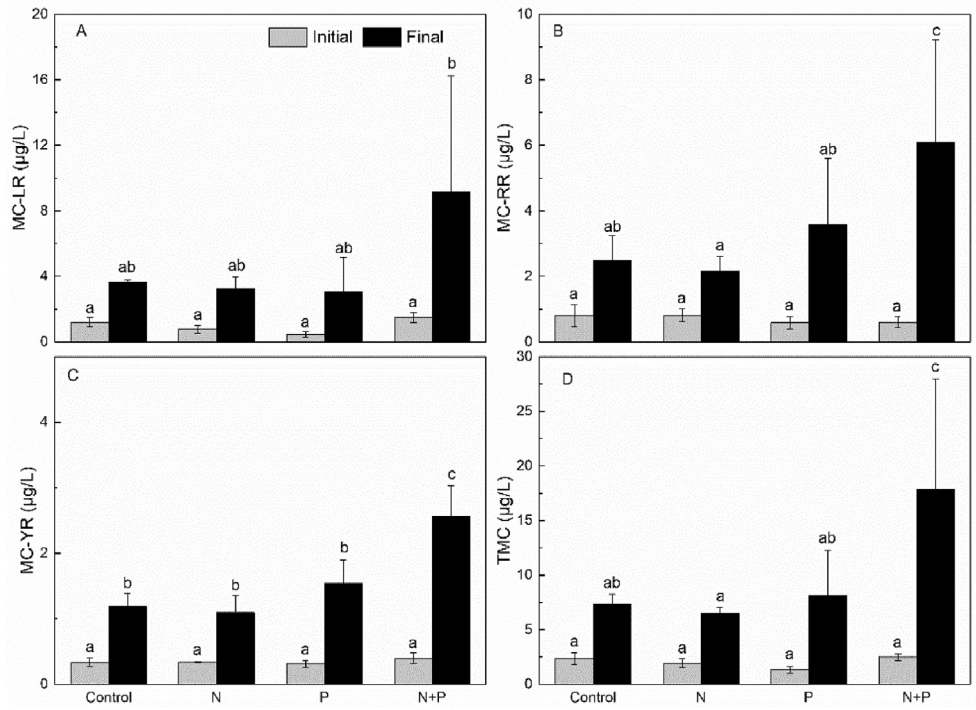


**Fig. 5.** Intracellular concentrations of (A) MC-LR, (B) MC-RR, (C) MC-YR, and (D) TMC from experimental treatments at the start and the end of bioassays. Incubation time was 7 days. Mean values are shown and error bars represent standard deviation (SD) of triplicates. Differences among treatments are shown with different letters based on ANOVA post-hoc tests. Note different y-axis scales.

Mean TKN concentrations were 1.066 mg/L at the start of the bioassay and increased in all treatments but the only statistically significant increase was in the N + P treatment, where the mean increase was to 3.304 mg/L (Fig. 3C). Mean ambient $NO_3^-$ concentration was 0.407 mg/L and the treatment spike increased it to 4.45 mg/L (Fig. 3D). $NO_3^-$ concentration decreased significantly ($P < 0.05$) in all treatments, but the decline was modest (accounting for 12.4%) in the N treatment compared to the N + P treatment (accounting for 67%; Fig. 3D), suggesting that $NO_3^-$ drawdown by phytoplankton was dependent on the presence of a P supply. $NH_4^+$ composed only 7.5% of the TKN in the initial incubation water, and concentrations declined over time in all treatments except N + P; similar to TKN, $NH_4^+$ increased significantly ($P < 0.05$) by the end of the incubation compared to other treatments (Fig. 3E).

### 3.3. Bioassay Chl-a and dissolved MC responses

Initial mean Chl-*a* concentrations ranged from 19.79 μg/L to 39.01 μg/L in the bioassays (Fig. 4A). The only treatment that resulted in a statistically significant increase ($P < 0.05$) was N + P (Fig. 4A). Generally similar results also were observed for dissolved MC as measured by ELISA; mean MC concentrations were less than 0.5 μg/L in all treatments at the start and end of the incubations with the exception of N + P on day 7, when the average concentration reached as high as 3.57 μg/L (Fig. 4B).

### 3.4. Bioassay intracellular MC responses determined by HPLC

The intracellular concentrations of MC-LR, MC-RR, MC-YR, and TMC responded very similarly in all treatments; only the N + P treatment resulted in significant increases by the end of experiment with the average values of 9.16 μg/L, 6.10 μg/L, 2.57 μg/L, and 17.82 μg/L, respectively (Fig. 5A–D). During the study period, MC-LR and MC-RR were dominant variants, followed by MC-YR. The relative abundances of MC-LR, MC-RR, and MC-YR changed very little over the course of the experiment (1% or less).

In addition, we evaluated variations in MC/Chl-*a*, which were calculated as the MC concentration normalized by the Chl-*a* concentration for each carboy. For dissolved MC, a significant increase ($P < 0.05$) was observed only in the N + P treatment (Fig. 6A). There were no significant differences ($P > 0.05$) among various treatments for MC-LR/Chl-*a* (Fig. 6B). For MC-RR/Chl-*a*, MC-YR/Chl-*a*, and TMC/Chl-*a* (Fig. 6C–E), the only significant increase ($P < 0.05$) was observed in the P treatment, which was different from the responses exhibited by MC concentrations (Fig. 5).

### 3.5. Field survey in the summer

There were obvious differences in environmental variables between the surface and bottom water samples during the summer field survey. WT, pH, DO, and Chl-*a* were significantly lower

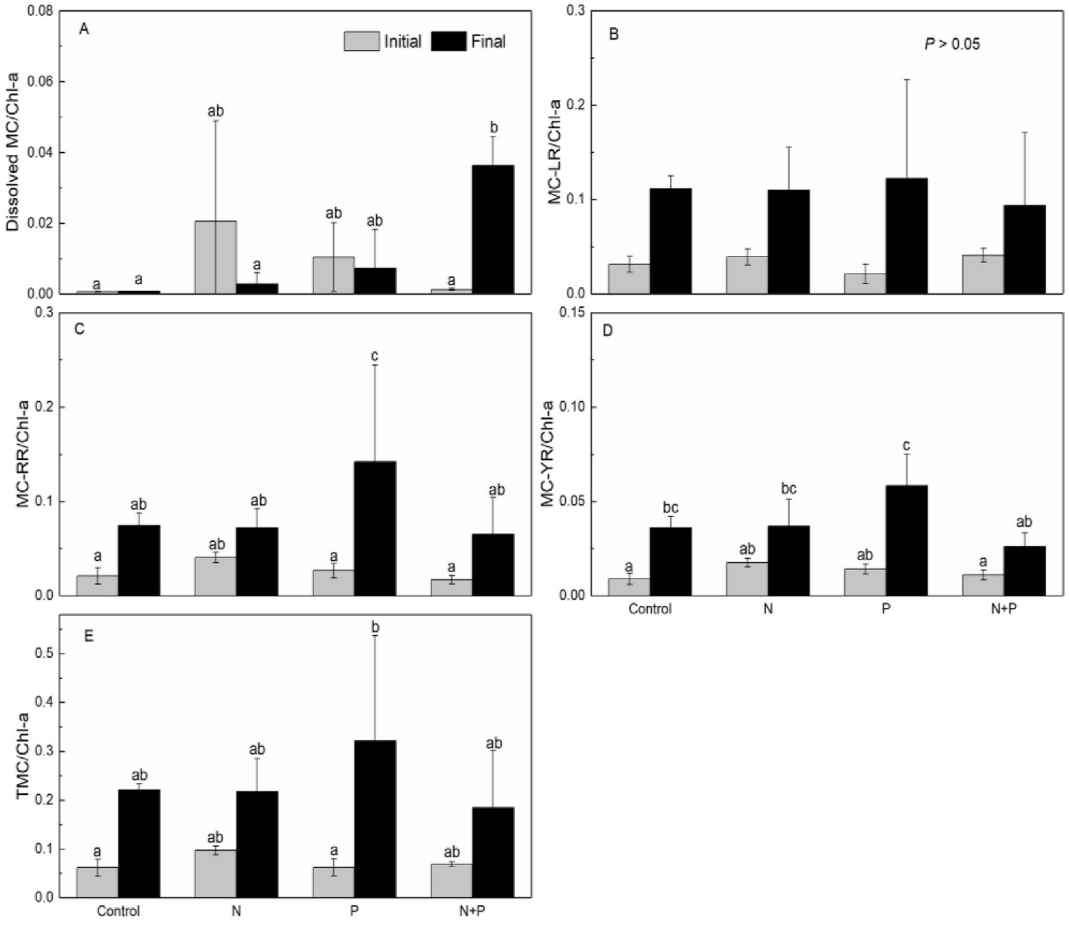


**Fig. 6.** Variations in (A) dissolved MC/Chl-*a*, (B) MC-LR/Chl-*a*, (C) MC-RR/Chl-*a*, (D) MC-YR/Chl-*a*, and (E) TMC/Chl-*a* from experimental treatments at the start and the end of bioassays. Incubation time was 7 days. Mean values are shown and error bars represent standard deviation (SD) of triplicates. Differences among treatments are shown with different letters based on ANOVA post-hoc tests. Note different y-axis scales.

**Table 1**
General environmental conditions for the surface and bottom water samples in these two sampling sites for July and August in 2017. Values are shown as mean ± SD. WT, EC, and dMC indicate water temperature, electrical conductivity, and dissolved MC, respectively. Statistically significant differences at the 0.01 level and 0.05 level are indicated with ** and *, respectively.

| Parameters | 1 top | 1 bottom | 2 top | 2 bottom | *P* value |
|---|---|---|---|---|---|
| WT (°C) | 24.9 ± 1.5 | 21.0 ± 2.1 | 25.4 ± 1.4 | 19.9 ± 2.3 | 0.021* |
| pH | 8.80 ± 0.1 | 8.37 ± 0.45 | 8.87 ± 0.19 | 7.93 ± 0.24 | 0.022* |
| DO (mg/L) | 10.33 ± 0.97 | 2.33 ± 2.45 | 11.68 ± 1.25 | 0.42 ± 0.13 | 0.006** |
| Cond (μS/cm) | 520 ± 12 | 577 ± 50 | 506 ± 13 | 557 ± 15 | 0.019* |
| Turbidity (NTU) | 9.4 ± 3.9 | 16.7 ± 16.6 | 11.0 ± 5.5 | 4.6 ± 0.5 | 0.049* |
| TP (mg/L) | 0.04 ± 0.02 | 0.06 ± 0.04 | 0.06 ± 0.02 | 0.06 ± 0.01 | 0.501 |
| SRP (mg/L) | 0.003 ± 0.001 | 0.018 ± 0.030 | 0.004 ± 0.001 | 0.015 ± 0.011 | 0.291 |
| TKN (mg/L) | 1.10 ± 0.11 | 1.07 ± 0.06 | 1.00 ± 0.11 | 0.88 ± 0.14 | 0.119 |
| $NO_3^-$ (mg/L) | 0.04 ± 0.06 | 0.16 ± 0.10 | 0.08 ± 0.06 | 0.21 ± 0.06 | 0.024* |
| $NH_4^+$ (mg/L) | 0.01 ± 0.01 | 0.10 ± 0.15 | 0.02 ± 0.01 | 0.20 ± 0.12 | 0.016* |
| Chl-*a* (μg/L) | 58.3 ± 14.8 | 39.4 ± 9.3 | 63.5 ± 17.1 | 15.7 ± 9.3 | 0.015* |
| dMC (μg/L) | 0.54 ± 0.98 | 0.22 ± 0.32 | 0.22 ± 0.26 | 0.49 ± 0.51 | 0.828 |
| MC-LR (μg/L) | 0.33 ± 0.38 | 0.26 ± 0.32 | 0.27 ± 0.32 | 0.15 ± 0.18 | 0.803 |
| MC-RR (μg/L) | 1.83 ± 1.51 | 1.50 ± 0.33 | 2.11 ± 0.98 | 1.91 ± 0.85 | 0.104 |
| MC-YR (μg/L) | 0.93 ± 0.31 | 0.55 ± 0.10 | 0.96 ± 0.40 | 0.48 ± 0.38 | 0.878 |
| Intracellular TMC (μg/L) | 3.08 ± 1.98 | 2.31 ± 0.63 | 3.35 ± 1.21 | 2.54 ± 1.09 | 0.724 |

($P < 0.05$) in bottom samples than in the surface water (Table 1). In contrast, $NO_3^-$ and $NH_4^+$ concentrations were significantly higher ($P < 0.05$) in bottom samples than in surface water. No significant differences were found in TP, SRP, and TKN between surface and bottom samples (Table 1). For intracellular MC, MC-RR was dominant with relative abundance of 60.1% in the surface water and 70.3% in the bottom, followed by MC-YR (31.6% and 21.6, respectively) and MC-LR (8.3% and 8.1%, respectively).

## 4. Discussion

Spring Lake, a eutrophic lake in west Michigan (USA), historically experienced very high summer TP concentrations in the photic zone (up to 300 μg/L), the majority of which was derived from internal P loading (Steinman et al., 2004), which resulted in very intense cyanobacterial blooms. An alum treatment conducted in 2005 helped reduce internal P loading by two orders of magnitude and summer TP concentrations declined as well (Steinman and Ogdahl, 2008), although lake-wide mean values remained relatively high at 30–50 μg/L (Steinman and Ogdahl, 2012), with a few readings in the deeper regions of the lake exceeding 900 μg/L (Steinman et al., 2018). Persistent high P concentrations may ultimately result in secondary N-limitation, whereby N limitation is an unnatural condition that has arisen due to excessive P loading (Havens, 1995). Our study examined this possibility; we addressed this data gap by conducting a 7-day nutrient addition experiment to evaluate the impact of nutrient limitation on phytoplankton growth and MC production in Spring Lake.

The development of cyanobacteria bloom is a complex process. Cyanobacterial blooms are influenced not only by nutrients, but also by meteorological and hydrological conditions (Yang et al., 2017). During our experimental period, the water temperature varied from 22.0 °C to 23.7 °C, which is within the optimal range for the growth of cyanobacteria, providing favorable conditions for the proliferation of algae and the production of MC (Boopathi and Ki, 2014). There were significant increases in DO and pH in the N + P treatment compared to the other treatments and the control. Increased photosynthetic activity in the N + P treatment is the most likely explanation for the increases in pH due to the uptake of carbon dioxide ($CO_2$) (Liu et al., 2011). In turn, higher pH conditions also provide a competitive advantage for some specific cyanobacteria because of their strong carbon-concentrating abilities compared to eukaryotic phytoplankton species (Yu et al., 2015). Blooms of phytoplankton also release high amounts of oxygen during the day because of photosynthesis but over longer time periods, hypoxic conditions tend to prevail in eutrophic lakes, which is attributed to the consumption of oxygen by cyanobacterial respiration and heterotrophic mineralization of abundant algal detritus (Paerl et al., 2006).

Our incubation period of 7 days may have gone too long given the very low concentrations of SRP at the end of the experiment. The significant increases in the concentrations of TKN and $NH_4^+$ in the N + P treatment at the end of bioassay were unexpected. It is possible that reduced forms of N contributed to the growth of non-$N_2$-fixing cyanobacteria instead of $N_2$-fixing taxa (Paerl et al., 2014). Besides, high $NH_4^+$ concentrations are required for the toxin-producing genes (Kuniyoshi et al., 2011).

Furthermore, our results showed that the combination of N and P greatly promoted the production of dissolved and particulate MC (Figs. 4 and 5). The final concentrations of intracellular and extracellular MC in N + P treatment were 3.57 μg/L and 17.82 μg/L, respectively. These concentrations fall below the US EPA threshold of 20 μg/L for high probability of acute health effects in recreational waters, but are above the low and moderate probabilities (https://www.epa.gov/nutrient-policy-data/guidelines-and-recommendations). MC-LR and -RR were the dominant variants; the concentrations of MC variants increased significantly between the initial and final times of the bioassay, while no changes were observed in the composition of MC and the proportions of each MC variant. The presence and dominance of MC-LR, which is considered to be the most toxic MC analogue (Gupta et al., 2003), is of potential concern to residents of the lake, as it may threaten human health and expose them to other environmental risks.

Results from our nutrient addition bioassay experiment indicated that phytoplankton growth and increased MC were mainly caused by the combined effects of N and P inputs. We did not analyze phytoplankton taxonomic composition, so it is unknown what group of algae were responsible for the increased biomass in the N + P treatment. The increased production of MC in the N + P treatment suggests cyanobacteria growth was responsible for the increased Chl-a. This is supported, indirectly, by the MC/Chl-a ratios (Fig. 5). Regardless of MC analogue, the ratios are no different in the N + P treatment compared to other treatments, suggesting the greater MC production in the N + P treatment was due to more MC-producing cells (i.e., cyanobacteria) than due to greater MC production per cell. Of course, this still does not indicate which cyanobacteria taxa were responsible for the MC production. Enrichment with only N or only P appeared to have no significant

influence on phytoplankton growth and MC production. This suggests a dual nutrient management strategy is needed for this lake, which heretofore has focused solely on P.

Our field survey revealed some significant reductions in TP (site 1: 0.06 vs 0.246; site 2: 0.06 vs 1.055; unit: mg/L) and SRP concentrations (site 1: 0.175 vs 0.018; site 2: 0.015 vs 0.930; unit: mg/L) in the bottom samples compared with values measured in 2016 (Steinman et al., 2018). However, the current SRP concentrations in the bottom waters at Sites 1 and 2 were still ten times higher than those in the water column, similar to the results found for $NO_3^-$ and $NH_4^+$ concentrations. These observations suggest that internal nutrient loading from lake sediment has returned to the water column and is contributing N and P in Spring Lake. Internal nutrient loading can play a key role in contributing to cyanobacterial blooms and potential MC production. According to a previous field study in 2006, the mean summer concentration of MC in Spring Lake was 0.057 μg/L (Xie et al., 2012) while in the current study, the average concentrations of dissolved and particulate MC were 0.38 μg/L and 3.22 μg/L in the surface and 0.36 μg/L and 2.42 μg/L in the bottom, respectively. High Chl-a concentrations were also observed in the surface water with a mean value of 60.9 μg/L. Thus, the significant increase in MC concentrations may be attributed to the toxin-producing algae being more abundant during the survey. More investigations to explore the dynamics of sediment nutrients may be helpful in explaining the high concentrations of N and P in the bottom waters.

## 5. Conclusion

The current study demonstrated that the enrichment with either N or P addition alone does not significantly increase phytoplankton growth and also did not lead to increased production of MC in these treatments. However, enrichment with a combination of both N and P can lead to a marked increase in biomass in the Spring Lake phytoplankton community, as measured by chlorophyll *a*, and an associated increase in MC concentration. Recently, a strategy of reducing input loading of both N and P has been recommended as an efficient method to control cyanobacterial blooms in freshwater systems (Paerl et al., 2016). The results of this study contribute to the notion that phytoplankton and MC are limited by both N and P, which underpins the importance of a dual nutrient reduction strategy in eutrophic lakes.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.06.047.

## References

APHA, 1994. Water Environment Federation (1998) Standard Methods for the Examination of Water and Wastewater. American Public Health Association, Washington, DC.
APHA, 1998. Standard methods for the evaluation of water and wastewater. In: 20th American Public Health Association, Washington, DC.
Boopathi, T., Ki, J.-S., 2014. Impact of environmental factors on the regulation of cyanotoxin production. Toxins 6, 1951–1978.
Bormans, M., Maršálek, B., Jančula, D., 2016. Controlling internal phosphorus loading in lakes by physical methods to reduce cyanobacterial blooms: A review. Aquat. Ecol. 50, 407–422.
Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Karl, E., Karl, E., Lancelot, C., Gene, E., Gene, E., 2009. Controlling eutrophication: Nitrogen and phosphorus. Science 123, 1014–1015.
Deng, J., Qin, B., Paerl, H.W., Zhang, Y., Wu, P., Ma, J., Chen, Y., 2014. Effects of nutrients, temperature and their interactions on spring phytoplankton community succession in Lake Taihu, China. PLoS One 9, e113960.
Elser, J.J., Marzolf, E.R., Goldman, C.R., 1990. Phosphorus and nitrogen limitation of phytoplankton growth in the freshwaters of North America: A review and critique of experimental enrichments. Can. J. Fish. Aquat. Sci. 47, 1468–1477.
Graham, J.L., Jones, J.R., Jones, S.B., Downing, J.A., Clevenger, T.E., 2004. Environmental factors influencing microcystin distribution and concentration in the Midwestern United States. Water Res. 38, 4395–4404.
Gupta, N., Pant, S.C., Vijayaraghavan, R., Rao, P.V.L., 2003. Comparative toxicity evaluation of cyanobacterial cyclic peptide toxin microcystin variants (LR, RR, YR) in mice. Toxicology 188, 285–296.
Harke, M.J., Steffen, M.M., Gobler, C.J., Otten, T.G., Wilhelm, S.W., Wood, S.A., Paerl, H.W., 2016. A review of the global ecology, genomics, and biogeography of the toxic cyanobacterium, Microcystis spp. Harmful Algae 54, 4–20.
Havens, K.E., 1995. Secondary nitrogen limitation in a subtropical lake impacted by non-point source agricultural pollution. Environ. Pollut. 89, 241–246.
Horst, G.P., Sarnelle, O., White, J.D., Hamilton, S.K., Kaul, R.B., Bressie, J.D., 2014. Nitrogen availability increases the toxin quota of a harmful cyanobacterium, Microcystis aeruginosa. Water Res. 54, 188–198.
Kuniyoshi, T.M., Gonzalez, A., Lopez-Gomollon, S., Valladares, A., Bes, M.T., Fillat, M.F., Peleato, M.L., 2011. 2-oxoglutarate enhances NtcA binding activity to promoter regions of the microcystin synthesis gene cluster. FEBS Lett. 585, 3921–3926.
Lürling, M., Mackay, E., Reitzel, K., Spears, B.M., 2016. Editorial – a critical perspective on geo-engineering for eutrophication management in lakes. Water Res. 97, 1–10.
Lee, T.A., Rollwagen-Bollens, G., Bollens, S.M., Faber-Hammond, J.J., 2015. Environmental influence on cyanobacteria abundance and microcystin toxin production in a shallow temperate lake. Ecotoxicol. Environ. Saf. 114, 318–325.
Levy, S., 2017. Microcystis rising: Why phosphorus reduction isn't enough to stop CyanoHABs. Environ. Health Perspect. 125.
Liu, X., Lu, X., Chen, Y., 2011. The effects of temperature and nutrient ratios on Microcystis blooms in Lake Taihu, China: An 11-year investigation. Harmful Algae 10, 337–343.
Monchamp, M.E., Pick, F.R., Beisner, B.E., Maranger, R., 2014. Nitrogen forms influence microcystin concentration and composition via changes in cyanobacterial community structure. PLoS One 9, e85573.
Paerl, H.W., Scott, J.T., McCarthy, M.J., Newell, S.E., Gardner, W.S., Havens, K.E., Hoffman, D.K., Wilhelm, S.W., Wurtsbaugh, W.A., 2016. It takes two to tango: When and where dual nutrient (N & P) reductions are needed to protect lakes and downstream ecosystems. Environ. Sci. Technol. 50, 10805–10813.
Paerl, H.W., Valdes, L.M., Peierls, B.L., Adolf, J.E., Harding, L.J.W., 2006. Anthropogenic and climatic influences on the eutrophication of large estuarine ecosystems. Limnol. Oceanogr. 51, 448–462.
Paerl, H.W., Xu, H., Hall, N.S., Rossignol, K.L., Joyner, A.R., Zhu, G., Qin, B., 2015. Nutrient limitation dynamics examined on a multi-annual scale in Lake Taihu, China: Implications for controlling eutrophication and harmful algal blooms. J. Freshw. Ecol. 30, 5–24.
Paerl, H.W., Xu, H., Hall, N.S., Zhu, G., Qin, B., Wu, Y., Rossignol, K.L., Dong, L., McCarthy, M.J., Joyner, A.R., 2014. Controlling cyanobacterial blooms in hypertrophic Lake Taihu, China: Will nitrogen reductions cause replacement of non-N2 fixing by N2 fixing taxa? PLoS One 9, e113123.
Smith, V.H., Wood, S.A., McBride, C.G., Atalah, J., Hamilton, D.P., Abell, J., 2016. Phosphorus and nitrogen loading restraints are essential for successful eutrophication control of Lake Rotorua, New Zealand. Inland Waters 6, 273–283.
Steinman, A., Abdimalik, M., Ogdahl, M.E., Oudsema, M., 2016. Understanding planktonic vs. benthic algal response to manipulation of nutrients and light in a eutrophic lake. Lake Reservoir Manag. 32, 402–409.
Steinman, A., Rediske, R., Reddy, K., 2004. The reduction of internal phosphorus loading using alum in Spring Lake, Michigan. J. Environ. Qual. 33, 2040–2048.
Steinman, A.D., Hassett, M.C., Oudsema, M., Rediske, R., 2018. Alum efficacy 11 years following treatment: Phosphorus and macroinvertebrates. Lake Reservoir

Manag. 34, 167–181.
Steinman, A.D., Isely, E.S., Thompson, K., 2015. Stormwater runoff to an impaired lake: Impacts and solutions. Environ. Monit. Assess. 187, 549.
Steinman, A.D., Ogdahl, M., 2008. Ecological effects after an alum treatment in Spring Lake, Michigan. J. Environ. Qual. 37, 22–29.
Steinman, A.D., Ogdahl, M.E., 2012. Macroinvertebrate response and internal phosphorus loading in a Michigan lake after alum treatment. J. Environ. Qual. 41, 1540–1548.
Su, X., Xue, Q., Steinman, A., Zhao, Y., Xie, L., 2015. Spatiotemporal dynamics of microcystin variants and relationships with environmental parameters in Lake Taihu, China. Toxins 7, 3224.
Tank, J.L., Dodds, W.K., 2003. Nutrient limitation of epilithic and epixylic biofilms in ten North American streams. Freshw. Biol. 48, 1031–1049.
Xie, L., Rediske, R.R., Hong, Y., O'Keefe, J., Gillett, N.D., Dyble, J., Steinman, A.D., 2012. The role of environmental parameters in the structure of phytoplankton assemblages and cyanobacteria toxins in two hypereutrophic lakes. Hydrobiologia 691, 255–268.
Xie, L.Q., Xie, P., Tang, H.J., 2003. Enhancement of dissolved phosphorus release from sediment to lake water by Microcystis blooms—an enclosure experiment in a hyper-eutrophic, subtropical Chinese lake. Environ. Pollut. 122, 391–399.
Xu, H., Paerl, H.W., Qin, B., Zhu, G., Gaoa, G., 2010. Nitrogen and phosphorus inputs control phytoplankton growth in eutrophic Lake Taihu, China. Limnol. Oceanogr. 55, 420–432.
Xu, H., Paerl, H.W., Qin, B., Zhu, G., Hall, N.S., Wu, Y., 2015. Determining critical nutrient thresholds needed to control harmful cyanobacterial blooms in eutrophic Lake Taihu, China. Environ. Sci. Technol. 49, 1051–1059.
Xu, H., Paerl, H.W., Zhu, G., Qin, B., Hall, N.S., Zhu, M., 2017. Long-term nutrient trends and harmful cyanobacterial bloom potential in hypertrophic Lake Taihu, China. Hydrobiologia 787, 229–242.
Xu, H., Zhu, G., Qin, B., Paerl, H.W., 2013. Growth response of Microcystis spp. to iron enrichment in different regions of Lake Taihu, China. Hydrobiologia 700, 187–202.
Yan, Y., Dai, R., Liu, Y., Gao, J., Wu, X., 2015. Comparative effects of inorganic and organic nitrogen on the growth and microcystin production of Microcystis aeruginosa. World J. Microbiol. Biotechnol. 31, 763–772.
Yang, J.R., Lv, H., Isabwe, A., Liu, L., Yu, X., Chen, H., Yang, J., 2017. Disturbance-induced phytoplankton regime shifts and recovery of cyanobacteria dominance in two subtropical reservoirs. Water Res. 120, 52–63.
Yu, L., Kong, F., Shi, X., Yang, Z., Zhang, M., Yu, Y., 2015. Effects of elevated CO2 on dynamics of microcystin-producing and non-microcystin-producing strains during Microcystis blooms. J. Environ. Sci. 27, 251–258.