Evaluating the contribution of physicochemical parameters to two common off-flavor compounds in a drinking water reservoir

by

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Abstract

Off-flavors, such as 2-methylisoborneol (MIB) and geosmin, are compounds that produce an earthy or musty taste and odor, which can negatively impact drinking water treatment and aquaculture products. Although these compounds were first identified as secondary metabolites of actinomycetes, cyanobacteria are considered to be the primary cause of off-flavors in freshwater lakes and ponds. Humans can detect off-flavors at very low concentrations (10 and 30 ng/L for MIB and geosmin, respectively), and, although not a health risk, off-flavors can promote distrust between consumers and water utilities. Removal of these compounds requires a large financial investment and is not guaranteed. Controlling ecological factors that promote off-flavor production may be more cost-effective in addition to improving the ecosystem as a whole. Due to their ability to produce toxins, cyanobacteria have been under particular scrutiny, and environmental factors promoting cyanobacterial blooms are relatively well known. Using some accepted paradigms surrounding cyanobacterial production, I conducted two limnocorral experiments that manipulated physicochemical conditions aimed at influencing phytoplankton community structure and, thereby, off-flavor production. The first experiment was conducted during fall 2013 and consisted of an unbalanced factorial design with four nitrogen-to-phosphorus (N:P) ratios and three nitrogen levels. I found that the addition of both N and P promoted MIB and effects were associated with cyanobacterial and diatom biovolume. The second experiment was a complete factorial design conducted during summer 2014 using the nutrient concentrations from the fall experiment that were found to best promote off-flavor. I

also manipulated limnocorral mixing frequency to potentially affect growth and off-flavor production of diatoms, taxa not usually associated with off-flavor production. I found that MIB was positively correlated with diatoms, and negatively correlated with green algae and cryptophytes. I also found that geosmin was positively correlated with cyanobacteria. MIB and geosmin showed no concurrent trends throughout the second experiment, suggesting that different management approaches are required for each off-flavor compound. In general, off-flavors increased in both experiments under elevated nutrient conditions. Diatom biovolume was consistently correlated with MIB production, but contributions of cyanobacterial biovolume to off-flavors appeared to vary seasonally. These results indicate that lake managers should consider year-round control of nutrient loading and mixing patterns to better eliminate MIB and geosmin.

Acknowledgments

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Introduction

Off-flavors, such as 2-methylisoborneol (MIB) and geosmin can be a nuisance for lake managers, particularly in drinking water reservoirs, as these compounds result in an earthy or musty taste and odor that can be detected by humans at very low concentrations (10 and 30 ng/L, respectively). Although MIB and geosmin have not been associated with adverse health effects (Dionigi *et al.*, 1993) and are therefore only regulated as voluntary secondary standards by the Environmental Protection Agency (EPA), (You, 2012) these compounds are the cause of numerous complaints for drinking water facilities. Off-flavors are also commonly found in aquaculture either through exposure to skin or gills or through direct consumption of off-flavor producing organisms (Tucker, 2000). Off-flavor fish can lower product value, raise prices in non-off-flavor fish, and decrease consumer appeal (Tucker, 2000). In both industries, off-flavors can be economically damaging and can impact product reputation, consumer trust and future relationships (Hanson *et. al*, 2009; Srinivasan and Sorial, 2011). Currently, the only known methods of off-flavor removal in drinking water include oxidation by ozone (Glaze *et al*., 1990) and adsorption by activated carbon (Dabrowski *et al*., 2005). However, ozone oxidation has been known to produce harmful disinfection by-products (DPBs), (Srinivasan and Sorial, 2011) and activated carbon becomes ineffective with greater concentrations of organic matter in source water (Pirbazari *et al*., 1993; Newcombe *et al.*, 2002a; Newcombe *et al.*, 2002b). Moreover, both of these methods can incur considerable expense to water utilities. A financially practical treatment method with less potential for restriction has yet to be discovered (Srinivasan and Sorial, 2011). Furthermore, off-flavors may be indicative of nutrient loading problems at the

watershed level, (Davis *et al*., 2004) but exact causes and the conditions under which off-flavors are produced in the environment are unclear (Jüttner and Watson, 2007).

Several organisms have been identified as producers of off-flavor compounds, including eukaryotes such as fungi (Breheret *et al*. 1999), and amoebae, (Hayes *et al*., 1991) but off-flavors are most commonly attributed to filamentous heterotrophic bacteria, particularly actinomycetes, and filamentous photoautotrophic cyanobacteria (Jüttner and Watson, 2007). Although off-flavors were originally discovered in actinomycete cultures (Gerber, 1968), the most frequent source of off-flavor production is now generally attributed to cyanobacterial metabolism and, primarily, cyanobacterial degradation (Tabachek and Yurkowski, 1976; Durrer *et al*., 1999). Cyanobacterial contribution to off-flavor concentration is especially prevalent in warm, nutrient-rich waters (Paerl and Huisman, 2008). Cyanobacteria have been under considerable scrutiny since the mid-1970s when they were discovered to produce harmful toxic secondary metabolites (Carmichael, 1981). Although extensive research has been performed on the occurrence of cyanotoxins, predicting their occurrence remains unclear, especially in large, dynamic waterbodies (Watson *et al*., 2008). Due to the limited health risks posed by off-flavor compounds, even less is known regarding their production. As with cyanotoxins, off-flavors seem to be more difficult to predict across a broad spatial range as shown by Dzialowski *et al.* (2009), which demonstrated that up to 94% of geosmin occurrence could be attributed to environmental variables, such as total phosphorus concentration or cyanobacterial biovolume, within a given reservoir compared to 35% across all reservoirs sampled. Despite similarities, studies that have looked at cyanotoxins and off-flavor compounds have found that these by-products do not seem to coincide and cannot be adequately predicted by traditional models (Watson *et al*., 2008).

Although off-flavors have been studied since the 1950s, research has been limited to correlative observations with phytoplankton community composition or culture isolation. Very few studies have manipulated environmental variables *in situ* to determine effects on off-flavors. There are several environmental factors known to promote cyanobacterial dominance including elevated nitrogen (Elser *et al*., 2009) and phosphorus (Downing *et al*., 2001), low N:P ratios (Smith, 1983), reduced mixing (Visser *et al*., 1996), and elevated temperatures (Paerl and Huisman, 2008). Cyanobacteria have also been thought to have a competitive edge over other phytoplankton taxa under low N:P given their ability to fix atmospheric nitrogen using specialized cells called heterocysts (Stewart, 1980). Assuming cyanobacteria produced MIB and geosmin in a local drinking water reservoir in which off-flavors had been reported, I conducted a limnocorral experiment adjusting factors thought to favor cyanobacterial growth based on prior studies. I manipulated three nitrogen concentrations based on the findings of Downing *et al.*, (2001) which showed an increase in cyanobacterial biomass with elevated nitrogen. I also adjusted four N:P levels based on the findings of Smith (1983), which showed that cyanobacteria can be found more frequently at N:P (by mass) of 29 or below.

In order to properly manage water bodies at the watershed level, it is necessary to identify nutrient levels at which off-flavor is most abundantly produced. Due to the limited data available linking nutrient concentration and off-flavors, I chose a broad range of concentrations for my nitrogen levels which reflect conditions found in (1) mesotrophic lakes, (2) eutrophic reservoirs, and (3) hypereutrophic aquaculture ponds rarely seen in the field to assess directional effects on off-flavors. Using the findings of Smith, (1983) I chose four N:P ratios above and below the 29:1 threshold (cyanobacteria are thought to be common below this threshold) that covered a broad range (from 2:1 to 90:1) in an attempt to see directional effects of off-flavor across an N:P

gradient. I predicted that the highest concentrations of both cyanobacterial biomass and off-flavor would be found under high nitrogen levels and low N:P.

Methods

This experiment was conducted in a drinking water reservoir located in Opelika, Alabama, which had recently reported MIB and geosmin concentrations of up to 380 and 78ng/L (much higher than human detection levels of 10 and 30 ng/L), respectively during the summer of 2013. Saugahatchee Lake is a shallow (maximum depth = 8m) polymictic and eutrophic (total nitrogen (TN) = 300µg/L; total phosphorus (TP) = 30µg/L, N:P = 10:1, by mass) reservoir. The experiment was conducted during November and December 2013 when off-flavors were still present in the lake at low levels (>10ng/L for both MIB and geosmin).

Total N and TN:TP ratios were manipulated throughout a 47-day experiment in 3,000L clear, polyethylene limnocorrals that were sealed at the bottom, open to the atmosphere and suspended from floating PVC frames. Thirty-six enclosures were filled on 26 October 2013 by pumping lake water through a 75µm sieve to remove large zooplankton. Four randomly chosen enclosures were sampled on 28 October before treatments were added to retrieve baseline data, which helped determine initial off-flavor concentrations in the enclosures and the fertilizer regime for treatments. I was simultaneously interested in the effects of both nutrient (N) concentration and N:P. Therefore, nine treatments were randomly assigned to the enclosures with four replicates per treatment. The experiment consisted of four N:P (by mass) treatments including: (1) 2:1, (2) 10:1 (ambient), (3) 33:1, and (4) 90:1, within three N treatments including: (1) 300 µg/L (low), (2) 1,000 µg/L (medium), (3) 3,000 µg/L (high). Due to the nature of the ambient nutrients in the lake (i.e., at low and medium N levels, I could not extract P from enclosures to achieve a higher N:P ratio), I had two N:P treatments (10:1 and 2:1) in the low nitrogen enclosures, three N:P

treatments (10:1, 2:1, and 33:1) in the medium N enclosures, and all four N:P treatments in the high N enclosures (Table 1). Enclosures were fertilized with differing amounts of $NaNO_3$ and $NaPO_4$, to increase N and P, respectively. At the start of the experiment, ambient enclosures (low N, 10:1 N:P) received no additional nutrients, while the low N, 2:1 N:P treatment received P only. The medium N, 33:1 N:P treatment and high N, 90:1 N:P treatments received N only. All other treatments received various amounts of both N and P. Enclosures were fertilized weekly with 10% of the original treatment concentration to maintain N and N:P over time. Deionized water was added to ambient enclosures in lieu of fertilizers.

Treatments were established 4 November 2013 and sampling took place every other week from 5 November to 4 December and weekly from 4 December until 16 December. Depth-integrated samples (2.0 m depth) were collected for nutrients (TN, TP), phytoplankton biovolume and species composition, and MIB and geosmin using a rigid tube sampler. Temperature was taken at 0.5 m, 1 m and 2 m with a Hydrolab in each enclosure. TN and TP were measured using ultraviolet (TN) and colorimetric (TP) standard methods (Gross and Boyd, 1998). Preserved phytoplankton samples (1% Lugol's) were enumerated and identified to genus using an inverted microscope (Utermöhl, 1958). Cells were counted (25 fields per sample from 100-400X) and measured using Nikon Image software to estimate biovolume for each species. Based on existing diversity during the experiment, phytoplankton were grouped into four taxa including cryptophytes, cyanobacteria (blue-green algae), diatoms, and chlorophytes (green algae). Phytoplankton were counted for the pre-treatment date (28 October 2013), a mid-experiment date (17 November 2013), and the final date (16 December 2013). MIB and geosmin samples were stored in parafilm-sealed 20 ml glass vials without air bubbles and analyzed by solid-phase microextraction and gas chromatography/mass spectrometry (American Public Health Association, 2012).

Three enclosures were damaged during the study and not included in the analysis. Analytical processes could not distinguish differences in geosmin below 50ng/L, and geosmin data were not included in the analysis. All data were log-transformed to meet normality and constant variance assumptions. The effects of N levels, N:P, and their interaction on total phytoplankton biovolume were tested using repeated-measures analysis of variance (ANOVA factors = N level and N:P, repeated measure = time) across all dates excluding pre-treatment samples. I then used each combination of N-level and N:P to form a single categorical variable labeled "treatment" (ANOVA factors = treatment; repeated measure = time) to run a Tukey's post-hoc test for total phytoplankton biovolume over time, which identified two unique subsets on the basis of nutrient treatments (group 1: N or P or neither; group 2: both N and P). To explicitly test whether treatments with or without MIB tracked phytoplankton biovolume, I used a linear contrast to compare concentrations for the two subsets identified through previously mentioned post-hoc testing results. A mixed effects linear regression model was used to determine the relationship among biovolume of phytoplankton taxa and MIB (response = MIB, predictors = diatoms + cyanobacteria + cryptophytes + green algae, random effect = time). A linear regression was used to determine the relationship between diatom biovolume and MIB on the final date of the experiment (16 December 2013). The relationship between cyanobacterial biovolume and MIB was assessed using a quadratic regression as residuals were not normally distributed. Pre-treatment phytoplankton counts were excluded from the model.

Results

Throughout the experiment, I was able to maintain established N and P concentrations (Fig. 1A and 1B). N:P decreased slightly over time in the high N:P treatments (90:1 and 33:1) since small differences in total N or total P can affect N:P. However, N:P maintained the same rank order over time (Fig. 1C). Average temperature for three depths ranged from 18.6^{o}C on 28 October 2014 to 9.7^{o}C on 16 December 2014. Nitrogen level, N:P, and their interaction all had significant effects on total phytoplankton biovolume over time (N level – $F_{2,24} = 79.82$, $P<0.0001$; N:P – $F_{3,24} = 24.19$, $P = 0.0001$; N level × N:P – $F_{3,24} = 15.25$, $P < 0.0001$) (Fig. 2A). Post-hoc testing revealed two unique subsets showing an increase in phytoplankton biovolume in treatments where both N and P were added (Medium N, 10:1 N:P; Medium N, 2:1 N:P; High N, 10:1 N:P; High N, 2:1 N:P; and High N 33:1 N:P) compared to treatments where only N, only P, or neither were added (Low N, 10:1 N:P; Low N, 2:1 N:P; Medium N, 33:1 N:P; and High N, 90:1 N:P) (Treatment global test: $F_{8,23} = 28.62$, $P<0.0001$). This indicated that by the end of the experiment, treatments where only one nutrient was added were limited by N or P. Based on the results of the post-hoc testing of total biovolume over time, I ran a contrast for MIB (Response = MIB, fixed effects = High N, 33:1 N:P + High N, 10:1 N:P + High N, 2:1 N:P + Medium N, 10:1 N:P + Medium N, 2:1 N:P, random effects = time) showing that MIB was also significantly higher in treatments that had both nutrients added than treatments that had one or no nutrients added ($T_{62} = 9.49$, $P<0.0001$) (Fig. 2B).

The mixed effects linear regression, showed a significant correlation between MIB and diatom biovolume ($T_{59} = 6.13$, $P<0.00001$), and between MIB and cyanobacterial biovolume

(T_{59} = 3.96, *P*=0.0002) (Fig. 3B), but only a marginally significant relationship between MIB and cryptophyte biovolume (T_{59} = 1.79, *P*=0.079). Given that both diatom and cyanobacterial biovolume were strongly correlated with MIB, contrasts were run to determine differences between treatment subsets that had been previously identified by post-hoc testing. Both diatom (T_{62} = 4.90, *P*<0.0001) and cyanobacterial (T_{62} = 3.53, *P*<0.0001) biovolume were significantly increased when both nutrients were added. On the final date of the experiment, diatom biovolume exhibited the strongest relationship with MIB (R^2 = 0.65, $F_{1,31}$ = 55.88, *P*<0.0001) (Fig. 3A), and the relationship between cyanobacterial biovolume and MIB was slightly weaker (R^2 = 0.56, $F_{2,31}$ = 18.65, *P*<0.0001) (Fig. 3B). Even though both taxa split into the two unique subsets identified through the phytoplankton and MIB analyses, cyanobacterial biovolume exhibited a general downward trend over time in all treatments (Fig. 4A). Diatom biovolume generally increased in treatments with N and P, but decreased or remained constant in treatments with only N, only P or neither (Fig. 4B).

Discussion

Nutrient limitation is often a significant driver of phytoplankton competition and dominance in aquatic systems. It is commonly accepted that some nitrogen-fixing cyanobacteria will dominate under low N:P (Schindler, 1977). Throughout this experiment, I expected to see cyanobacteria dominate species composition, especially in low N:P (2:1) enclosures or at high nitrogen levels (Downing *et al.*, 2001). However, cyanobacteria were generally rare at all N levels, decreased over time, and became the least dominant taxa in all treatments by the end of the experiment (Fig. 5). As the experiment was conducted in November and December, the decrease in cyanobacteria and limitations in growth may be attributed to colder temperature, higher rates of thermal-mediated mixing (Visser *et al*., 1996), and reduced light. Percent composition of diatoms did not differ by treatment, but, unlike cyanobacteria, demonstrated an increase over the course of the experiment. The lack of compositional differences between treatments may be attributed to other factors, such as limiting micronutrients (i.e., silica) that were not measured (Schrader and Blevins, 2001). These patterns support predictions from seasonal manipulations and phytoplankton succession models (Sommer et al., 1986; Visser et al., 1996).

Although N level, N:P and their interaction were significantly related to MIB concentration, the greatest predictor of MIB appeared to be the addition of both N and P. I cannot adequately compare our findings to those of Smith (1983) as I did not see cyanobacterial dominance in any of the enclosures, but a majority of the treatments that consisted of N:P (by mass) < 29 produced a greater amount of MIB with the exception of the low nitrogen treatments.

One way to test the findings of Smith (1983) on MIB production would be to conduct the experiment during warmer months when cyanobacteria are more likely to dominate or to design a complete factorial experiment manipulating N and P concentrations and N:P. Because MIB generally grouped into treatments that had either been fertilized with both N and P or those that had been fertilized with one or no nutrients, it is difficult to determine whether specific nutrient levels or individual N:P ratios truly had an effect. However, it is clear that MIB concentrations increased when both N and P were added to the system.

A possible mechanism contributing to the observed treatment differences in MIB may be cyanobacterial decay as lake temperatures cooled. After isolating *Fischerella muscicola*, Wu and Jüttner (1988) found geosmin at higher concentrations within the intracellular fraction, causing a period of high off-flavor occurrence during bloom die-off.

Nutrient loading has also been attributed to cyanobacterial death and corresponding off-flavor concentration increases, but MIB and geosmin do not always follow similar patterns (Ma *et al*., 2013). In general, MIB seems to be less prevalent than geosmin in the literature, which has resulted in fewer studies looking at specific production sources. Similarly, diatoms tend to be a less common problem, especially in aquaculture where many off-flavor studies take place, which, given the results of this study, may be further reason for the lack of information surrounding MIB.

Another potential mechanism for MIB production throughout this study may be linked to diatoms. *Synedra* spp. was the most common diatom observed during the experiment. Although rarely reported in the literature, this study supports others which have linked *Synedra* and MIB. (Sugiura *et al*., 2004; Sugiura *et al*., 1998; Izaguirre and Taylor, 1998; Schrader *et al*., 2011; Xu *et al*., 2010) Even though *Synedra* has not been shown to directly produce MIB, it has been identified as a substrate for the growth of *Streptomyces* spp., an MIB-producing actinomycete

(Sugiura *et al*., 1994). However, there has only been one study that explored the mechanism behind off-flavor compounds and diatoms (Sugiura et al., 1994), while many of the other known studies, have only been able to identify a correlation between these two variables. Additional research is necessary to understand the relationship among diatoms, actinomycetes, and off-flavor production.

Actinomycetes were not measured in this study and may have been a factor for MIB increases, but many studies have reported conflicting results when evaluating environmental parameters and off-flavor production by actinomycetes. Additionally, most of these studies have been focused solely on geosmin rather than on MIB and geosmin. In general, actinomycete biomass tends to increase under elevated nutrients (both N and P) (Wood *et al*., 1985). Although geosmin production has been observed at both high and low N (Blevins *et al*., 1995; Schrader *et al*., 2013), this off-flavor compound has only been detected in systems with high P concentrations (Schrader and Blevins, 2001). Despite reported patterns for N, P, actinomycete biomass, and geosmin concentrations, a strong correlation across waterbodies that vary in nutrient concentrations has yet to be determined (Schrader *et al*., 2013; Schrader and Blevins, 2001). Temperature has also been shown to have an effect on geosmin production by actinomycetes, but reports have been conflicting. For example, Wood *et al.* (1985) found that actinomycetes best produced geosmin at 30 $^{\circ}$C, but will still occur at 15 $^{\circ}$C. However, Aoyoma *et al.* (1993) reported lower optimal temperatures for geosmin production (15 $^{\circ}$C), but higher temperatures for actinomycete growth (30 $^{\circ}$C). Temperature may also have an interactive effect with pH as Blevins *et al.* (1995) showed similar results for optimal actinomycete growth temperatures, but a higher propensity for actinomycetes to produce geosmin at 35 $^{\circ}$C under pH 9.

There is some evidence showing off-flavor production by actinomyceters are absent under anaerobic conditions (Wood *et al*., 1985). Given the time of year, increased mixing, and

oxygen availability during this study actinomycetes may have been more able to contribute to increases in MIB than other times of the year when thermal mixing is reduced. However, with the discrepancies in the literature regarding optimal conditions for actinomycete growth, and the lack of information regarding actinomycetes and MIB, it is necessary for research efforts to become more focused on off-flavor contribution by actinomycetes before a mechanism for off-flavor production can be determined in similar study systems. Although mechanisms and specific sources of MIB production could not be detected from this study, the contribution of nitrogen and phosphorus addition is clear and further supports the need for nutrient management.

Table and Figures

Table 1. Description of the unbalanced factorial design using four nitrogen: phosphorus within three nitrogen concentrations. Herein triangles represent the ambient (300 μg/L) nitrogen treatments, diamonds represent the medium (1,000 μg/L) nitrogen treatments, and circles represent the high (3,000 μg/L) nitrogen treatments. Grey symbols indicate 2:1 N:P treatments, white indicates 10:1 N:P treatments, black indicates 33:1 N:P treatments, and black and white symbols indicate the 90:1 N:P treatment.

		N:P (by mass)			
		2:1	10:1	33:1	90:1
Nitrogen (ug/L)	300	△	▲	N/A	N/A
	1000	◇	◆	◆	N/A
	3000	○	●	●	◑

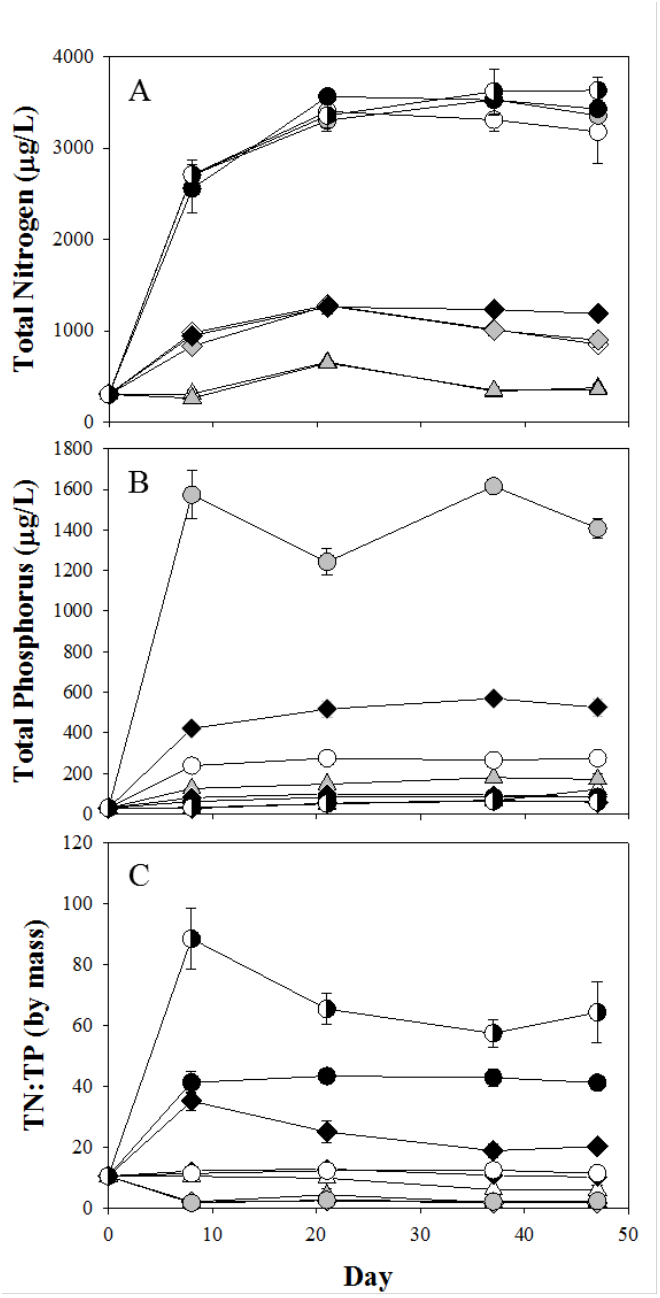

Figure 1. (A) Total nitrogen (µg/L), (B) total phosphorus (µg/L), and (C) total nitrogen: total phosphorus (by mass) over the 47-day experiment. Data represent means ± one standard error. Refer to Table 1 for symbol legends. Triangles represent the ambient (300 µg/L) nitrogen treatments, diamonds represent the medium (1,000 µg/L) nitrogen treatments, and circles represent the high (3,000 µg/L) nitrogen treatments. Grey symbols indicate 2:1 N:P treatments,

white indicates 10:1 N:P treatments, black indicates 33:1 N:P treatments, and black and white symbols indicate the 90:1 N:P treatment.

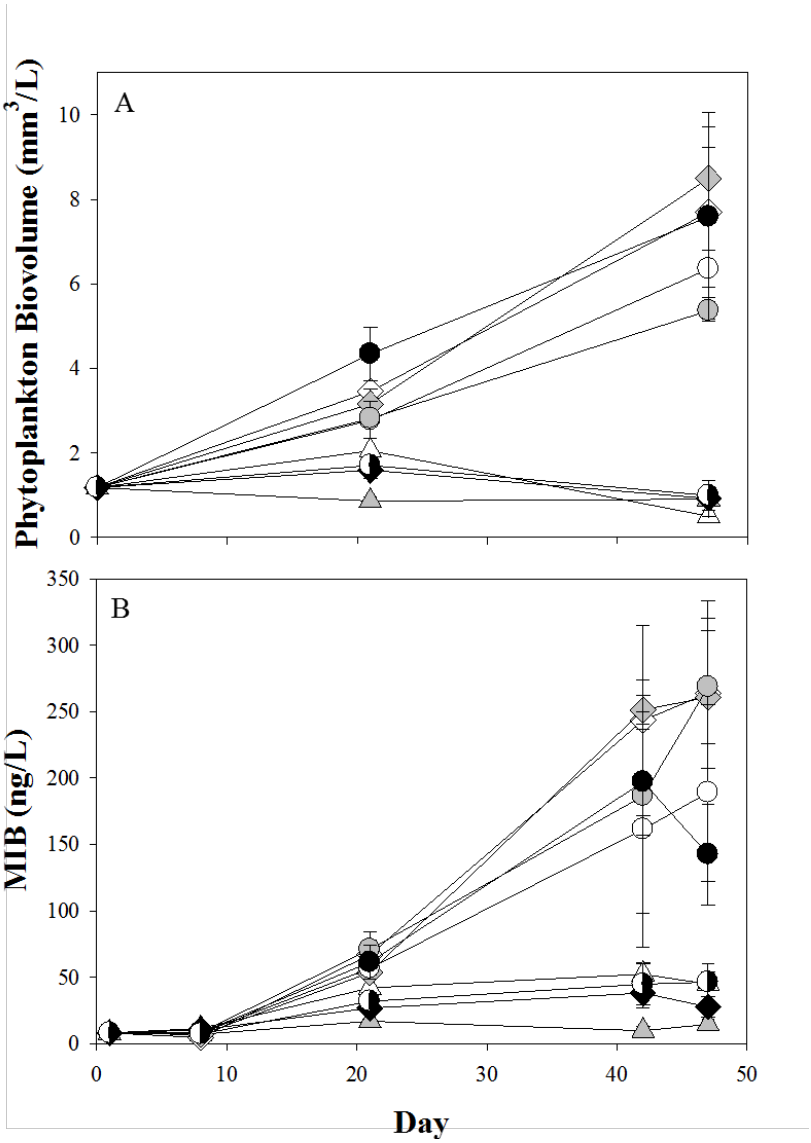

Figure 2. (A) Total phytoplankton biovolume (mm^3/L) and (B) 2-methylisoborneol (MIB) (ng/L) throughout the 47-day experiment. Data represent means ± one standard error. Refer to Table 1 for symbol legends. Triangles represent the ambient (300 µg/L) nitrogen treatments, diamonds represent the medium (1,000 µg/L) nitrogen treatments, and circles represent the high (3,000 µg/L) nitrogen treatments. Grey symbols indicate 2:1 N:P treatments, white indicates 10:1 N:P treatments, black indicates 33:1 N:P treatments, and black and white symbols indicate the 90:1

N:P treatment.

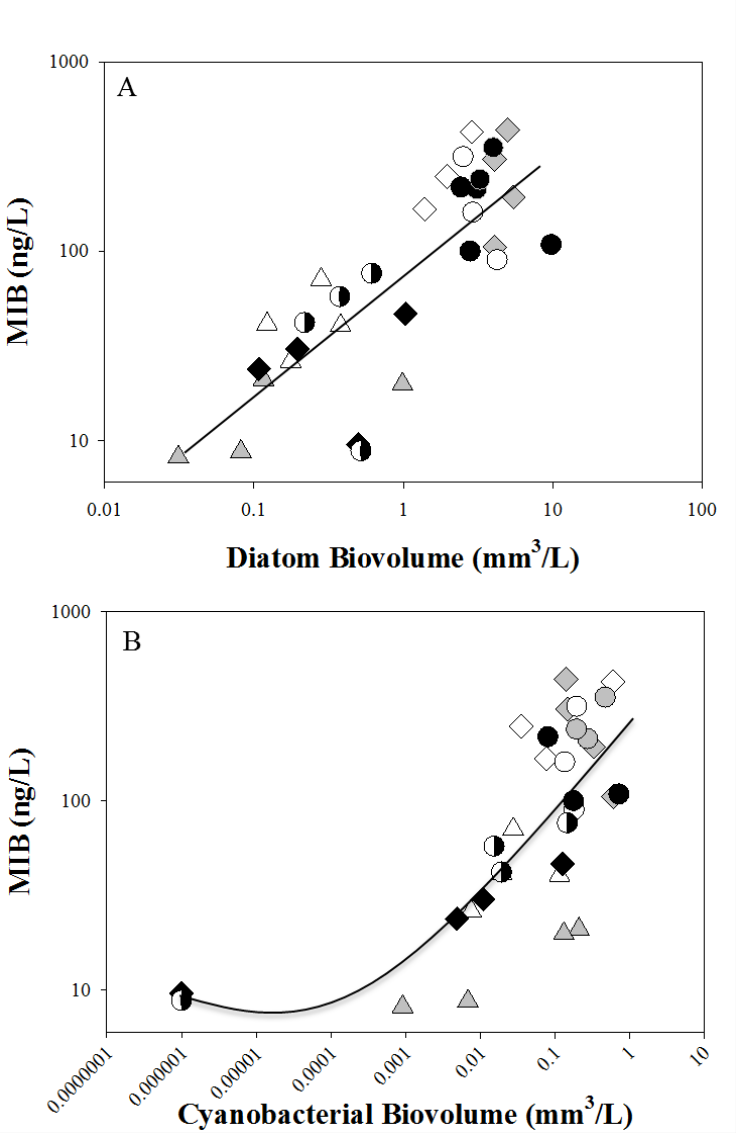

Figure 3. The relationship between (A) log 2-methylisoborneol (MIB) (ng/L) and log diatom biovolume (mm^3/L) (R^2 = 0.65, P < 0.0001) (B) log 2-methylisoborneol (MIB) (ng/L) and log cyanobacterial biovolume (mm^3/L) and log MIB (ng/L) (R^2 = 0.56, P < 0.0001) on the last day of the 47-day experiment (16 December 2013). Refer to Table 1 for symbol legends. Triangles represent the ambient (300 µg/L) nitrogen treatments, diamonds represent the medium (1,000 µg/L) nitrogen treatments, and circles represent the high (3,000 µg/L) nitrogen treatments. Grey

symbols indicate 2:1 N:P treatments, white indicates 10:1 N:P treatments, black indicates 33:1 N:P treatments, and black and white symbols indicate the 90:1 N:P treatment.

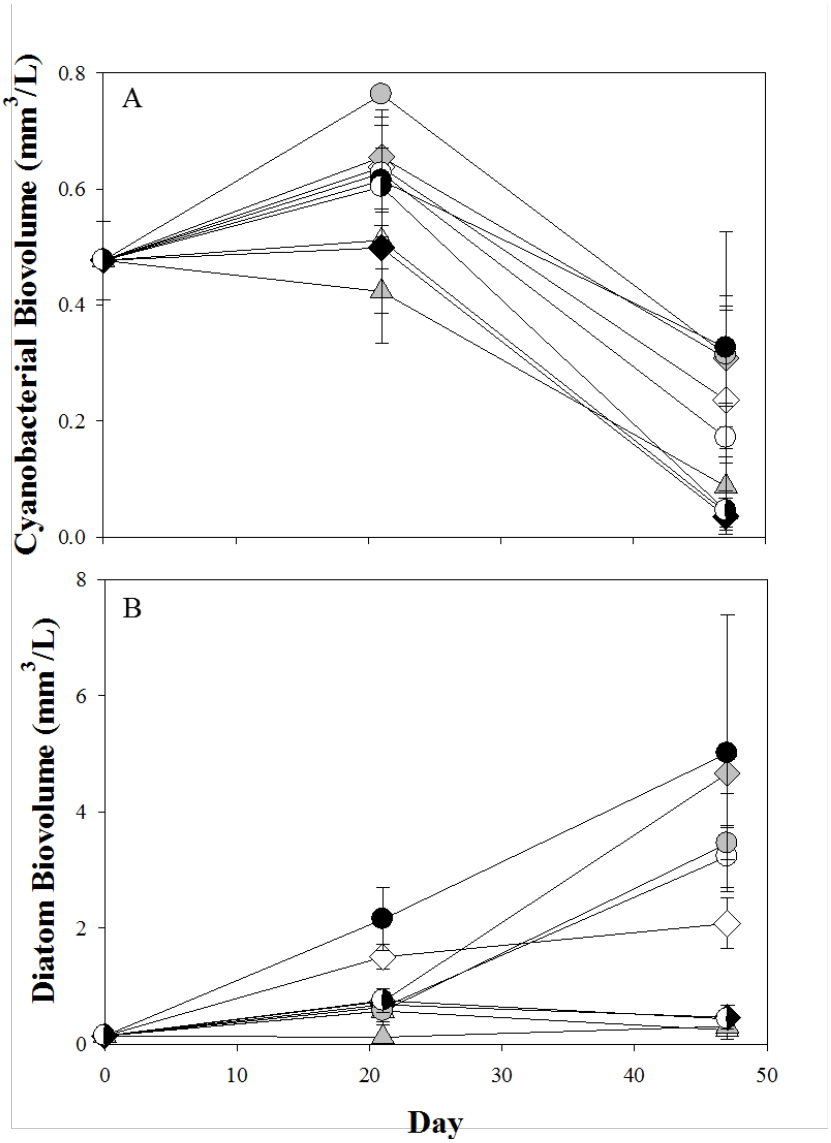

Figure 4. (A) Cyanobacterial biovolume (mm^3/L), and (B) diatom biovolume (mm^3/L) over the 47-day experiment. Data represent means ± one standard error. Refer to Table 1 for symbol legends. Triangles represent the ambient (300 µg/L) nitrogen treatments, diamonds represent the medium (1,000 µg/L) nitrogen treatments, and circles represent the high (3,000 µg/L) nitrogen treatments. Grey symbols indicate 2:1 N:P treatments, white indicates 10:1 N:P treatments, black indicates 33:1 N:P treatments, and black and white symbols indicate the 90:1 N:P treatment.

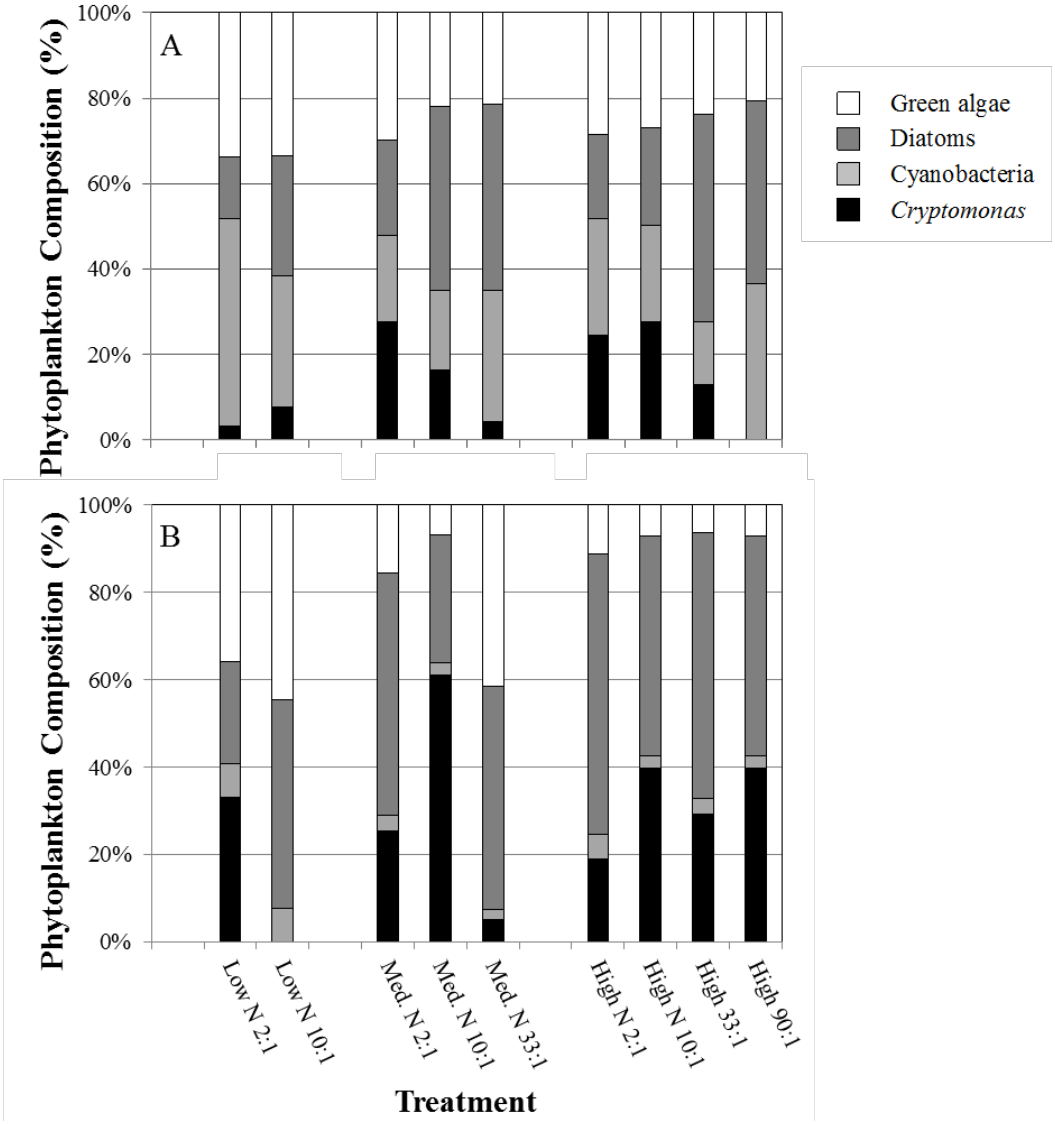

Figure 5. Phytoplankton relative abundance (% biovolume) by 2 dates per treatment. Low indicates the ambient (300 µg/L) nitrogen treatment, Med. represent the 1,000 µg/L nitrogen treatments, and High indicates the 3000 µg/L nitrogen treatments. Numbers following Low, Med, and High represent a N:P (by mass) of 2:1, 10:1, 33:1 or 90:1. Data represent mean percentage for green algae (white), diatoms (dark grey), cyanobacteria (light grey), and *Cryptomonas* (black).

Chapter 2: Lake mixing and nutrient addition influence MIB and geosmin in a drinking water reservoir

Introduction

2-Methylisoborneol (MIB) and geosmin, are organic compounds that produce earthy, musty tastes and odors in freshwater systems (Medsker *et al*., 1968) and are collectively referred to as off-flavors. These compounds can be a problem for drinking water and aquaculture facilities, since methods of removal are expensive and are potentially ineffective or dangerous for human consumption (Srinivasan and Sorial, 2011; Pirbazari *et al*., 1993). MIB and geosmin were initially thought to be produced primarily by actinomycetes, a gram-positive microbe commonly found in soils (Gerber, 1968). However, MIB and geosmin were identified in isolated colonies of filamentous cyanobacteria (Tabacheck and Yurkowski, 1976), which have come under particular scrutiny for their ability to produce toxic secondary metabolites (Carmichael and Mahmood, 1984). Since then, cyanobacteria have been commonly attributed as the primary source of off-flavors, (Jüttner and Watson, 2007), and are the focus of many studies on MIB and geosmin outbreaks. Some studies have indicated that diatoms, particularly *Synedra* spp., can contribute to off-flavor production, but evidence has been mostly correlational (Olsen *et al*., In prep; Izaguirre and Taylor, 1998; Schrader *et al*., 2011). Other studies have suggested that diatoms do not directly produce off-flavor compounds, but can provide substrate and carbon for actinomycete growth, thus enabling actinomycetes to produce MIB and geosmin (Sugiura *et al*., 1998; Sugiura *et al*, 2004).

Despite the lack of understanding regarding biological causes of off-flavors, efforts associated with off-flavor management have focused on engineering approaches to remove these

compounds. However, ultimate environmental factors that mediate off-flavor outbreaks still remain relatively unknown (Srinivasan and Sorial, 2011). Some laboratory-based studies have manipulated variables such as nutrients, light intensity, and temperature using isolates of known off-flavor producing organisms (Saadoun *et al*., 2001, Zhang *et al.*, 2009). Additionally, several observational studies have monitored aquatic parameters including algal biovolume, water chemistry, temperature, and weather events, (Uwins *et al*., 2007; Izaguirre *et al*., 1982; Martin *et al*., 1987) but these variables can be difficult to manipulate parameters *in situ*. One recent study conducted by Olsen *et al*. (in prep) that manipulated nitrogen concentration and nitrogen-to-phosphorus ratios (N:P) in limnocorrals in an Alabama reservoir found that both nitrogen and phosphorus were co-limiting in the production of MIB. As with previous observational studies, toward the end of their experiment Olsen *et al*. (in prep) found a strong relationship between MIB and cyanobacteria ($R^2 = 0.56$). However, the relationship between diatoms, and MIB was stronger ($R^2 = 0.65$).

Limnological processes and phytoplankton communities may vary seasonally (Mazumder *et al*, 1990), which likely affect off-flavor occurrences (Westerhoff *et al*, 2005). Like cyanobacterial toxins, off-flavor events are difficult to predict and may depend on changing chemical, biological, and physical conditions in lakes. When developing models to best predict off-flavors in Taihu Lake, China, Qi *et al*. (2012) found that cyanobacterial biomass was the best predictor during warmer months, where the diatom, *Synedra*, was a better predictor of off-flavors during cooler months. The primary driver of seasonal differences involves changes in temperature, which influences water chemistry and water movement in lakes. Summer stratification and high temperatures favor cyanobacterial blooms (Pearl and Huisman 2008) but generally excludes diatoms, which rely on lake mixing for movement to the surface given that they lack flagella or buoyancy-regulation vacuoles (Sommer *et al*., 1986).

Many past studies that have investigated off-flavor events have focused on geosmin (Billica *et al*., 2010; Lauderdale *et al*., 2004; Saadoun *et al*., 2001), since biosynthetic pathways are better known for this compound than for MIB (Jüttner and Watson, 2007). Most studies that have addressed both MIB and geosmin either focused on removal methods (Geldenhuys *et al*., 1996; Cook *et al*., 2001) or on monitoring programs evaluating off-flavor outbreaks. Few studies have hypothesized why one compound should be favored over the other (Izaguirre and Taylor, 2007).

Saugahatchee Lake in Opelika, AL is a local drinking water reservoir. After complaints of a high occurrence of off-flavor compounds during summer and fall 2013, Olsen *et al*. (In prep.) found that MIB increased under elevated N and P concentrations and, in late fall, was primarily correlated with cyanobacterial and diatom biovolume. In this study I continue to investigate differences in off-flavor production of two compounds (MIB and geosmin) between diatoms and cyanobacteria by manipulating nutrients and mixing in a mesocosom-field experiment during the summer of 2014 when the reservoir was fully stratified. Treatments were chosen based on the alternating conditions that favor diatoms (mixing) and certain cyanobacterial species (high nutrients and no-mixing).

Methods

Nutrients (nitrogen and phosphorus) and mixing were manipulated in a fully-factorial design throughout a 28-day experiment in 4,300L limnocorrals composed of clear polyethylene. Enclosures were open to the atmosphere, sealed at the bottom and suspended by floating PVC frames in Saugahatchee Lake, Opelika, Alabama. I chose to run the experiment during the summer (24 July 2014 through 21 August 2014) when the lake was stratified to manipulate mixing conditions and see effects of stratified versus mixed conditions. Saugahatchee Lake is a polymictic and eutrophic (TN = 300µg/L; TP = 30µg/L, N:P = 10:1 [by mass]) reservoir with a maximum depth of 8 m. Sixteen enclosures were filled by pumping lake water through a 75 µm mesh sieve to remove large-bodied zooplankton. I randomly chose four enclosures to sample before treatments were established for baseline data. The concentrations of off-flavor compounds measured at the start of the experiment were low (MIB=8 ng/L and geosmin=12 ng/L). One of four treatments was randomly assigned to enclosures in a 2 × 2 factorial design with four replicates per treatment. Treatments included a (1) control (no mixing and no additional nutrients) (2) mixing only (3) nutrient addition only, and (4) mixing and nutrient addition. Each replicate of the two mixing treatments (treatments 2 and 4) was thoroughly mixed, using the circular frame of a zooplankton net wrapped in clear, polyethylene to create a solid disk. Holes were punctured in the polyethylene to aid in sinking. The mixing disk was suspended at 3 points by rope, which was used to pull the device through the water column. Mixing began on 26 July and took place every other day generally before noon by slowly dropping the disk to the bottom of the appropriate enclosures (3.5 m depth) and quickly pulling the device through the water column. This process was repeated three times per enclosure. The mixing device was rinsed in

the reservoir between treatments 2 (mixing only) and 4 (mixing and nutrient addition) to minimize contamination. The mixing device was not used for the other two treatments. Nitrogen and phosphorus concentrations in nutrient treatments (treatment 3 and 4) were chosen based on the results of Olsen *et al*. (In prep), which showed that MIB exhibited a significant increase when N was at 1,000 μg/L and P was at 100 μg/L (N:P = 10). Nutrients were added once to relevant enclosures on 26 July 2014. Enclosures were not fertilized again throughout the remainder of the experiment.

Enclosures were sampled weekly throughout the duration of the experiment with a depth integrated tube sampler (2 m). Samples were brought to the lab and processed for nutrients (TN and TP), phytoplankton biovolume and composition, and MIB and geosmin concentration. Nutrient concentration was determined by spectrophotometry using ultraviolet (TN) and colorimetric (TP) standard methods (Gross and Boyd, 1998). Phytoplankton samples were preserved in 1% Lugol's and were identified to genus and enumerated using inverted microscopy (Utermöhl, 1958). Twenty-five fields within each phytoplankton sample were counted using 100-400X; cells were measured using Nikon Image to calculate average algal biovolume for each genus. Phytoplankton were counted for the pre-treatment date (24 July 2014), the first date after treatments were applied (31 July 2014), mid-experiment (15 August 2014), and at the experiment's end (21 August 2014). Whole-water was stored in glass vials sealed with parafilm and analyzed through solid phase micro-extraction using gas chromatography/mass spectrometry (Standard Methods 6040-D).

One enclosure was damaged during the study and not included in the analysis. Nutrient data for 15 August 2015 are not presented due to sample contamination. All data were log-transformed to meet normality assumptions. A repeated measures analysis of variance (ANOVA) was used to examine the effects of nutrients, mixing, and their interaction on phytoplankton

biovolume. Tukey's post-hoc testing on phytoplankton revealed two unique subsets among treatments. To explicitly test whether off-flavor patterns tracked changes in phytoplankton biovolume, I used a linear contrast to compare concentrations for these two subsets for both MIB and geosmin. Phytoplankton were categorized into four groups including cryptophytes, cyanobacteria, diatoms, and green algae. I used a mixed model to determine the effects of individual phytoplankton taxa on MIB and geosmin concentration (response variables = MIB or geosmin, predictors = diatoms + cryptophytes + green algae + cyanobacteria, random effect = time). A simple linear regression was used to determine if patterns in MIB and geosmin concentrations were similar over time.

Results

After treatments had been applied, phytoplankton biovolume was highest in the two treatments with added nutrients (treatments 3 and 4) (repeated measures ANOVA $F_{1,12} = 34.256$, $P<0.0001$) (Fig. 1A). Mixing only or the interaction of nutrients and mixing did not have an effect on phytoplankton biovolume. Post-hoc testing revealed two statistically different subsets for phytoplankton biovolume over time including the control and mixing only treatments (treatments 1 and 2) or the nutrient only and mixing and nutrient addition treatments (treatments 3 and 4) ($F_{3,11} = 11.34$, $P=0.001$).

Added nutrients ($F_{1,11} = 40.73$, $P<0.0001$) and mixing ($F_{1,11} = 6.69$, $P=0.025$) significantly affected MIB over time while their interaction had a marginal effect ($F_{1,11} = 4.51$, $P=0.057$). Three subsets were derived from post-hoc tests that indicated differences among the control and mixing only treatments (treatments 1 and 2), which were significantly lower than the mixing only and nutrient only treatments (treatments 2 and 3). Nutrient addition and mixing (treatment 4) had the highest effect on MIB concentration (Fig. 1B). Geosmin was elevated in treatments where nutrients were added regardless of mixing ($F_{1, 11}=72.06$, $P<0.0001$) (Fig. 1C). Post-hoc testing for geosmin reflected the global test revealing two subsets consisting of the control and mixing only treatments, which were significantly lower than the nutrient only and mixing and nutrient treatment (Tukey's test $F_{3,11} = 26.51$, $P<0.0001$). Interestingly, MIB and geosmin patterns differed over time (Figs. 1B and C) where the highest concentration for geosmin was seen in the nutrient (treatment 3) and nutrient and mixing (treatment 4) treatments immediately after treatments were added, but concentrations decreased in all enclosures over

time, so that by the end of the experiment geosmin concentrations in all treatments neared 10 ng/L or below and were no longer significantly different. Geosmin patterns contrasted with MIB given that MIB did not start to demonstrate differences until the third week of the experiment and highest concentrations were observed at the end of the experiment in enclosures that received nutrients and were mixed (treatment 4). A simple linear regression used to compare the two off-flavor compounds showed that there was no significant correlation between geosmin and MIB over time ($F_{1,59} = 1.49$, P=0.227, $R^2 = 0.025$).

When examining the contribution of individual phytoplankton taxa to MIB using a mixed effects model, I found that each phytoplankton taxa, except cyanobacteria, correlated strongly with MIB. Diatoms were the only taxa that corresponded positively with MIB ($T_{38} = 3.74$, P=0.0006) (Fig. 2A). The relationship between cryptophytes and MIB ($T_{38} = 4.58$, P=0.0001) green algae and MIB ($T_{38} = 3.99$, P=0.0003) were stronger than those observed with diatoms. Although cryptophyte and green algae coefficients were among the strongest, they were also negative, indicating that biovolume was decreasing with rising MIB concentration (Figs. 2B and C).

Cyanobacteria were the only algal group that had a significant effect on geosmin concentration ($T_{38} = 2.56$, P=0.015). Trends in cyanobacterial biovolume were similar to total phytoplankton biovolume, (Fig. 2D) whereas geosmin concentrations dropped over time in all treatments after an initial increase (Fig. 1C)

Discussion

After establishing the treatments, cyanobacteria dominated all enclosures; especially where nutrients had been added (Fig. 3). Although cyanobacteria continued to dominate the treatment where nutrients only were added, diatoms dominated the other three treatments by the end of the experiment. Relative diatom abundance increased in all enclosures that had been mixed. It was surprising to see an increase in diatoms in the control enclosures that were not mixed or were given additional nutrients. One possible explanation to the increase in diatoms under non-mixing conditions could be the severe rain events experienced during the week of August 11th. Throughout this week, water was flowing over the reservoir's dam and possible bag movement by flow may have disrupted diatoms, and previous studies have shown a relationship between diatoms and heavy rainfall events (Pearsall, 1923; Breton *et al*., 2006; Kilham *et al*., 1996). The fact that diatoms did not increase in the nutrient only treatment during the rainfall event may indicate that cyanobacteria are better able to compete with diatoms when nutrients are high regardless of heavy rain events.

Diatoms were the only phytoplankton taxa that had a positive relationship with MIB over time. Added nutrients and mixing (treatment 4) caused the greatest increase in MIB by the end of the experiment. Concomitantly diatoms also showed an increase in this treatment. There was also a slight increase in MIB for the nutrient-only enclosures, although not statistically different from the mixing only treatment. An increase in diatoms was not seen in the nutrient-only enclosures; in fact, diatoms were rarely present in these enclosures throughout the last two weeks of the

experiment. Diatoms increased in the mixing only and control treatments throughout the duration of the experiment.

One explanation for the discrepancies among treatment patterns for phytoplankton taxa and MIB could be other off-flavor producing organisms, such as actinomycetes, which were not measured in this study. Although actinomycetes were the first organism to be associated with off-flavors (Medesker, 1968), cyanobacteria have been thought to be the biggest contributor of off-flavors in large aquatic systems (Jüttner and Watson, 2007). Several studies have looked at environmental contributions to actinomycete growth and off-flavor production, but no consensus has been reached on the potential for nutrients to increase off-flavor production by actinomycetes (Wood *et al.*, 1985; Schrader *et al.*, 2013; Schrader and Blevins, 2001). Given that actinomycetes are important decomposers, added nutrients would indirectly influence actinomycetes through increased producer and consumer detritus. Few, if any studies, have studied the effect of mixing on actinomycetes, but one study by Allgaier and Grossart (2006) found that actinomycete biomass peaked during the fall and spring, corresponding to typical lake mixing events. Additionally, actinomycetes have been found to use diatoms and cyanobacteria as substrate for growth (Sugiura *et al.*, 1994), which may explain the positive correlation between diatoms and MIB, as diatoms have not been shown to directly produce off-flavors.

Geosmin was markedly higher in the mixing only (treatment 3) and nutrient addition and mixing (treatment 4) treatments immediately after treatments were applied and decreased to less than 20 ng/L in all treatments by the end of the experiment. Cyanobacteria was the only phytoplankton taxa that correlated with geosmin over time, and percent composition shows that cyanobacteria were dominant in both of these treatments throughout the experiment (Fig. 3), but percent composition decreased in treatment 4 and began to get replaced by diatoms near the end

of the experiment. Although there is an increase in cyanobacterial dominance throughout the experiment, there is a relationship between cyanobacteria and geosmin ($F_{1, 43} = 15.48$, $P<0.0001$, $R^2= 0.26$), which may indicate that total cyanobacterial biomass is the best predictor of geosmin. After an initial increase post-nutrient application, phosphorus levels decreased in all treatments (Fig. 4A). Nitrogen generally exhibited a similar pattern as phosphorus, but increased considerably in the mixing only treatment (Fig. 4B). As *Cylindrospermopsis spp.* was the dominant phytoplankter (>99%) in nutrient only enclosures, it is possible that the increase seen in nitrogen and N:P is an effect of nitrogen fixation (Presing *et al*., 1996, Chislock *et al.*, 2014), made even more evident by an increase in cyanobacterial biovolume during the previous week (Fig. 2D). However, I did not see a rise in geosmin toward the end of the experiment. Similar effects were seen in Peterson *et al*., (1995) where geosmin exhibited an increase when nitrogen fixation was inhibited. This may indicate that the form of nitrogen available to cyanobacteria may be an important determinant in off-flavor production.

MIB and geosmin dynamics were not consistent throughout this experiment and few studies have addressed the ecological trends of these two compounds. Although I did not find a correlation between MIB and geosmin concentrations, both compounds increased when exposed to added nutrients and mixing, and geosmin increased with elevated nutrients only. These results indicate that lake management strategies may need to include a combination of approaches that minimize year-round nutrient inputs and stratification patterns to control the production of both compounds

Figures

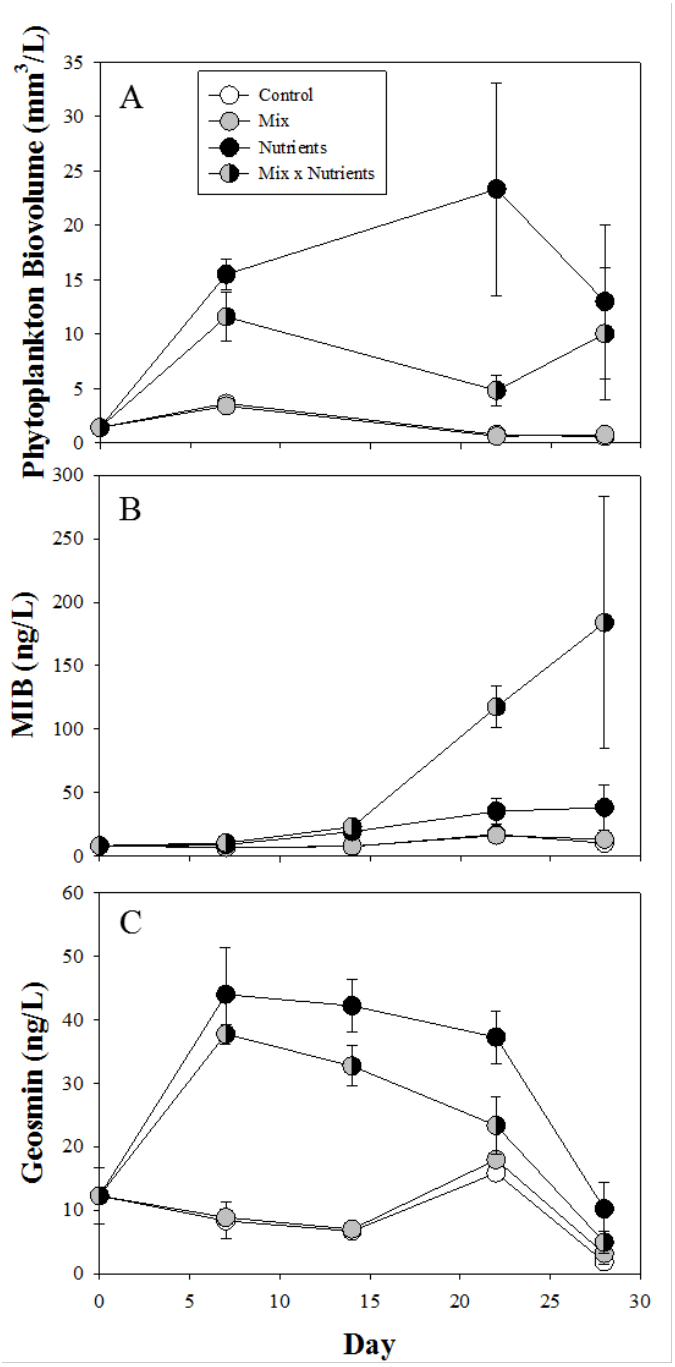

Figure 6. (A) Phytoplankton biovolume (mm^3/L), (B) 2-methylisoborneol (MIB) (ng/L), and (C) geosmin (ng/L) over the 28-day experiment. Data represent means ± one standard error. White circles indicate the

control treatment, grey circles represent the mixing only treatment, black circles represent the nutrient only treatment, and black and grey circles indicate the mixing and nutrient treatment.

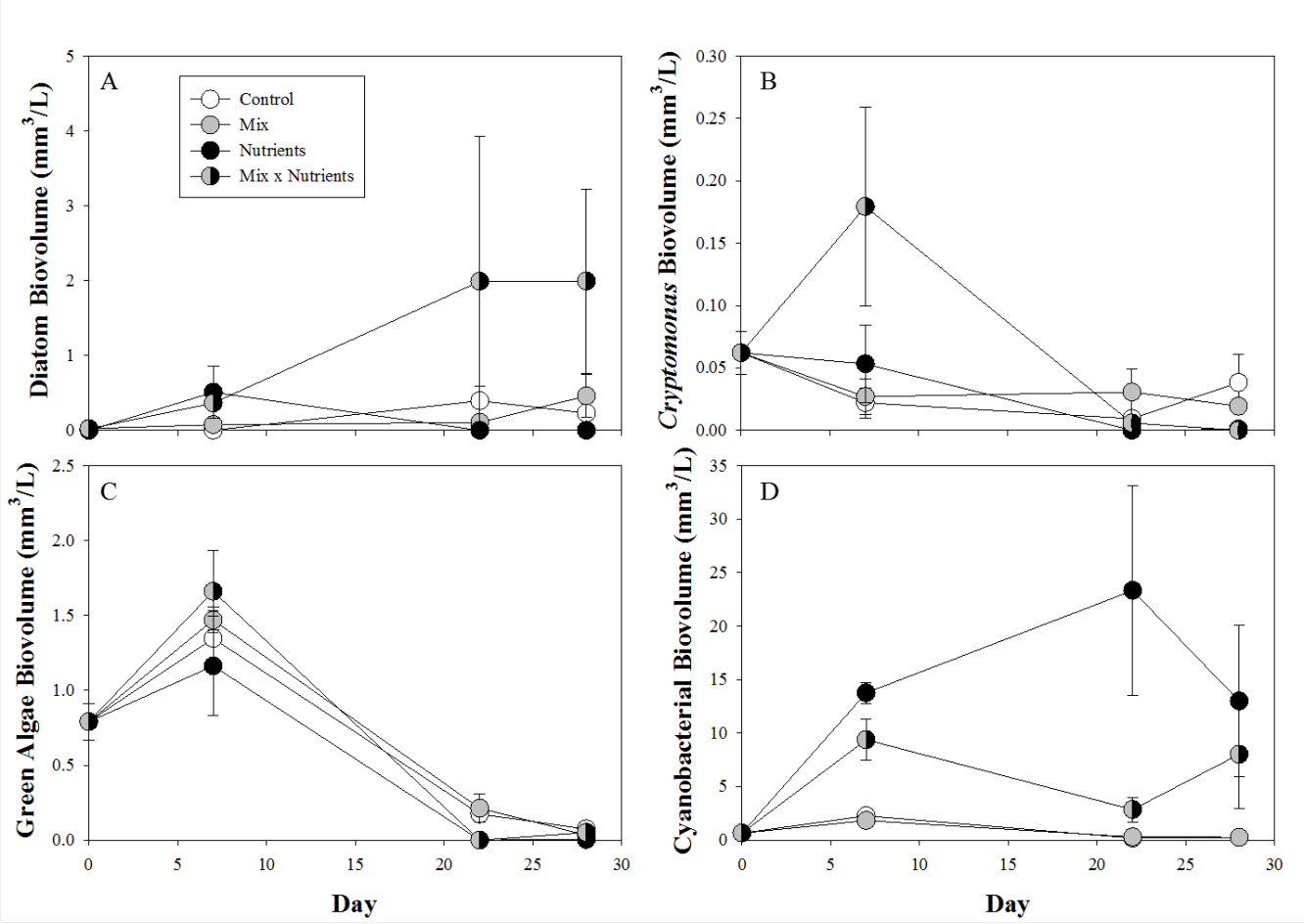

Figure 7. (A) Diatom biovolume (mm^3/L), (B) *Cryptomonas* biovolume (mm^3/L), (C) green alga biovolume (mm^3/L), and (D) cyanobacterial biovolume (mm3/L) over the 28-day experiment. Data represent means ± one standard error. White circles indicate the control treatment, grey circles represent the mixing only treatment, black circles represent the nutrient only treatment, and black and grey circles indicate the mixing and nutrient treatment.

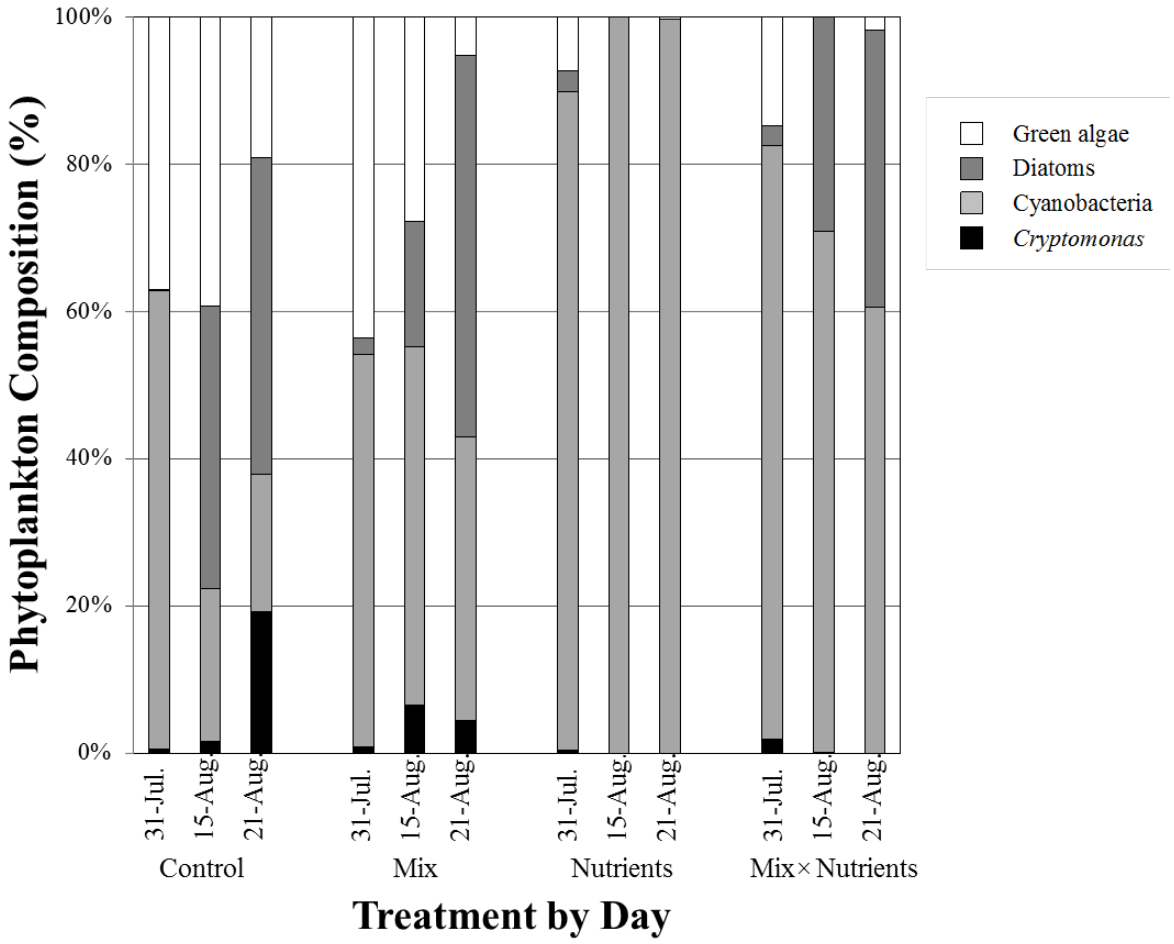

Figure 8. Phytoplankton community composition for 3 dates per treatment. Data represent mean percentage for green algae (white), diatoms (dark grey), cyanobacteria (light grey), and *Cryptomonas* (black).

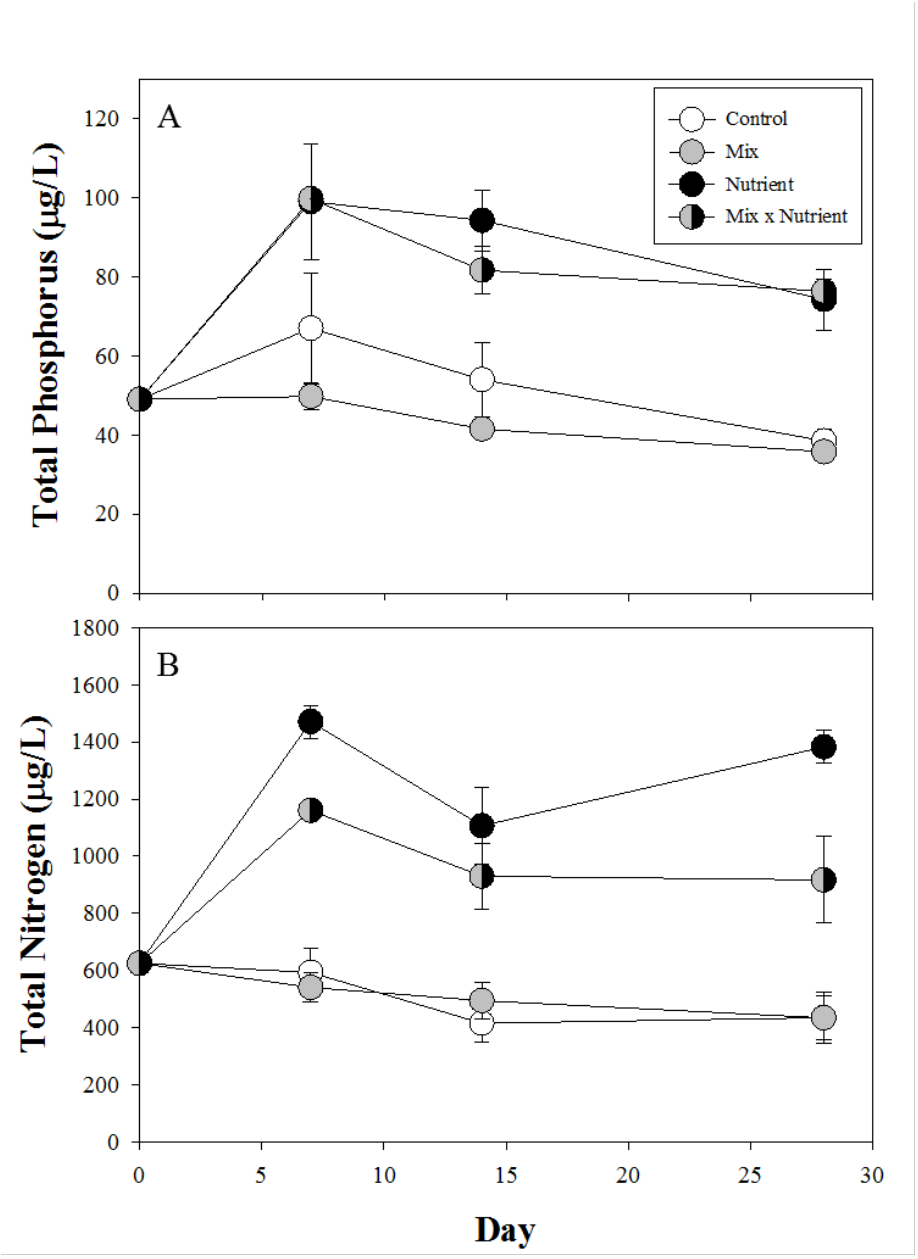

Figure 9. (A) Phosphorus and (B) nitrogen concentrations (µg/L) over the 28-day experiment. Data represent means ± one standard error. White circles indicate the control treatment, grey circles represent the mixing only treatment, black circles represent the nutrient only treatment, and black and grey circles indicate the mixing and nutrient treatment.

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