An analysis of the cyanobacterium *Gloeotrichia* in Silver Lake

FINAL REPORT

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Introduction

Globally, humans are driving lakes into eutrophication at an accelerated pace, threatening both the ecology of the lakes and the economies they support (Dodds et al. 2008, Keeler et al. 2012). Communities whose economies are centered on waterfront development and recreation, activities which account for $12.5 billion annually in Michigan (Austin and Steinman 2015), are particularly affected by eutrophication and its associated undesirable water quality. Nuisance algal blooms, commonly formed by cyanobacteria, are a typical symptom of eutrophication that elicit a range of concerns, including degraded aesthetics, fish kills due to depleted dissolved oxygen (Paerl 1988), and potential human health risks from exposure to toxins.

Located in Oceana County, Michigan, Silver Lake is a 672-acre lake situated along the eastern shore of Lake Michigan. A recent nutrient budget study revealed that Silver Lake is eutrophic based on average total phosphorus and total nitrogen concentrations (Brennan et al. 2015). Septic systems were identified as the primary source of phosphorus, but not nitrogen, to the lake (Brennan et al. 2015). Nuisance algal blooms and fish (common carp) kills in recent years have raised concerns among lake property owners, as Silver Lake is a popular tourist destination and economic engine in the area. Although the year-round population is less than 1,000 residents, the summer population is typically over 20,000 (DeJong 2015). Silver Lake State Park, located directly on the shore of Silver Lake, received over 770,000 visitors during 2014 (DeJong 2015). The degradation of Silver Lake's water quality poses a threat to this important revenue source.

The nuisance cyanobacterium *Gloeotrichia* has been observed in Silver Lake over the past several years, at times forming dense surface scums. Like many cyanobacteria species, *Gloeotrichia* forms akinetes, or dormant cells, that settle onto lake sediments where they can remain viable for years to decades (Livingstone and Jaworski 1980, Wood et al. 2009). The akinetes germinate during spring, as temperatures warm and light increases, and form colonies. When mature, these colonies can detach from the sediment and float to the surface, where they disperse throughout the water column and can be seen by the naked eye. *Gloeotrichia* colonies may be concentrated by wind and water currents on downwind shores, only to be dispersed again when the wind shifts (Wynne at al. 2011). *Gloeotrichia* can form heterocysts, allowing it to fix atmospheric nitrogen, providing it a competitive advantage over other algal species in lakes where nitrogen limits algal growth. Furthermore, *Gloeotrichia* can transfer a substantial amount of phosphorus from the sediment into the water column during recruitment (Istvanovics et al. 1993, Pettersson et al. 1993). *Gloeotrichia* also has been found to significantly increase growth rates of other phytoplankton species, including *Microcystis*, by putatively releasing dissolved phosphorus and nitrogen into the water column (Carey and Rengefors 2010, Carey et al. 2014a, Carey et al. 2014b).

Not only does *Gloeotrichia* form nuisance blooms, it also may pose human health and ecological concerns through its ability to form cyanotoxins. Even at low densities, *Gloeotrichia* has been associated with skin rashes and irritations and thus has the potential to be a recreational nuisance (Backer 2002, Carey et al. 2012). More concerning is the ability of *G. echinulata* to produce the hepatotoxin microcystin-LR (MC-LR) at a level potentially threatening to human health, if bloom conditions occur (Carey et al. 2007, Carey et al. 2012). Exposure to low levels of MC-LR can be disruptive to food webs by inhibiting growth of algae (Pietsch et al. 2001) and macrophytes (Pflugmacher 2002), and has been linked to shifts in zooplankton community structure (Kotak et al. 1996).

We studied the seasonal phenology of the phytoplankton community and cyanotoxin production in Silver Lake, with a particular focus on the cyanobacterium *Gloeotrichia*, through a combination of field surveys and controlled experiments. Specifically, we 1) monitored phytoplankton community composition, microcystin production, and general water quality parameters throughout the growing season (April-October) and 2) conducted nitrogen (N) and phosphorus (P) bioassay studies to identify if either nutrient limits phytoplankton growth in Silver Lake and how N and P affect community composition and cyanotoxin production. We related our results to environmental data to determine what factors are important in shaping the phytoplankton community and influencing toxin production. Our study complements a concurrent nutrient budget study funded by the Silver Lake Improvement Board (SLIB) and the U.S. Geological Survey (USGS) (Brennan et al. 2015). The nutrient source information generated from the SLIB study, conducted by the USGS and AWRI, will be used to guide the implementation of best management practices to alleviate the undesirable cyanobacterial blooms that have been plaguing Silver Lake.

Methods

Study Site

Silver Lake is a 672-acre natural inland lake located along the eastern shore of Lake Michigan in Oceana County, Michigan (Figure 1). A 2,000-acre sand dune formation along Silver Lake's west shore separates the lake from Lake Michigan. Silver Lake's maximum depth is 6.7 m (Groves et al. 2013). The Silver Lake watershed drains approximately 65 km^2 and the primary tributary to the lake is Hunter Creek. The lake discharges to Lake Michigan through Silver Creek. Land use in the watershed is dominated by forest (38%) and agriculture (26%). Although only 9% of the watershed is developed, the shoreline of the lake is mostly developed on the east and north shores. The remaining shoreline consists of mature forest and sand dunes, which are part of Silver Lake State Park (Brennan et al. 2015).

Bimonthly monitoring was conducted at two locations (Middle, South) in Silver Lake that were chosen to align with the concurrent Silver Lake nutrient study (Brennan et al. 2015) (Figure 1). The Middle location is the historic deep basin location sampled in previous studies. The bioassay incubation location was along the north shore of the lake (Figure 1).

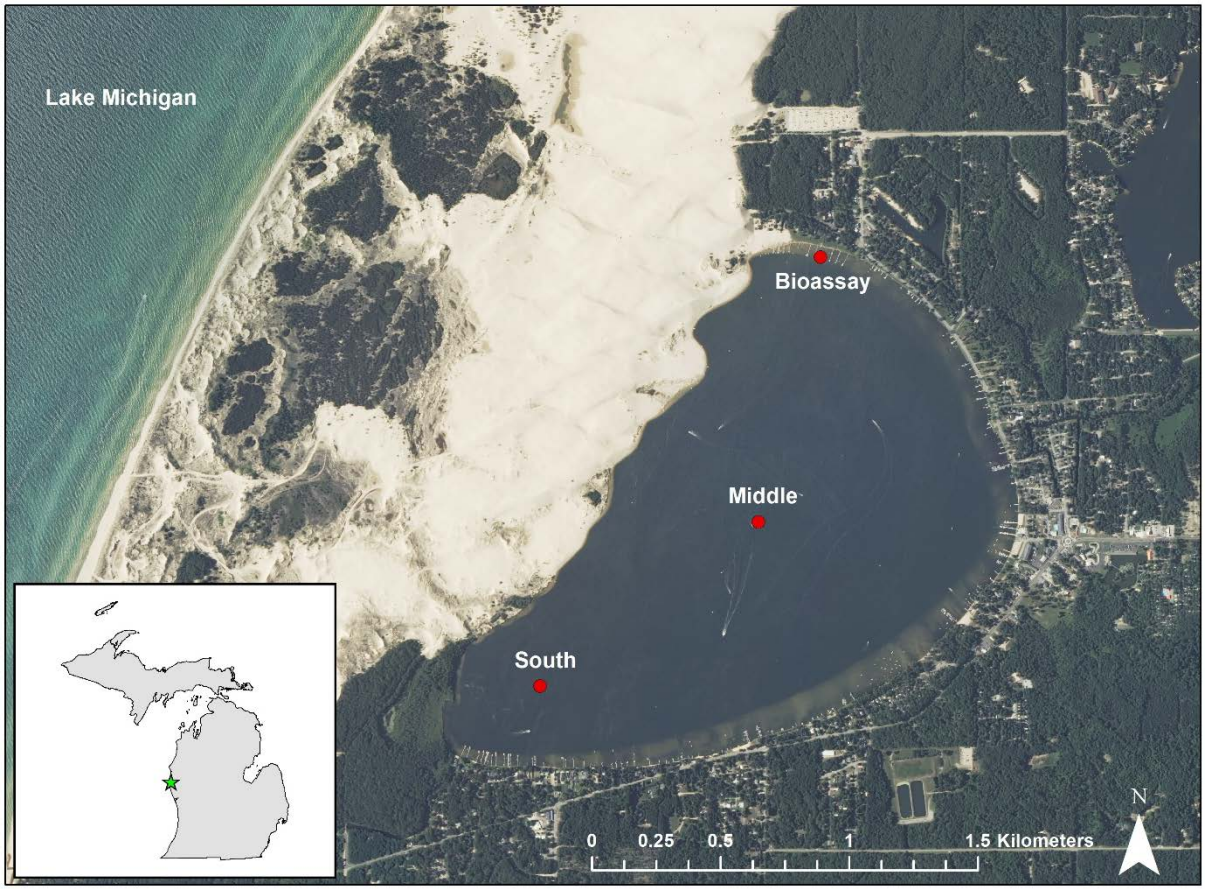


Figure 1. Orthophotograph of Silver Lake (2010, National Agricultural Inventory Program [NAIP]) with locations of bimonthly monitoring locations and bioassay incubation (red dots). Inset map shows the location of Silver Lake within Michigan.

Bimonthly Monitoring

Phytoplankton was collected bimonthly at two locations (Middle, South) in Silver Lake from April through October, 2014 (n = 28). Two water samples for phytoplankton community structure and microcystin analysis were collected at each site with a 1-m depth-integrated sampler and dispensed into separate 4L glass jars: 1) near the water surface to 1 m depth and 2) approximately 1 m above the sediment surface. At the time of water collection, general water quality measurements (dissolved oxygen [DO], temperature, pH, specific conductance, turbidity, total dissolved solids [TDS], redox potential [ORP], chlorophyll *a*, and cyanobacteria [phycocyanin pigment]) were taken at the surface, middle, and bottom of the water column with a YSI 6600 sonde. Samples were transported back to the AWRI lab on ice.

Upon return to the lab, sample jars were gently inverted 50 times to ensure a well-mixed sample before subsampling for phytoplankton identification and microcystin analysis. A 1L subsample for phytoplankton identification was poured into an opaque bottle and preserved with formalin to a final concentration of 4%; subsamples were concentrated in 1L glass graduated cylinders by allowing them to settle for at least 48 hours before the supernatant was removed. The concentrated phytoplankton sample (<125 mL) was quantitatively transferred to an opaque sample bottle. An additional 2 mL of

formalin was added to each concentrated sample upon transfer; samples remained stored in opaque bottles until processing for microscopic analysis. Analytical methods are described below.

A second 1L subsample for microcystin analysis was poured into an amber glass bottle and stored at 4°C. Cyanotoxin samples were prepared according to a modified method previously described by Fastner et al. (1998) and Dyble et al. (2008). Within 24 hours of collection, 50-100 mL was removed after mixing and filtered onto a Whatman glass GF/A glass microfiber filter. The filters were transferred to 10 mL marked vials and placed in an ultralow freezer for a minimum of 12 hours. After freezing, the filters were lyophilized for a minimum of 8 hours. The filters then were extracted by adding 5 mL of 75% methanol and 25% water, and sonicating for 3 minutes in a 15 mL glass centrifuge tube. The extracts were centrifuged and the supernatant was transferred to a second 15 mL glass centrifuge tube. The extraction procedure was repeated two additional times and the combined extracts were taken to dryness under nitrogen and brought up to 2 mL in methanol. The extracts were filtered using a 0.25 µm GF syringe filter, and placed in a 2 mL autosampler vial containing 40 ng/mL of Nodularin as an internal standard. Analytical methods are described below.

Nutrient Bioassays

Nutrient bioassays (Biddanda et al. 2008) were conducted 11-14 August 2014 and 22-25 September 2014, to complement the bioassay study that was performed during summer 2013 as part of the Silver Lake nutrient study (Brennan et al. 2015). On each occasion, twelve (12) experimental 10-L carboys were filled with natural phytoplankton collected from the Middle site (Figure 1; prior sampling indicated this site was representative of overall lake conditions), and exposed to 4 nutrient treatments, each replicated 3×: nitrogen (N) addition (10× ambient concentration, as potassium nitrate [KNO_3]); phosphorus (P) addition (10× ambient concentration, as monopotassium phosphate [KH_2PO_4]); N and P together (each 10× ambient concentration); and a control (no nutrient additions) (Table 1). Carboys were suspended from a PVC frame near the dock of a willing homeowner (Figure 1) and incubated for 4 days.

Table 1. Nutrient concentrations used in bioassay experiment. Ambient concentrations were based on data collected by the U.S. Geological Survey as part of the Silver Lake nutrient study on 11 June 2013 (Brennan et al. 2015).

Nutrient Concentrations, mg/L		
Nutrient	**Ambient**	**Target concentration**
NO_3-N	0.126	1.26 (10×)
SRP	0.004	0.04 (10×)

At the start of each experiment, subsamples were collected from each carboy (n=12) for analysis of algal parameters (i.e., community structure, chlorophyll *a*, microcystin content). The nutrients were then added to the carboys and immediately subsampled again (n=12) for nutrient concentrations (total phosphorus [TP], soluble reactive phosphorus [SRP], nitrate (NO_3), nitrite (NO_2), ammonium (NH_3), total Kjeldahl nitrogen [TKN]) to determine initial conditions. At the end of the 4-day incubation, each carboy (n=12) was subsampled again for all algal and nutrient parameters. Prior to each subsampling, carboys were gently inverted 30 times to ensure well-mixed subsamples. Subsamples for SRP, NO_2, and NO_3

analysis were immediately syringe-filtered through 0.45-μm membrane filters; subsamples for NH_3 and TKN analysis were preserved with H_2SO_4. Subsamples were placed on ice until arrival at the AWRI lab.

General water quality parameters (see above) were measured at the Middle site at the time of water collection, at the start of the bioassay (dockside), and at the end of the bioassay (dockside).

Upon return to the lab, subsamples for phytoplankton community structure and microcystin determination were processed as described above. Subsamples for chlorophyll *a* analysis were filtered onto GFF filters in a darkened room and frozen until analysis.

Laboratory Analysis

Phytoplankton analysis was done using a two-step approach, which was slightly modified from the standard protocol for Great Lakes phytoplankton analysis (EPA 2010) to increase the likelihood that we would detect the species that form large floating colonies (e.g., *Gloeotrichia*). In the first step, the concentrated sample was inverted by hand for 60 seconds before a subsample was loaded into the counting chamber. All identification and enumeration of preserved phytoplankton samples was done in a Palmer-Maloney counting chamber (0.1 mL) at 400X using a Leica DM LB2 compound microscope. A total of 300 algal units (cells, filaments, or colonies) were counted and identified to the lowest possible taxonomic level. The biovolume of dominant taxa (i.e., all taxa that composed >5% of a sample's biovolume) was estimated by measuring 10 individuals and applying the appropriate mathematical formulae for their geometric shape (Hillebrand et al. 1999). The biovolume of less abundant taxa was estimated by using biovolumes obtained from organisms collected in nearby Muskegon Lake (Gillett and Steinman 2011). Only organisms with intact cell contents were included in the biovolume estimation. In the second step of phytoplankton analysis, each sample was settled in a glass beaker for at least 24 hours before all visible colonies of *Gloeotrichia* were counted with a magnifying glass. The sample was also examined for individual *Gloeotrichia* filaments but they were not encountered. To estimate colony size, number of filaments, and biovolume, all visible colonies were transferred to a Palmer-Maloney counting cell and measured under the microscope. The measurements for multiple *Gloeotrichia* colonies per sample were averaged before their biovolumes (μm^3/mL) were included with the rest of the phytoplankton species.

Microcystin concentration was determined using liquid chromatography-mass spectrometry (LC-MS) to yield quantitative results for three major congeners of microcystin (LR, RR, and YR). This approach has been used to measure cyanotoxins in west Michigan Lakes (Xie et al. 2011, 2012) and is not subject to cross reactivity between congeners (Metcalf et al. 2002). Extracts were analyzed using a Thermo Surveyor MSQ according to the conditions in Table 2. The column used was a Phenomenex Kinetex 100mm x 3.0mm C18 2.6u 100A and the standard curve was 100/50/25/10/5 ng/mL.

Table 2. Conditions for the analysis of microcystin using liquid chromatography-mass spectrometry (LC-MS).

MS Detector Parameters

Probe temp: 500°C	Mass	Span	Time (min)	Dwell (min)	Polarity	Cone (V)
SIM1 - Microcystin RR	519.99	0.20	5-12	0.20	POS	80
SIM2 – Internal standard	825.72	0.20	5-12	0.20	POS	80
SIM3 - Microcystin LR	995.95	0.20	5-12	0.20	POS	80
SIM4 – Microcystin YR	1045.96	0.20	5-12	0.20	POS	80

HPLC Conditions

	Time (min)	Flow (mL/min)	A	B	C
A) HPLC Water	0	0.40	90	0	10
B) Acetonitrile	1	0.40	90	0	10
C) Acetonitrile (0.10% formic acid)	6	0.40	0	90	10
	12	0.40	0	90	10
	12.01	0.40	90	0	10
	20	0.40	90	0	10

The nutrients SRP, TP, NO_2, NH_3, and TKN were analyzed on a SEAL AQ2 discrete automated analyzer (EPA 1993); NO_3 was analyzed on a Dionex ICS 2100 Ion Chromatograph (APHA 1992). Chlorophyll a samples were analyzed on a Shimadzu UV-1601 spectrophotometer (APHA 1992). Values below detection were calculated as ½ the detection limit.

Data Analysis

Bimonthly monitoring

We compared microcystin concentration and phytoplankton biovolume between sampling sites and depths by using an independent sample t-test (i.e., site 1 vs. site 2) or paired t-test (i.e., top vs. bottom samples at each site). When the assumptions of the parametric t-test were not met, we used its non-parametric alternative (e.g., Mann-Whitney U test or Wilcoxon test). T-tests were used to compare sample richness and diversity between depths. Initially, we analyzed microcystin concentration and phytoplankton biovolume with a repeated measures ANOVA and found no significant difference between sites when using Date as the error term (F-value=1.51, p=0.24). As a consequence, we ran a one-way ANOVA to assess differences in microcystin concentration and phytoplankton biovolume among sampling events (n = 13); the two sites and two sampling depths were pooled for this analysis. Test assumptions were verified by examining biovolume data for normal distribution and equal variance among treatments. Statistically significant (α=0.05) results were further analyzed with Tukey HSD test to identify significant differences between pairs of treatments. If test assumptions were not met, we used the non-parametric Kruskal-Wallis test for comparison of all treatments and the Kruskal multiple comparison test for pairwise comparisons among treatments.

Non-metric multidimensional scaling (NMDS) ordination was used to evaluate spatial (between sites) and temporal (among bimonthly events) patterns in phytoplankton community structure. NMDS used species relative abundance and the Bray-Curtis similarity coefficient (Bray and Curtis 1957) after exclusion of rare taxa (< 1% of sample's total biovolume) and log-transformation of the data to down-weight the effect of dominant species. To evaluate the goodness-of-fit between the calculated (plotted) and the actual (from the similarity matrix) distances among the samples, the stress value was estimated. A stress value <0.20 indicates a good ordination (Clarke 1993). The NMDS function was specified to run with 20 random starts in search of optimal solution with the lowest stress value.

To test if different sites, depths, and sampling events resulted in different species compositions, samples were analyzed with analysis of similarity (ANOSIM; Clarke 1993). This method tests for significant differences between two or more groups using the rank order of the samples' dissimilarity matrix. Dissimilarity values were calculated with the Bray-Curtis similarity coefficient. If two groups had very different species compositions, the dissimilarities between the groups would be larger than those within the groups. This was evaluated with the R statistic, which varies between -1 and 1, with values close to 0 indicating random grouping. The statistical significance (α=0.05) of the R statistic was evaluated with 999 permutations. If there was a significant difference in the species composition between sites, depths, and among sampling events, indicator species analysis (Dufrene and Legendre 1997) was performed to find the species most responsible for the differences. Indicator taxa were those that were more abundant and had a higher probability of occurrence in one particular site, depth, or sampling event ($\alpha \leq 0.05$).

Bioassay

One-way analysis of variance (ANOVA), as described above, was used to assess differences in microcystin concentration, chlorophyll *a* concentration, total biovolume, and algal division biovolume among the four treatments (control, C; nitrogen, N; phosphorus, P; and nitrogen+phosphorus, N+P). Separate ANOVAs were run to compare treatments before nutrient additions (i.e., in order to evaluate whether there might be any systemic differences among them) and on the biovolume differences between before and after nutrient additions (biovolume after nutrient additions minus biovolume

before nutrient additions). Richness and diversity among treatments were compared in the same manner as biovolume.

Results

Bimonthly Monitoring

Water quality exhibited seasonal variation that is typical for northern temperate lakes (Figure 2). Silver Lake is polymictic, meaning that its shallow depth allows for frequent mixing of the water column during the ice-free period, thus preventing thermal stratification. Water temperatures throughout the monitoring period reflected this phenomenon (Figure 2). However, hypoxia (DO concentrations < 2 mg/L) was measured on 2 occasions at the South site and on 3 occasions (6 occasions < 5 mg/L) at the Middle site, indicating that despite the lake's tendency to mix, there can be times of very low DO. Mean chlorophyll *a* levels ranged between 6.3 and 11.9 µg/L (Table 3), suggestive of mesotrophic to eutrophic conditions. A summary of average general water quality parameters during the monitoring period is provided in Table 3.

Concentrations of the three main microcystin congeners (RR, LR, and YR), as well as total microcystins, were not significantly different between the two monitoring sites or between the two sampling depths at each site; therefore, data are presented as pooled means (i.e., both sites and both sampling depths) for each monitoring date (Figure 3). Seasonal variation in microcystin concentration was evident within all of the congeners, with the concentrations peaking during September and quickly declining thereafter. The lowest concentrations of all microcystin congeners were measured early in Spring. In general, concentrations were low during the April through June period and higher from mid-August through early October (Table 4, Figure 3).

Microcystin-LR, the most toxic of the microcystin congeners, is a known hepatotoxin and is possibly carcinogenic to humans (EPA 2015). The U.S. EPA issued a Health Advisory (HA) in 2015 defining 10-day limits of total microcystin, using microcystin-LR as a surrogate, that are considered protective of non-carcinogenic adverse effects over a 10-day exposure to microcystins in drinking water. Although Silver Lake is not a drinking water source, we list these limits as a precautionary measure: 0.3 µg/L for infants through pre-school age children and 1.6 µg/L for school-age children through adults (EPA 2015). Drinking water is considered the primary route of human exposure to microcystins, but recreational use on affected waters also is a concern. As a consequence, the World Health Organization (WHO) has issued guidelines for recreational exposure, including a low probability of acute health effects when microcystin-LR concentration is <10 µg/L, moderate risk at 10-20 µg/L, high risk at 20-2,000 µg/L, and very high risk at >2,000 µg/L (WHO 2003).

In Silver Lake, all microcystin-LR concentrations were well below the WHO low-risk guidance for recreational exposure and none exceeded the EPA drinking water advisory for adults. Microcystin-LR concentrations were >0.3 µg/L (i.e., EPA HA for infants and young children) in early June and from mid-

August through early October (Figure 3), though it is highly unlikely that any person could ingest a sufficient volume of lake water to cause adverse health effects from microcystin at such low concentrations.

In contrast to microcystin concentration, no seasonal trends were evident in phytoplankton biovolume (Figure 4); analysis of pooled biovolume data revealed no significant biovolume differences ($p>0.05$) among the bimonthly samples. Mean phytoplankton biovolume was not significantly different between the South and Middle sites over the monitoring period. Biovolume was significantly greater at the surface (8.474×10^6 μm^3/mL) than near the bottom (5.314×10^6 μm^3/mL, $p \leq 0.05$) at the Middle site, but the two sampling depths were statistically similar at the South site (Table 5).

Phytoplankton biovolume was dominated by diatoms (7.093×10^6 μm^3/mL), followed by much lower abundances of cyanobacteria (0.385×10^6 μm^3/mL) and chlorophytes (0.103×10^6 μm^3/mL) (Table 5). Diatoms had higher, but not significantly different, biovolumes in bottom samples at the South site, but at the Middle site they were significantly higher in top samples (7.781×10^6 μm^3/mL) compared to bottom samples (4.691×10^6 μm^3/mL). There also were significant differences ($p \leq 0.05$) in biovolume between top and bottom samples at the South site for cyanobacteria, cryptophytes, and chrysophytes, although the absolute biovolumes in these algal classes were very low (Table 5).

A total of 85 phytoplankton species were identified, which contributed to an average sample richness of 17 species (range 6-29) (Table 5). The average Simpson diversity was 2.95 (range 1.24-5.06, Table 5). The most diverse group were the chlorophytes (33 species), followed by diatoms (26 species), cyanobacteria (16 species), euglenoids (3), dinoflagellates (3), cryptophytes (2), and chrysophytes (2). Species richness was similar at each site and depth, varying between 16 and 17 species. Of the 10 most abundant taxa, 6 were diatoms, 3 were cyanobacteria, and 1 was a dinoflagellate (Table 6). Three of the most abundant diatoms were chain-forming taxa: *Fragilaria crotonensis* Kitton, *Aulacoseira islandica* (Müller) Simonsen, and *Aulacoseira granulata* (Ehrenberg) Simonsen.

Gloeotrichia echinulata Richter was observed only rarely (6 out of 52 samples; Table 6), with a mean biovolume of 3,465 μm^3/mL (range 736 - 10,261 μm^3/mL) in those samples and with a density of 1-4 colonies/L. Across all samples (i.e., including those where the taxon was not observed; n=52), average *Gloeotrichia* biovolume declined to 400 μm^3/mL (range 0-10,261 μm^3/mL). The 6 samples with *Gloeotrichia* colonies were collected on 19 June (South top and Middle bottom), 14 July (Middle top), 4 August (South top and bottom), and 18 September (South top). Each colony had on average 432 filaments (range 100-1,070) and was 738 µm in diameter (range 500-1,100 µm). This low species occurrence did not allow for any rigorous statistical analyses or comparisons between sites, depths, or months.

The NMDS plot revealed that species composition was more similar in space than time (Figure 5). That is, the two sites clustered closely to each other within any particular sampling month, but the months varied in ordination space. Species composition gradually changed over time with the April-June (i.e., 4-6) samples clustering close to each other, the July and August samples (i.e., 7-8) distant from the other months, and September-October (i.e., 9-10) samples close in space and returning to near the June

sample space. In general, the early- and late-season samples were separated along the second axis on the ordination plot (Figure 5). ANOSIM results revealed significant differences in species composition between bimonthly events ($R=0.55$, $p \leq 0.05$). The species responsible for these differences (highest indicator value and significant results from the indicator species analysis, $p \leq 0.05$, Table 7) were the diatom *Cymbella* spp. in April, the euglenophyte *Trachelomonas* spp. in May, the cyanobacterium *Gomphosphaeria lacustris* Chodat in July, another diatom *Cyclotella ocellata* Pantocsek in August, the chlorophyte *Crucigenia quadrata* Morren in September, and the cyanobacterium *Aphanocapsa delicatissima* West & West in October.

Table 3. Summary of general water quality parameters measured during bimonthly monitoring events, April-October 2014.

Site	Depth, m	Temp, °C		DO, mg/L		pH		SpCond, µS/cm		ORP, mV		TDS, g/L		Turbidity, NTU		Chl, µg/L		BGA, cells/mL	
	Mean	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
South	0.48	17.61	6.33	9.8	1.6	8.30	0.27	301	9	363	32	0.196	0.006	5.6	3.4	6.3	3.2	6,886	3,659
	2.44	17.49	6.24	9.7	1.7	8.26	0.24	301	10	364	30	0.196	0.006	5.9	3.7	8.2	2.3	7,981	5,078
	4.11	17.26	6.14	8.0	3.5	8.09	0.51	310	31	341	69	0.201	0.020	10.0	8.3	11.9	4.6	10,206	6,594
Middle	0.19	17.76	6.32	9.9	1.5	8.28	0.24	301	9	369	36	0.196	0.006	6.1	4.6	6.3	2.7	16,109	21,941
	3.06	17.57	6.19	9.7	1.6	8.25	0.26	301	9	369	36	0.196	0.006	6.4	4.5	8.5	2.6	9,897	6,481
	5.72	17.05	5.87	5.9	3.9	7.81	0.47	311	20	340	86	0.202	0.013	8.5	5.5	10.3	3.2	8,443	4,477

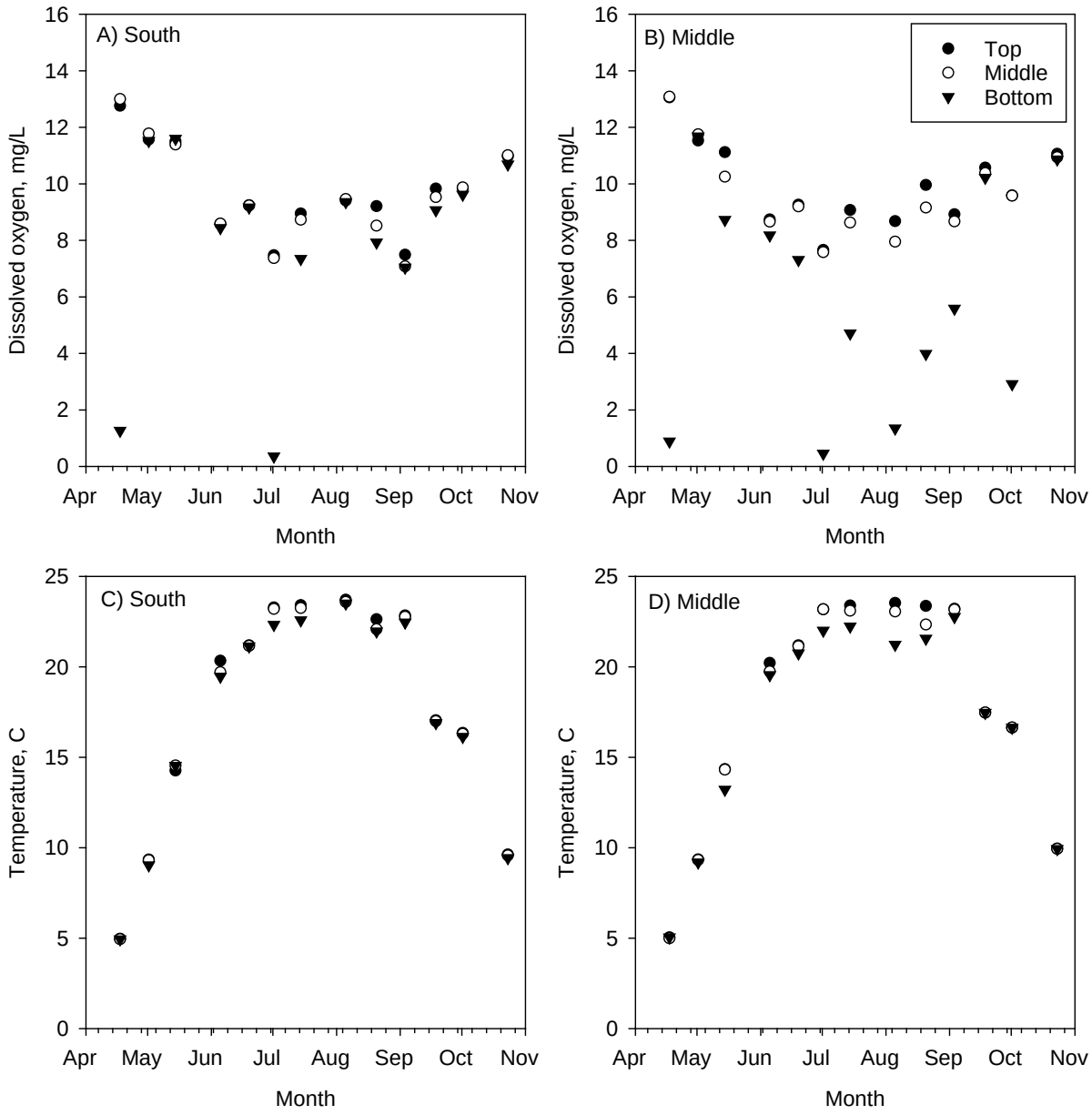


Figure 2. Dissolved oxygen concentrations and water temperature from bimonthly monitoring in Silver Lake, April-October 2014.

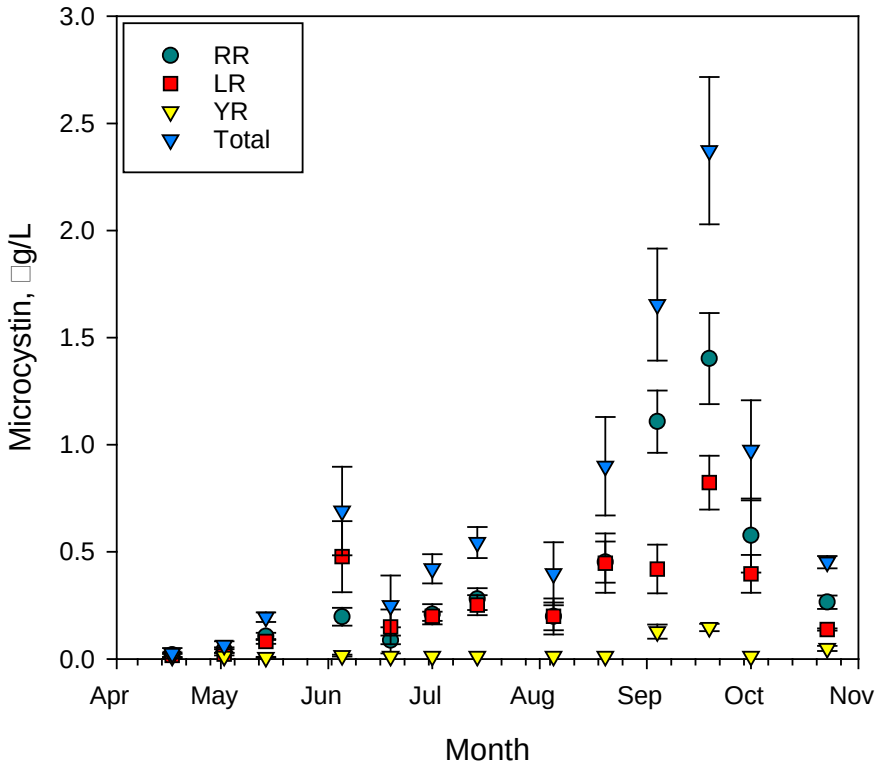


Figure 3. Mean (±SD) concentrations of microcystin congeners measured in Silver Lake during bimonthly monitoring events in 2014. Mean values include South and Middle sites and both sampling depths.

Table 4. Summary of statistical results for pairwise comparisons of microcystin concentrations through time for the bimonthly monitoring of Silver Lake, April-October 2014. Contrasting letters within a column (i.e., for each congener) indicate a statistically significant difference, with greater letters signifying greater concentration. A double-dash indicates no significant pairwise contrasts.

	Microcystin Congener			
Date	**RR**	**LR**	**YR**	**Total**
17 Apr	A	A	--	A
1 May	A,B	A,B	--	A,B
14 May	A,B,C	A,B,C	A	A,B,C
5 June	--	C,D	B	--
19 June	A,B	--	--	A,B,C
1 July	--	--	--	--
14 July	--	--	--	--
4 August	--	--	--	--
20 August	B,C,D	B,C,D	--	B,C,D
3 September	C,D	B,C,D	B	C,D
18 September	D	D	B	D
1 October	B,C,D	B,C,D	--	B,C,D
23 October	--	--	B	--
p-value	<0.001	<0.001	<0.001	<0.001

Table 5. Summary of sample richness, diversity, and biovolume. Mean (± SD) values are given for overall (n=52) and for each sampling depth per site (n=13) in Silver Lake. Different superscript letters represent significant contrasts ($p<0.05$) between top and bottom samples.

Variable	Overall Mean (± SD)	South Site Mean (± SD)		Middle Site Mean (± SD)	
		Top	Bottom	Top	Bottom
Biovolume (10^6 μm^3/mL)					
Total	7.66 (3.75)	7.61 (2.90)	9.23 (5.24)	8.48^b (3.19)	5.31^a (2.12)
Bacillariophyta	7.09 (3.75)	6.90 (2.72)	9.00 (5.25)	7.78^b (3.07)	4.69^a (2.19)
Chlorophyta	0.10 (0.11)	0.10 (0.11)	0.08 (0.09)	0.12 (0.10)	0.12 (0.15)
Chrysophyta	0.01 (0.01)	0^a (0.01)	0.02^b (0.02)	0 (0)	0.01 (0.01)
Cryptophyta	0 (0)	0^b (0)	0^a (0)	0.01 (0.01)	0 (0)
Cyanobacteria	0.39 (0.42)	0.49^b (0.36)	0.10^a (0.12)	0.54 (0.43)	0.41 (0.56)
Euglenophyta	0 (0.01)	0 (0)	0 (0.01)	0 (0)	0.01 (0.01)
Pyrrhophyta	0.06 (0.18)	0.12 (0.29)	0.03 (0.10)	0.03 (0.12)	0.07 (0.17)
Sample richness	17 (5.72)	16 (5.01)	17 (6.82)	17 (6.25)	17 (5.32)
Simpson diversity	2.95 (0.75)	3 (0.58)	2.86 (0.83)	2.71 (0.80)	3.23 (0.76)

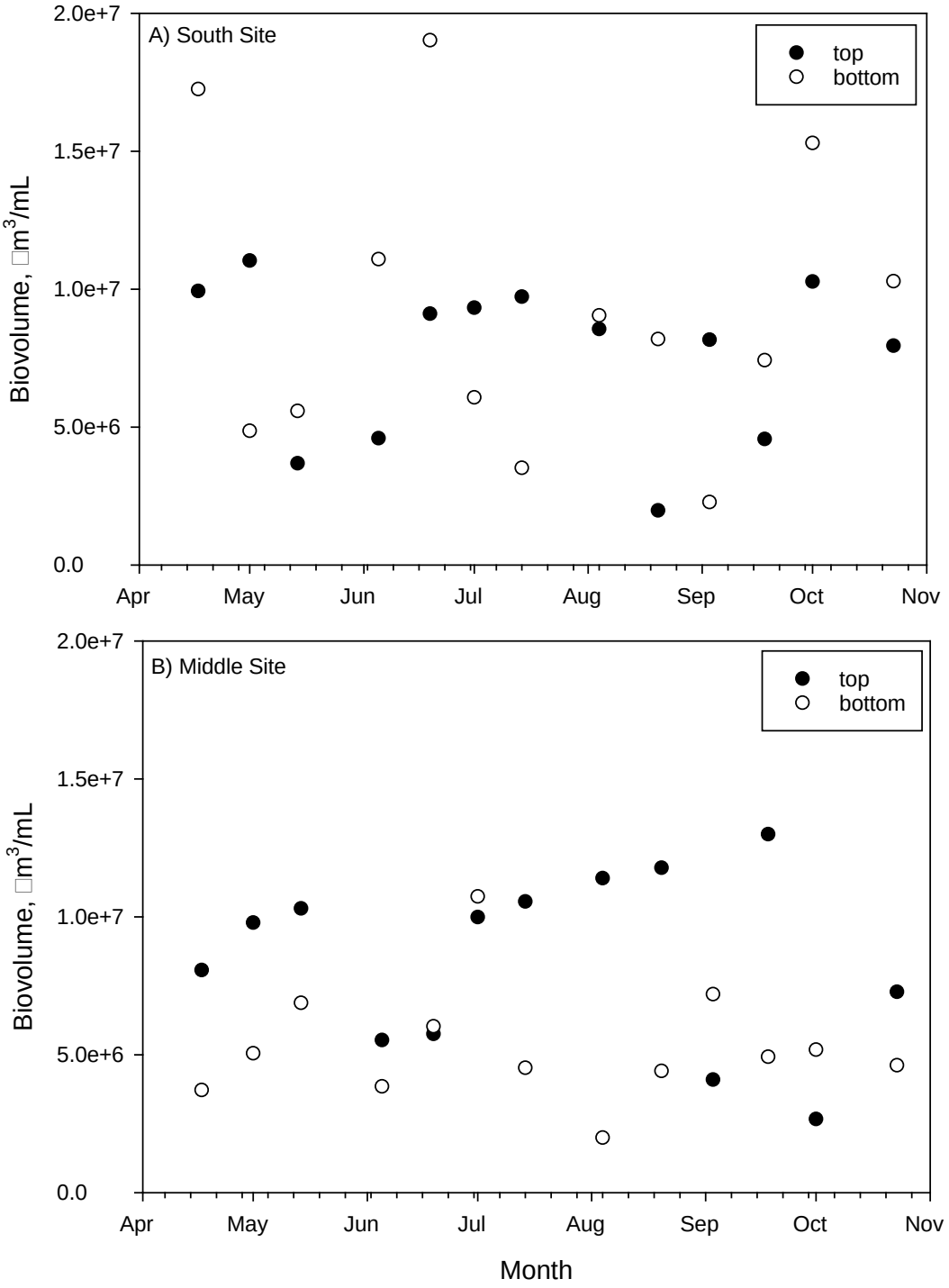


Figure 4. Phytoplankton biovolume measured in bimonthly monitoring samples collected from Silver Lake, April-October 2014.

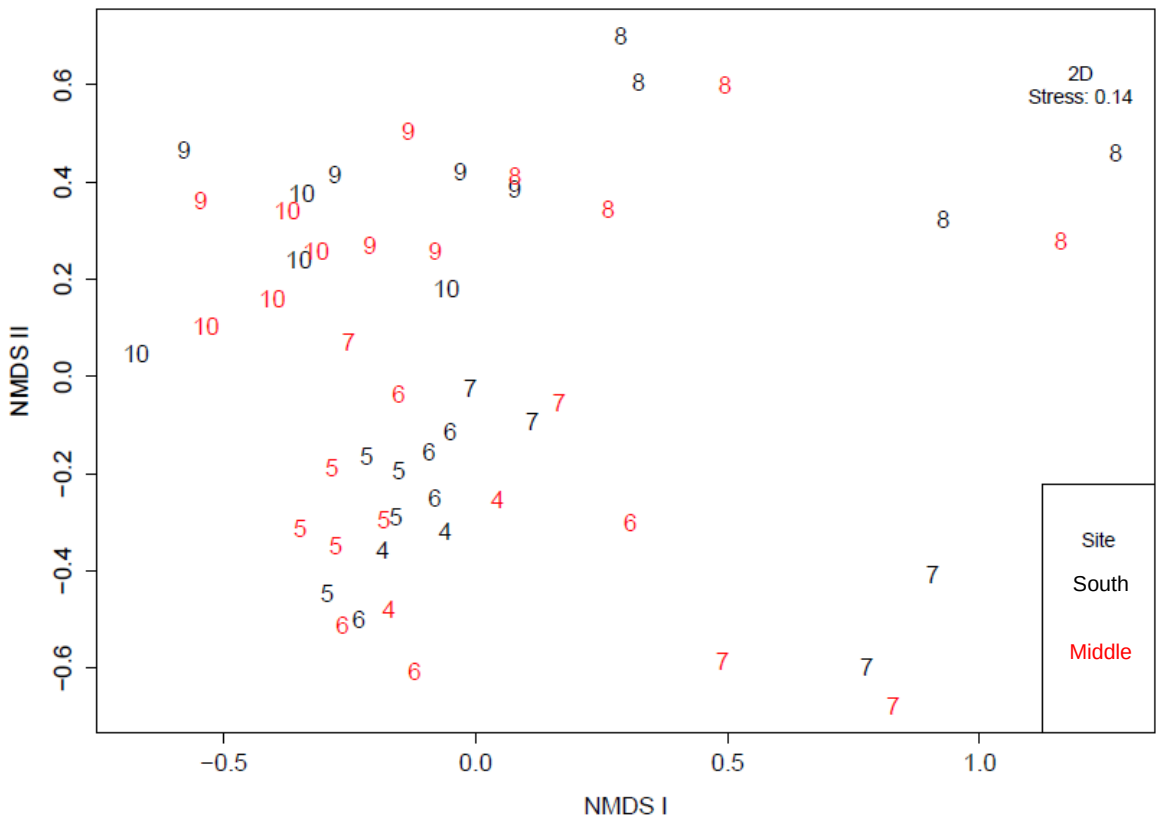


Figure 5. Non-metric multidimensional scaling plot (2 dimensions, stress value 0.14) of phytoplankton samples (n=52) from Silver Lake. Each number on the plot represents one sample with its corresponding sampling month (4-10, April-October) while the color represents sampling site (South, black; Middle, red). Bimonthly samples collected within the same month were grouped together for this analysis.

Table 6. Summary of biovolume (10^6 µm^3/mL) for the top 10 dominant taxa in Silver Lake. Summary data are also given for *Gloeotrichia echinulata* for comparison. freq, proportion of samples where species was present; SD, standard deviation; Min, minimum; Max, maximum.

Species (sorted by mean biovolume)	**Division**	**freq**	**Mean**	**SD**	**Median**	**Min**	**Max**
Aulacoseira islandica (Müller) Simonsen	Bacillariophyta	0.87	3.12	2.73	2.64	0.00	11.61
Aulacoseira granulata (Ehrenberg) Simonsen	Bacillariophyta	0.83	1.41	1.62	0.77	0.00	7.84
Stephanodiscus niagarae Ehr.	Bacillariophyta	0.75	0.88	1.49	0.57	0.00	8.64
Asterionella formosa Hassall	Bacillariophyta	0.56	0.83	1.20	0.12	0.00	4.56
Cyclotella ocellata Pantocsek	Bacillariophyta	0.67	0.54	0.91	0.04	0.00	2.91
Fragilaria crotonensis Kitton	Bacillariophyta	0.94	0.26	0.33	0.14	0.00	1.41
Woronichinia naegeliana Elenkin	Cyanobacteria	0.67	0.10	0.17	0.04	0.00	0.96
Microcystis aeruginosa Kütz.	Cyanobacteria	0.56	0.09	0.13	0.01	0.00	0.48
Anabaena affinis Lemmermann	Cyanobacteria	0.27	0.07	0.25	0.00	0.00	1.73
Ceratium hirundinella (Müller) Dujardin	Pyrrhophyta	0.12	0.06	0.18	0.00	0.00	0.87
Gloeotrichia echinulata Richter	Cyanobacteria	0.12	0.0004	0.002	0.00	0.00	0.01

Table 7. Indicator species analysis results using month (April-October) as the grouping variable. Bimonthly samples collected within the same month were grouped together for this analysis.

Indicator species	**Month**	**Indicator value**	**Probability**
Cymbella spp.	April	0.68	0.001
Navicula spp.	April	0.38	0.007
Trachelomonas spp.	May	0.50	0.003
Asterionella formosa Hassall	May	0.49	0.001
Dinobryon sertularia Ehr.	May	0.48	0.003
Desmodesmus microspina (Chodat)Tsarenko	May	0.33	0.033
Gomphosphaeria lacustris Chodat	July	0.74	0.001
Sphaerocystis schroeteri Chodat	July	0.72	0.001
Stephanodiscus niagarae Ehr.	July	0.55	0.001
Anathece minutissima (W.West) Komárek Kastovsky & Jezberová	July	0.47	0.003
Fragilaria crotonensis Kitton	July	0.41	0.001
Cyclotella ocellata Pantocsek	August	0.71	0.001
Ankyra spp.	August	0.69	0.001
Oocystis borgei Snow	August	0.46	0.003
Oocystis parva West & West	August	0.45	0.006
Chlamydomonas mucicola Schmidle	August	0.40	0.006
Unknown chlorophyte	August	0.34	0.019
Crucigenia quadrata Morren	September	0.60	0.001
Quadrigula lacustris (Chodat) Smith	September	0.56	0.001
Pseudanabaena mucicola (Naumann & Huber-Pestalozzi) Schwabe	September	0.49	0.002
Rhodomonas minuta Skuja	September	0.47	0.013
Chroococcus limneticus Lemm.	September	0.41	0.003
Scenedesmus quadricauda (Turp.) Bréb.	September	0.39	0.006
Microcystis aeruginosa Kütz.	September	0.38	0.005
Aulacoseira granulata (Ehrenberg) Simonsen	September	0.31	0.037
Aphanocapsa delicatissima West & West	October	0.59	0.001
Chlamydomonas angulosa Dill	October	0.35	0.033
Closteriopsis longissima (Lemmermann) Lemmermann	October	0.30	0.046
Aulacoseira islandica (Müller) Simonsen	October	0.29	0.005

Bioassay

Nutrient concentrations measured in the carboys at the beginning and end of the bioassay experiments revealed complete uptake of the SRP (100%) that was added, as final concentrations were below the detection limit (5 µg/L), but only minimal NO_3 uptake (6-7%) was observed during either bioassay (Figure 6). Ambient concentrations of SRP, NH_3, NO_2, and NO_3 were very low or below the detection limit prior to nutrient additions for both bioassays (Table 8). In contrast, TP and chlorophyll *a* concentrations were high (33 and 23 µg/L, respectively) at the beginning of the September bioassay (Table 8) and indicative of eutrophic conditions.

Microcystin and chlorophyll *a* concentrations were affected by nutrient additions only during the August bioassay (Figures 7-9). Microcystin-RR was the only congener that responded significantly to nutrient additions during August, when the N+P treatment had a significantly greater increase in MC-RR concentration than the control and P treatments (Figure 7). Chlorophyll *a* concentration also increased significantly in the N+P treatment compared to the control during the August bioassay (Figure 9). There were no significant differences in microcystin or chlorophyll *a* concentration among treatments at the start of the bioassay experiments or at the end of the September bioassay.

The change in phytoplankton biovolume was not significantly different among nutrient treatments during either bioassay experiment (Figure 10). Although biovolume increased in all treatments from the start to end of the August bioassay, particularly for the N+P treatment, the overall changes were not statistically significant due to large variance (Figure 10). Most of the biovolume increase observed in the N+P treatment during the August bioassay was attributable to increases in chlorophytes (from 0.82 to 2.21 x 10^6 µm^3/mL) and cyanobacteria (from 0.82 to 2.36 x 10^6 µm^3/mL; Table 9). During both experiments, diatoms accounted for the greatest biovolume (18.07 x 10^6 µm^3/mL), followed by cyanobacteria (1.76 x 10^6 µm^3/mL) and chlorophytes (0.59 x 10^6 µm^3/mL) (Tables 9-10). There were no significant differences in total biovolume or algal division biovolume among treatments before nutrient additions, with the exception of significantly greater cyanobacteria biovolume in the N treatment at the beginning of the September bioassay (Table 10).

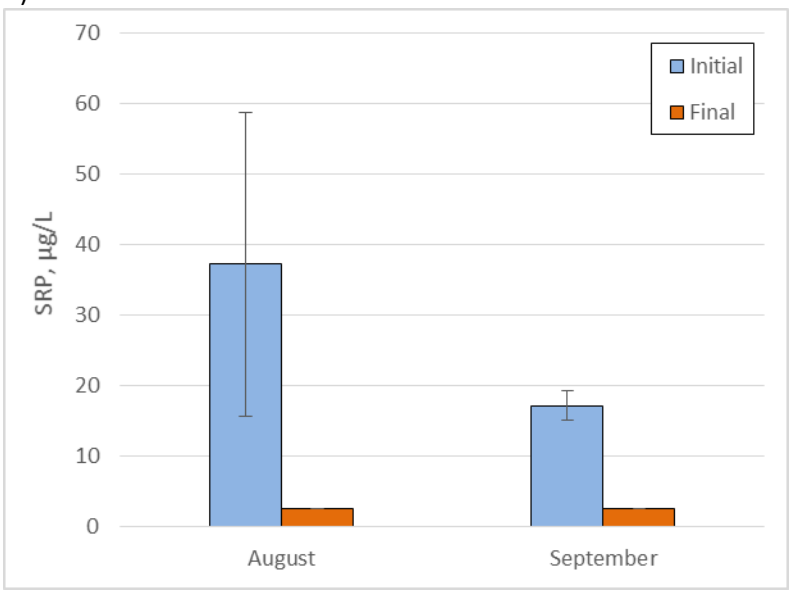
Gloeotrichia echinulata was detected during the August bioassay, but absent during the September bioassay. *Gloeotrichia* was found in 15 out of 24 samples in August, with a mean biovolume of 4,813 µm^3/mL (range 1,903-12,210 µm^3/mL) when it was present. Across all August bioassay samples (n=24), *Gloeotrichia* had a mean biovolume of 3,008 µm^3/mL (range 0-12,210 µm^3/mL). However, it was not one of the 10 most dominant taxa. In samples where *Gloeotrichia* was present, its density was 1-6 colonies/L. Each colony had on average of 309 filaments (range 185-450) and was 738 µm in diameter (range 563-1000 µm). *Gloeotrichia* was found in 8 samples before nutrient additions and 7 samples after nutrient additions (2 N, 3 P, and 2 N+P). Before nutrient additions, average *Gloeotrichia* biovolume was 5,811 µm^3/mL (range 1,903-12,210 µm^3/mL), 300 filaments per colony (range 240-400), and 783 µm in diameter (range 625-1,000 µm). After nutrient additions, average *Gloeotrichia* biovolume was 3,672 µm^3/mL (range 2,025-8,302 µm^3/mL), 318 filaments per colony (range 185-450) and 687 µm in diameter (range 563-850 µm). There were no significant differences ($p>0.05$) in *Gloeotrichia* biovolume, colony

size, number of filaments or colonies among treatments either before or after nutrient additions in the August experiment.

Table 8. Ambient nutrient and chlorophyll *a* concentrations measured in control carboys at the beginning of the bioassay experiments.

	SRP, µg/L		TP, µg/L		NH_3, mg/L		TKN, mg/L		NO_2, mg/L		NO_3, mg/L		Chl, µg/L	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
11 August 2014	<5	0	14.3	2.4	0.013	0.005	0.570	0.103	<0.006	0	<0.006	0	7.5	1.5
22 September 2014	<5	0	33.2	8.5	<0.01	0	0.948	0.264	<0.006	0	<0.006	0	23.3	9.2

A)



B)

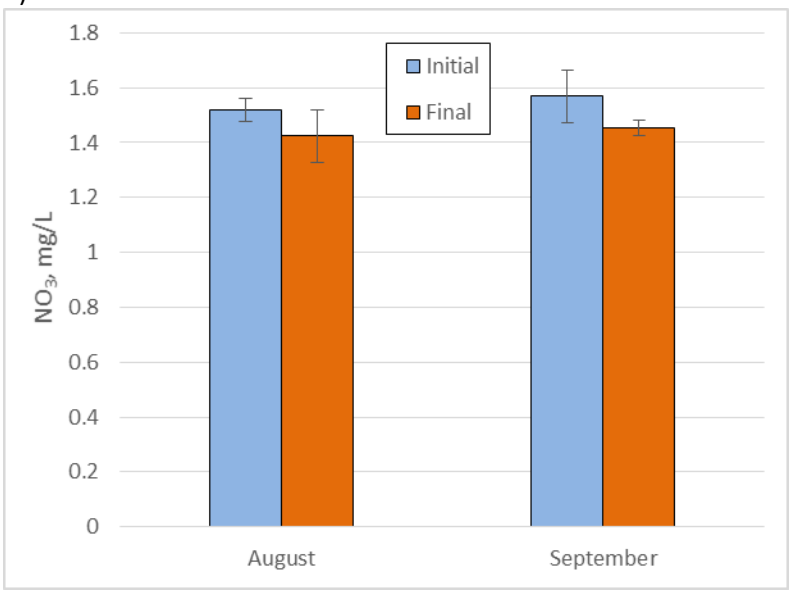


Figure 6. Soluble reactive phosphorus (SRP; A) and nitrate (NO_3; B) concentrations in nutrient treatment carboys at the beginning (i.e., immediately after nutrient spiking) and end of the bioassay experiments.

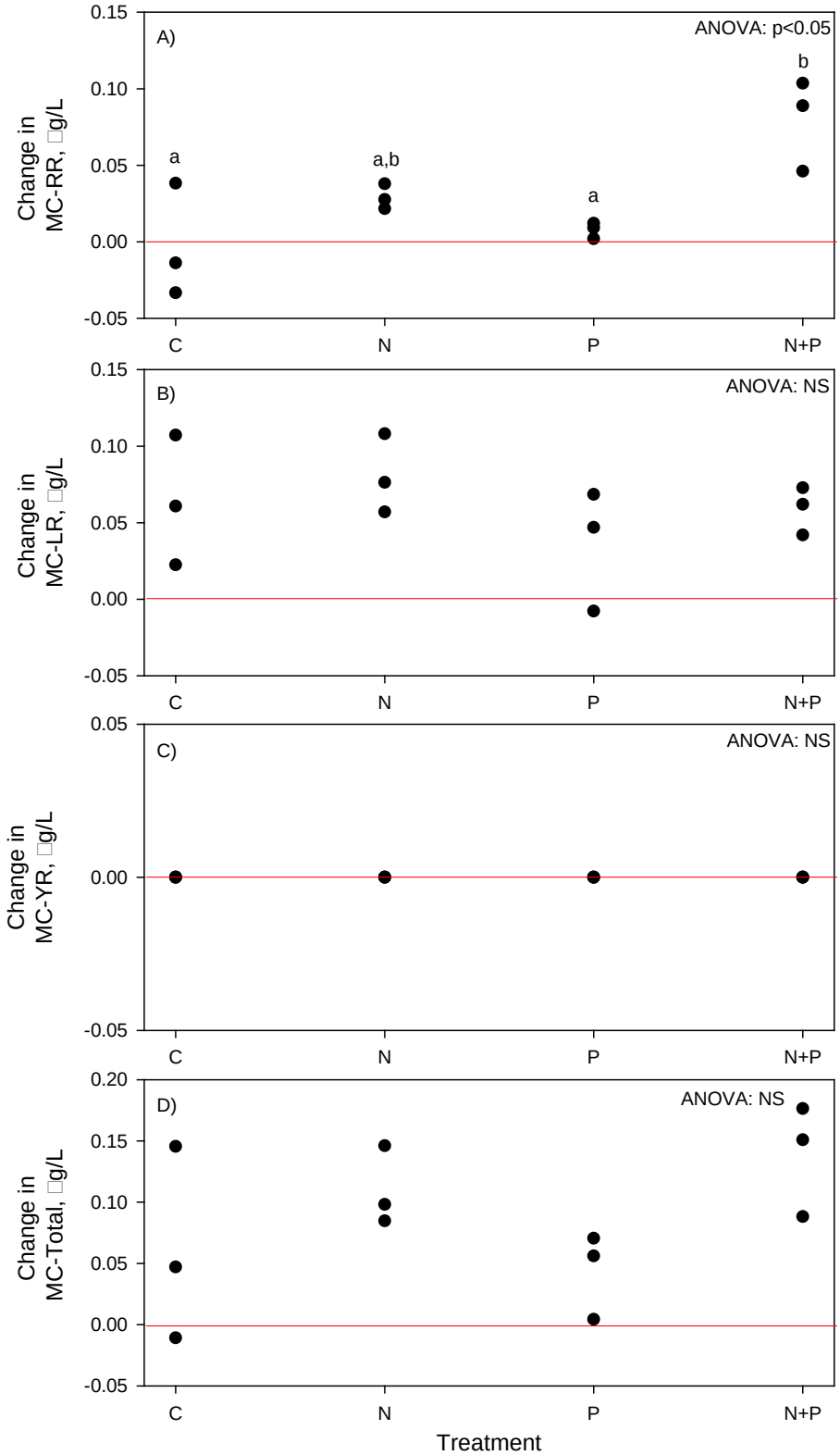


Figure 7. Dot plots of the change in microcystin concentration over the course of the 11-14 August 2014 bioassay (i.e., final concentration - initial concentration). The red line represents zero change in microcystin concentration. Different letters above data groups indicate statistically significant contrasts ($p<0.05$). Note different scales on the y-axes.

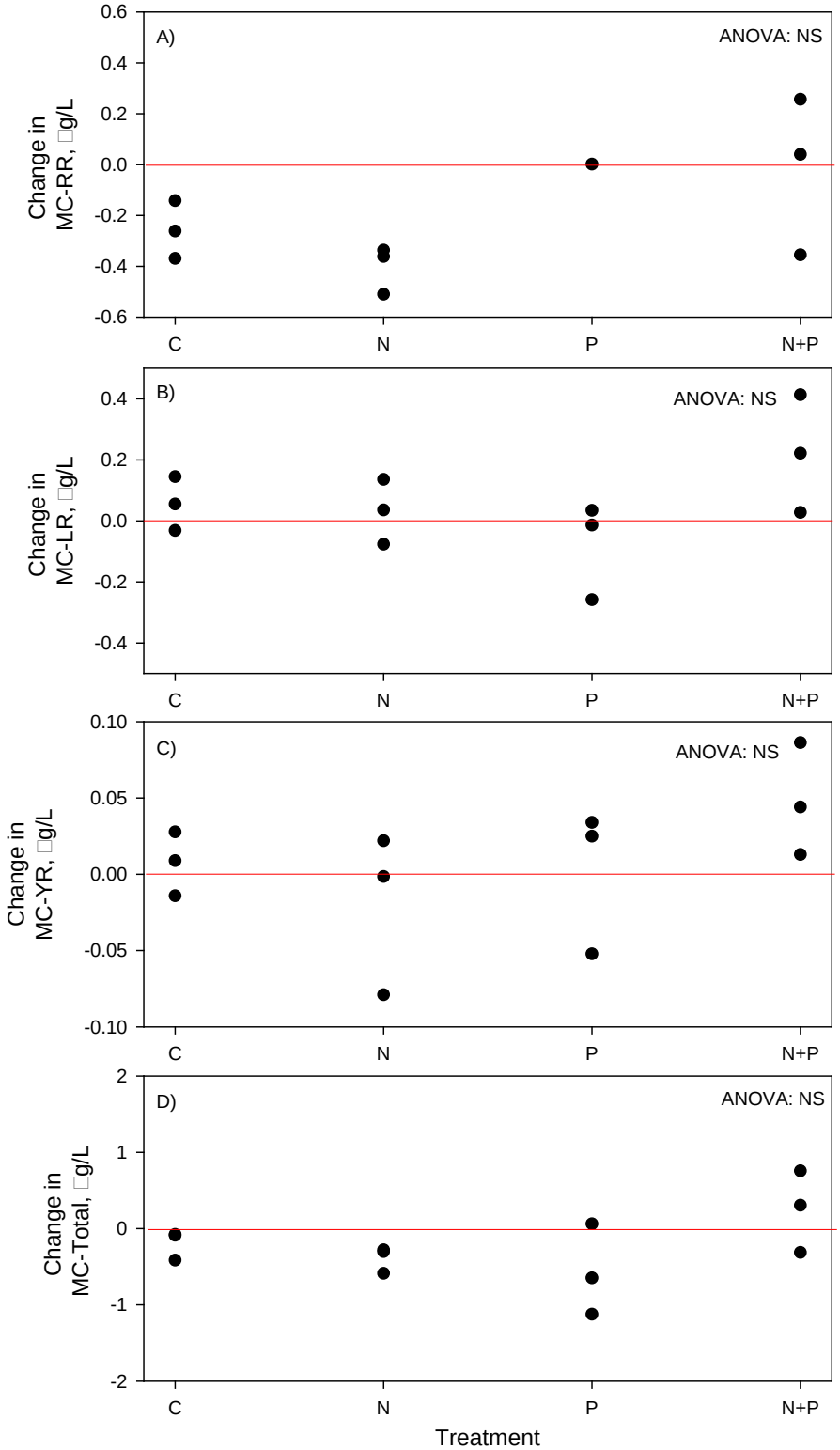
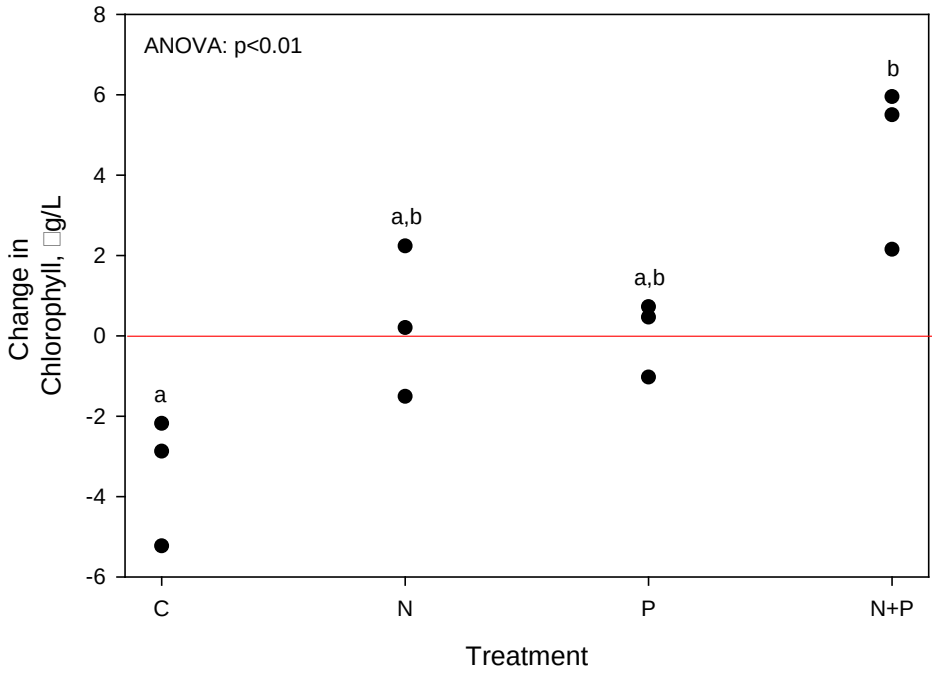


Figure 8. Dot plots of the change in microcystin concentration over the course of the 22-25 September 2014 bioassay (i.e., final concentration - initial concentration). The red line represents zero change in microcystin concentration. Note different scales on the y-axes.

A) August



B) September

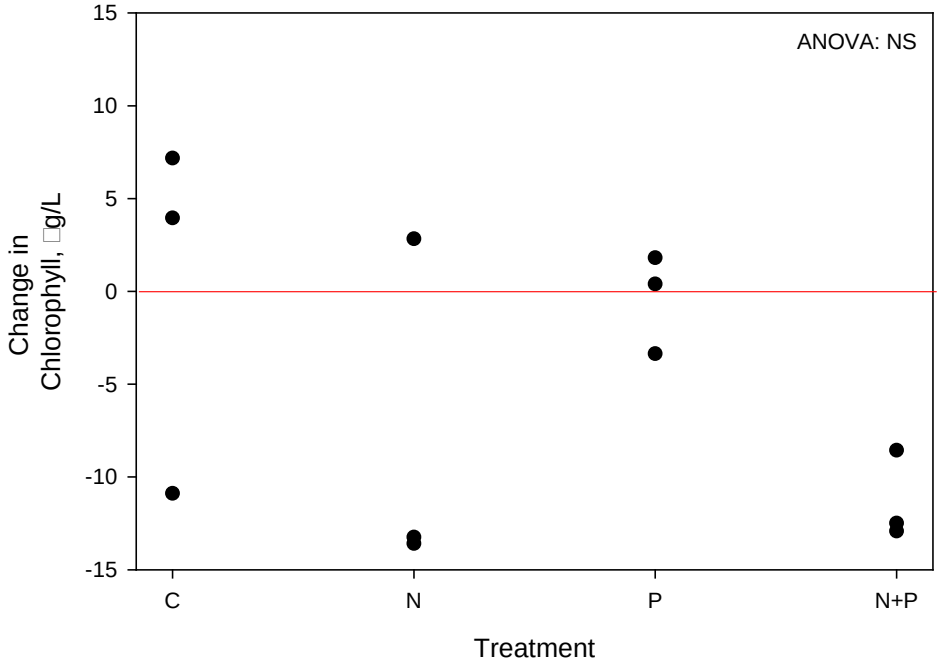


Figure 9. Dot plots of the change in chlorophyll *a* concentration over the course of the 11-14 August 2014 and 22-25 September 2014 bioassays (i.e., final concentration - initial concentration). The red line represents zero change in chlorophyll *a* concentration. Different letters above data groups indicate statistically significant contrasts ($p<0.05$). Note different scales on the y-axes.

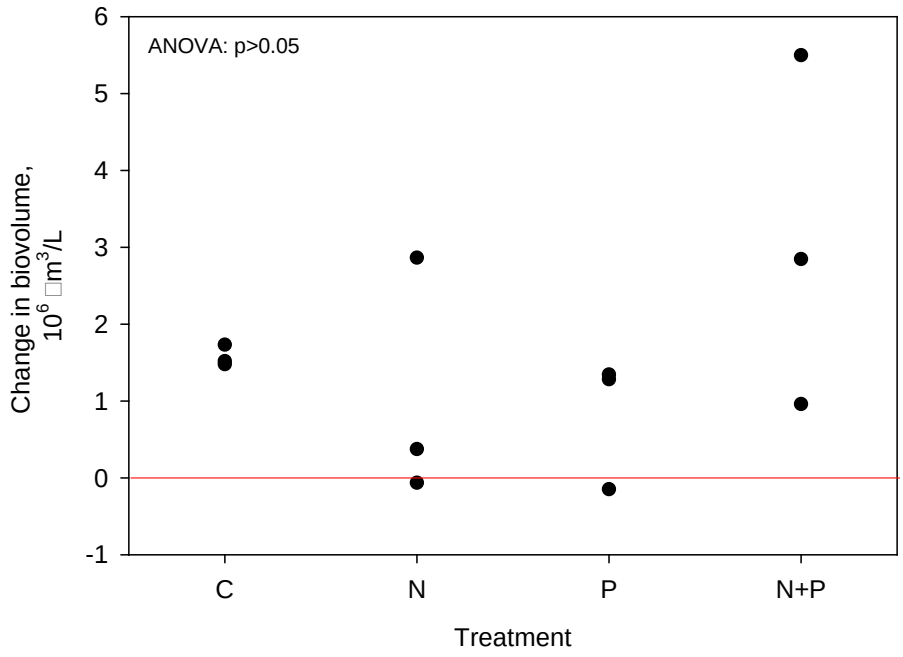
Table 9. Summary of mean (± SD) sample richness, diversity, and biovolume for first bioassay experiment (August, 2014) and treatment level (Control; N, nitrogen; P, phosphorus; N+P, nitrogen and phosphorus) before and after nutrient additions (3 replicates). Different superscript letters represent significant contrasts ($p<0.05$) among treatments within experiments before or after nutrient additions.

Variable	**Control**		**N**		**P**		**N+P**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
Biovolume (10^6 μm^3/mL):								
Total	2.53 (0.53)	4.10 (0.67)	2.32 (0.28)	3.29 (1.38)	2.71 (0.53)	3.54 (0.66)	3.71 (0.73)	6.80 (1.56)
Bacillariophyta	1.70 (0.31)	1.76 (0.64)	1.53 (0.03)	1.71 (0.25)	1.60 (0.27)	1.84 (0.58)	1.65 (0.24)	1.87 (0.13)
Chlorophyta	0.07 (0.03)	1.26 (0.27)	0.24 (0.35)	0.61 (0.96)	0.35 (0.41)	0.66 (1.00)	0.82 (0.90)	2.21 (1.91)
Cryptophyta	0 (0)	0^a (0)	0 (0)	0^a (0)	0 (0)	0^a (0)	0 (0)	0.03^b (0.00)
Cyanobacteria	0.41 (0.11)	0.85 (0.34)	0.50 (0.12)	0.83 (0.13)	0.48 (0.10)	1.03 (0.27)	0.82 (0.41)	2.36 (0.78)
Glaucophyta	0 (0)	0.10 (0.09)	0 (0)	0 (0)	0.07 (0.11)	0 (0)	0 (0)	0.20 (0.34)
Pyrrhophyta	0.34 (0.24)	0.14 (0.12)	0.05 (0.09)	0.14 (0.12)	0.23 (0.07)	0 (0)	0.42 (0.15)	0.12 (0.21)
Sample richness	21 (2.52)	24 (1.00)	18 (1.53)	23 (2.31)	23 (1.53)	20 (2.65)	25 (2.65)	22 (1.73)
Simpson diversity	2.19 (0.24)	3.85 (0.35)	2.33 (0.48)	2.80 (0.58)	2.70 (0.46)	3.18 (0.62)	3.34 (1.00)	3.77 (1.00)

Table 10. Summary of mean (± SD) sample richness, diversity, and biovolume for second bioassay experiment (September, 2014) and treatment level (Control; N, nitrogen; P, phosphorus; NP, nitrogen and phosphorus) before and after nutrient additions (3 replicates). Different superscript letters represent significant contrasts ($p<0.05$) among treatments within experiments before or after nutrient additions.

Variable	**Control**		**N**		**P**		**N+P**	
	Before	**After**	**Before**	**After**	**Before**	**After**	**Before**	**After**
Biovolume (10^6 μm^3/mL):								
Total	14.37 (1.11)	16.95 (1.16)	22.57 (4.23)	22.62 (4.31)	19.67 (5.24)	22.45 (3.00)	22.90 (12.89)	22.07 (4.12)
Bacillariophyta	12.39 (1.52)	14.93 (1.16)	19.12 (4.53)	19.79 (5.17)	18.27 (5.64)	19.67 (4.88)	20.92 (13.21)	19.46 (2.78)
Chlorophyta	0.41 (0.65)	0.15 (0.14)	0.21 (0.30)	0.95 (1.47)	0.22 (0.34)	0.94 (1.46)	0.52 (0.88)	1.36 (1.15)
Cryptophyta	0.01 (0.00)	0 (0)	0.01 (0.00)	0 (0)	0.01 (0.02)	0 (0)	0.00 (0.00)	0.01 (0.01)
Cyanobacteria	1.57^{a} (0.19)	1.86 (0.74)	3.24^{b} (0.53)	1.88 (1.07)	1.15^{a} (0.16)	1.83 (0.50)	1.46^{a} (0.29)	1.04 (0.56)
Glaucophyta	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Pyrrhophyta	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0.20 (0.35)
Sample richness	18 (3.06)	17 (1.53)	15 (4.93)	18 (2.08)	19 (3.51)	16 (2.52)	19 (6.66)	20 (1.73)
Simpson diversity	2.62 (0.75)	3.06 (0.23)	2.53 (0.55)	2.78 (0.38)	2.14 (0.55)	2.58 (0.83)	2.39 (0.84)	2.75 (0.43)

A) August



B) September

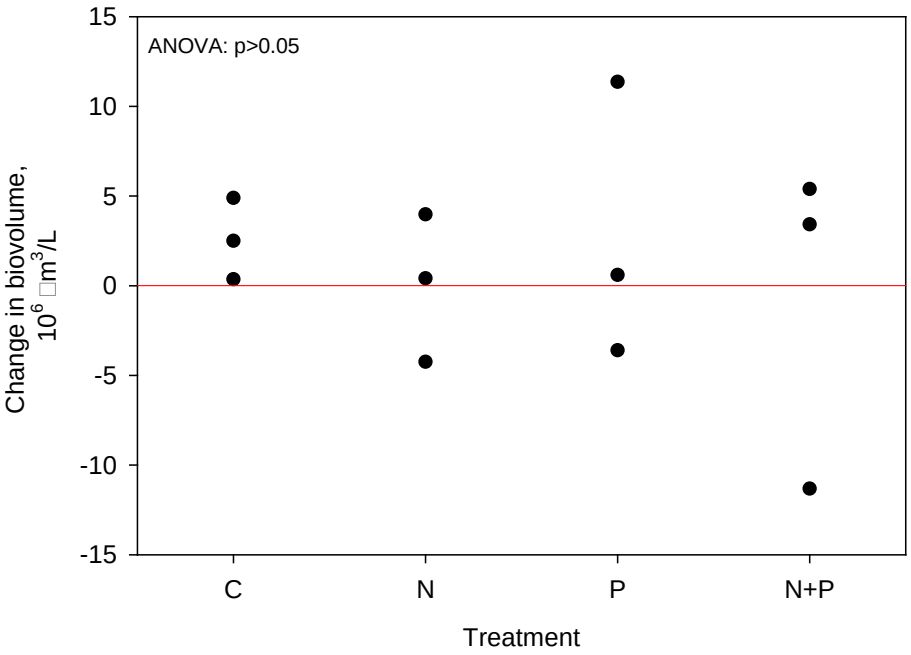


Figure 10. Dot plots of change in biovolume over the course of the 11-14 August 2014 and 22-25 September 2014 bioassays (i.e., final biovolume - initial biovolume). The red line represents zero change in biovolume.

Discussion

The citizenry of Silver Lake had anecdotally noted declining water quality conditions in Silver Lake over the past decade (authors, pers. obs.). After a massive fish kill and severe algal blooms, identified as *Gloeotrichia*, in the summers of 2011 and 2012, concerns escalated and it was determined that research was needed to identify the cause of these environmental impairments. A large-scale study was funded by the Silver Lake Improvement Board, with in-kind support from USGS and AWRI, to identify and model the nutrient sources to Silver Lake; however, that study did not address what factors may be responsible for the growth and development of *Gloeotrichia* in the lake, and its potential toxicity. *Gloeotrichia* is a taxon of concern—it can bloom in both oligotrophic and eutrophic lakes (Carey et al. 2014a), it produces macroscopic colonies that are easily visible to the naked eye and hence presents an aesthetic problem for lake users, it can facilitate the growth of other phytoplankton, and it is a known producer of the cyanotoxin microcystin (Carey et al. 2012).

While Gloeotrichia formed conspicuous blooms in 2011 and 2012, the occurrence and abundance of *Gloeotrichia* was quite low during our monitoring period in 2014. *Gloeotrichia* was found in only 12% of the bimonthly samples and when it was present, it was at very low densities (1-4 colonies/L). By comparison, scum-forming *Gloeotrichia* blooms have densities of up to 250 colonies/L (Carey and Rengefors 2010). Considerable interannual variability in *Gloeotrichia* recruitment from the sediment has been documented and attributed, in part, to regional climatic variability (Carey et al. 2014c). In particular, physical factors that affect lake mixing may be connected to *Gloeotrichia* recruitment; however, lag times make it difficult to detect associations between environmental factors and recruitment (Carey et al. 2014c). Furthermore, it was not possible to determine if *Gloeotrichia* is nutrient limited in Silver Lake due to its very low abundance. Despite the low occurrence of *Gloeotrichia* in 2014, it should be understood that its akinetes are present in Silver Lake sediments (Stamann 2015) and could result in future blooms in the water column during a year with high recruitment.

Despite the inconclusive results in terms of *Gloeotrichia*, our bimonthly sampling does provide some insights into general phytoplankton dynamics in this heavily used lake. Although no seasonal trends in phytoplankton biovolume were observed, a seasonal progression of species composition throughout the growing season was detected. In addition, a distinct seasonal trend was observed in microcystin concentration, with the highest values occurring from mid-August through early October and peak concentrations in mid-September. The toxin-producer *Microcystis aeruginosa* was identified as an indicator species in September, when microcystin concentrations were highest. Despite the noticeable trend in microcystin abundance in Silver Lake, concentrations remained far below the WHO thresholds for recreational water use, indicating users of the lake were not at risk to this cyanotoxin during our monitoring period.

Although thermal stratification was not evident during the study, low DO was measured near the lake bottom of the Middle site (i.e., the deeper of the sites) from July through early September. During the mid-September monitoring event, when the highest microcystin concentrations were measured, DO was near saturation throughout the water column, suggesting a mixing event was occurring at that time. Chlorophyll and TP concentrations measured at the beginning of the September bioassay, four days

after the bimonthly monitoring event, were also high. The relationships between water column mixing, TP concentrations, phytoplankton communities, and microcystin production have been well-documented (Diehl et al. 2002, Jäger et al. 2008, Rinta-Kanto et al. 2009, Lee et al. 2015, de Castro Medeiros et al. 2015). Our limited dataset from only one year of monitoring precludes our ability to definitively connect the early fall microcystin peak with mixing, but warrants further investigation. It appeared that this mixing event was short-lived, as DO declined again temporarily at the bottom of the Middle site in early October. Our results both from this study, and the nutrient budget study with USGS (Brennan et al. 2015), reveal that DO conditions in Silver Lake are dynamic and vary over short time scales.

Results from the two bioassay experiments were incongruous in terms of nutrient limitation. Although added P was completely taken up during both experiments, nutrient limitation was evidenced only during the August experiment, when N+P co-limitation was suggested by changes in microcystin-RR, chlorophyll *a*, and biovolume, though the latter was not significant. Added N was only minimally taken up, with a 6-7% decrease in concentration during both experiments. Ambient TP and chlorophyll *a* concentrations were 2- and 3-fold greater, respectively, at the start of the September bioassay than the August bioassay. It is possible that the already-elevated ambient TP, which was greater than the SRP addition, was sufficient to eliminate nutrient limitation during September. A similar bioassay conducted in July 2013 as part of the USGS nutrient study also indicated N+P co-limitation (Brennan et al. 2015). Taken together, these results point to a possible seasonal change in nutrient limitation, with N+P limitation occurring during summer and no nutrient limitation in early fall, coinciding with late-season mixing or additional external nutrient loading.

The seasonal changes observed in Silver Lake's phytoplankton community, its production of microcystin, and its nutrient limitation shed light on the dynamic nature of algae in the lake. Although no nuisance blooms or high microcystin concentrations were detected during this study, Silver Lake's eutrophic state is capable of supporting them. Given the high recreational value of Silver Lake, and the potential for future algal blooms, nutrient reduction strategies should be implemented in the watershed to lessen the nutrient loading to the lake. The concurrent Silver Lake nutrient study concluded that septic systems were the largest potentially controllable nutrient load to the lake (Brennan et al. 2015). Internal nutrient loading from the sediments was found not to be a significant source (Brennan et al. 2015). However, it should be noted that *Gloeotrichia* has been reported to contribute up to 2/3 of the total summer internal P load through translocation of P from the sediments into the water column (Barbiero and Welch 1992, Istanovics et al. 1993). This aspect of internal loading was not examined and should be considered in the future if *Gloeotrichia* blooms become a regular occurrence.

Acknowledgements

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