



# Eutrophication mediates a common off-flavor compound, 2-methylisoborneol, in a drinking water reservoir



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## ABSTRACT

Off-flavors, such as 2-methylisoborneol (MIB) and geosmin, cause drinking water to have earthy or musty tastes and odors. Humans can detect such compounds at minute concentrations (10 and 30 ng/L for MIB and geosmin, respectively), and, although not a health risk, off-flavors can promote consumer distrust. Removal of these compounds is costly and often unreliable or only suitable under certain conditions. Minimizing off-flavor production at the watershed-scale may be more cost-effective in addition to improving ecosystem health and aesthetics. Cyanobacteria are considered to be the primary drivers of off-flavors in freshwater systems. Due to their ability to produce toxins, cyanobacteria have been under particular scrutiny, and environmental factors promoting cyanobacterial blooms are relatively well-studied. Using this body of literature, we conducted a seven-week, limnocorral experiment where we manipulated nitrogen and nitrogen-to-phosphorus concentrations to influence phytoplankton community structure and off-flavor production. The addition of a single nutrient across broad ranges (nitrogen or phosphorus) had no effect on MIB. However, the addition of both nitrogen and phosphorus promoted high concentrations of MIB relative to treatments that received no nutrients (448% increase) or only nitrogen or phosphorus (722% increase). Interestingly, cyanobacteria waned during the experiment and were replaced by diatoms, which were the dominant taxa by the end of the experiment. Our findings clearly show that eutrophication affects MIB production, but mechanisms leading to the production of this compound may differ from what has been previously predicted.

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## 1. Introduction

Off-flavors, such as 2-methylisoborneol (MIB) and geosmin, can be a nuisance for water resource managers (Graham et al., 2010), 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) (Korth et al., 1992; Persson, 1980). 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) (U.S. EPA, 1994), these compounds regularly promote drinking water consumer complaints. Off-flavors can also negatively impact the marketability of aquaculture products (Tucker, 2000). In both industries, off-flavors can be economically damaging and can negatively impact product reputation, consumer trust, and future relationships.

Currently, existing methods for off-flavor removal in drinking water include oxidation using potassium permanganate, chlorine, and ozone (Glaze et al., 1990) as well as adsorption by activated carbon (Dabrowski et al., 2005). However, oxidation has been known to produce harmful or off-color disinfection by-products (Glaze et al., 1990; Srinivasan and Sorial, 2011), and activated carbon becomes less effective with greater concentrations of organic matter in source water (Pirbazari et al., 1993; Newcombe et al., 2002a,b). Moreover, both of these methods can incur considerable expense to water utilities. A financially practical and more efficient method of treatment has yet to be discovered (Srinivasan and Sorial, 2011). Furthermore, off-flavors may be indicative of problems at the watershed-level (Davies et al., 2004), but exact causes and the conditions under which off-flavors are produced in the environment are unclear (Jüttner and Watson, 2007). Finally, off-flavor management has focused primarily on treatment methods rather than the ultimate factors responsible for their production in nature. Instituting management plans that minimize the ecological conditions that favor off-flavor production may be a cost-effective

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solution for water resource managers.

Diverse organisms have been identified as off-flavor producers, including eukaryotes, such as fungi (Breheret et al., 1999) and amoebae (Hayes et al., 1991), but these compounds are most commonly attributed to photoautotrophic cyanobacteria and filamentous heterotrophic bacteria, particularly actinomycetes. 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, degradation (Tabachek and Yurkowski, 1976; Durrer et al., 1999). Warm, nutrient-rich waters are especially prone to cyanobacterial blooms and concomitant off-flavor events (Paerl and Huisman, 2008; Graham et al., 2010). Cyanobacteria have been under considerable scrutiny since the mid-1970s when they were discovered to produce potent secondary metabolites (Carmichael, 1981). Even though extensive research has been performed on the occurrence of cyanotoxins, predicting these compounds remains challenging, especially in large, dynamic waterbodies (Watson et al., 2008; Graham et al., 2010). 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 their apparent similarities, studies that have looked at cyanobacterial toxins and off-flavor compounds have found that these by-products rarely coincide and cannot be adequately predicted by conventional methods (Watson et al., 2008).

Although off-flavors have been studied for decades, the literature is often limited to laboratory-based studies focused on isolated cultures or correlative observations focused on water quality parameters, including phytoplankton community composition (Sugiura and Nakano, 2000; Schrader and Blevins, 2001; Wang et al., 2005; Westerhoff et al., 2005; Parinet et al., 2010; Li et al., 2012; Winston et al., 2014; Kehoe et al., 2015; Suurnäkki et al., 2015). We are not aware of any prior studies that have manipulated environmental variables, such as nutrient concentrations, in a drinking water reservoir using a replicated experimental design to determine their effects on off-flavors, including MIB, through changes in phytoplankton species composition. Given the importance of community- and ecosystem-level processes associated with the production of off-flavors and the critical need to understand the drivers of off-flavor dynamics in drinking water systems, empirical data linking patterns and processes are imperative.

There are several environmental factors known to promote cyanobacterial dominance including elevated nitrogen (Elser et al., 2009) and/or phosphorus (Downing et al., 2001), low nitrogen to phosphorus ratios (TN:TP) (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 TN:TP given the ability of some genera to fix atmospheric nitrogen (Stewart, 1980; Harris et al., 2014). Based on what we know about cyanobacterial growth and dominance, we conducted a field mesocosm experiment where two main factors that favor cyanobacteria, namely nitrogen concentration (Downing et al., 2001) and TN:TP (Smith, 1983), were manipulated to measure corresponding effects on MIB production.

Due to the limited data available linking nutrient concentration and MIB, a broad range of three nitrogen concentrations was targeted that reflect conditions found in (1) mesotrophic lakes (300 µg/L), (2) eutrophic reservoirs (1000 µg/L), and (3)

hypereutrophic aquaculture ponds (3000 µg/L) to assess directional effects on MIB. These levels were chosen using the Carlson Trophic State Index for phosphorus (Carlson, 1977) and the ambient TN:TP ratio found in the lake (10:1). Using the seminal findings of Smith (1983) related to TN:TP and cyanobacterial presence, four TN:TP ratios around a 29:1 threshold (cyanobacteria were shown to be rare above this threshold; Smith, 1983) that covered a broad range (2:1 to 90:1) were used in an attempt to determine how TN:TP influences MIB. Based on earlier studies (Smith, 1983; Downing et al., 2001), the highest concentrations of cyanobacteria and MIB were predicted to be observed under conditions with elevated nitrogen concentrations and low TN:TP.










## 2. Methods

This experiment was conducted in a drinking water reservoir located in the southeastern United States (Alabama), which experienced severe off-flavors problems during the summer of 2013 (MIB = 380 ng/L). The reservoir is relatively shallow (maximum depth = 8 m), dimictic, and mesotrophic (total nitrogen (TN) = 300 µg/L; total phosphorus (TP) = 30 µg/L, TN:TP = 10:1, by mass). The experiment was conducted during the months of November and December 2013 when off-flavors were still present and problematic (MIB > 10 ng/L). This reservoir and its surrounding watershed, like many other watersheds in the region, have been affected by a rapidly growing human population and subsequent urban development, and thus receive elevated loads of sediments and nutrients. Therefore, this drinking water reservoir provides an ideal study system for translational research that aids in future water resource management decisions.

Total N and TN:TP ratios were manipulated throughout a seven-week field experiment in 3000 L clear, polyethylene limnocorral mesocosms 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 75 µm sieve to remove large zooplankton and small fish. Four randomly chosen enclosures were sampled on 28 October 2013 (day 1) before treatments were added to retrieve baseline data for the mesocosms, which helped determine initial off-flavor concentrations in the enclosures and a fertilizer regime for the nine treatments (Table 1) that simultaneously manipulated TN and TN:TP. Thus, the experimental layout consisted of three TN levels (300 µg/L (low), 1000 µg/L (medium), and 3000 µg/L (high)) with four TN:TP (2:1, 10:1, 33:1, and 90:1; by mass) manipulations in an unbalanced factorial design. Nine treatments were randomly assigned to the enclosures with four replicates per treatment. Due

**Table 1**

Description of the unbalanced factorial design using four total nitrogen (TN): total phosphorus (TP) within three nitrogen concentrations. Herein triangles represent the ambient (300 µg/L) total nitrogen treatments, diamonds represent the medium (1000 µg/L) total nitrogen treatments, and circles represent the high (3000 µg/L) total nitrogen treatments. Gray shading indicates the 2:1 N:P treatments, white shading indicates the 10:1 N:P treatments, black shading indicates 33:1 the N:P treatments, and black and white shading indicates the 90:1 N:P treatment.

		TN:TP (by mass)			
		2:1	10:1	33:1	90:1
Total nitrogen (µg/L)	300			N/A	N/A
	1000				N/A
	3000				

to the nature of the ambient nutrients in the lake and that TP could not be extracted from the enclosures to achieve a higher TN:TP ratio, two TN:TP treatments (10:1 and 2:1) were used in the low TN treatment, three TN:TP treatments (10:1, 2:1, and 33:1) were used in the medium TN treatment, and all four TN:TP treatments were used in the high TN treatment (Table 1). Treatments were established by fertilizing enclosures with differing amounts of  $\text{NaNO}_3$  and/or  $\text{NaH}_2\text{PO}_4$ . At the start of the experiment, ambient enclosures (low TN, 10:1 TN:TP) received no additional nutrients, while the low TN, 2:1 TN:TP treatment received only phosphorus and the medium TN, 33:1 TN:TP treatment and high TN, 90:1 TN:TP treatments received only nitrogen. All other treatments received varying amounts of both TN and TP. Enclosures were fertilized weekly at a rate of 10% of the target TN and TP for each treatment to maintain TN and TN:TP over time (Table 1; Fig. 1) with  $\text{NaNO}_3$  and  $\text{NaH}_2\text{PO}_4$  dissolved in deionized water. Deionized water was added to ambient enclosures in lieu of fertilizers.

Treatments were established on 4 November 2013 (day 8) and

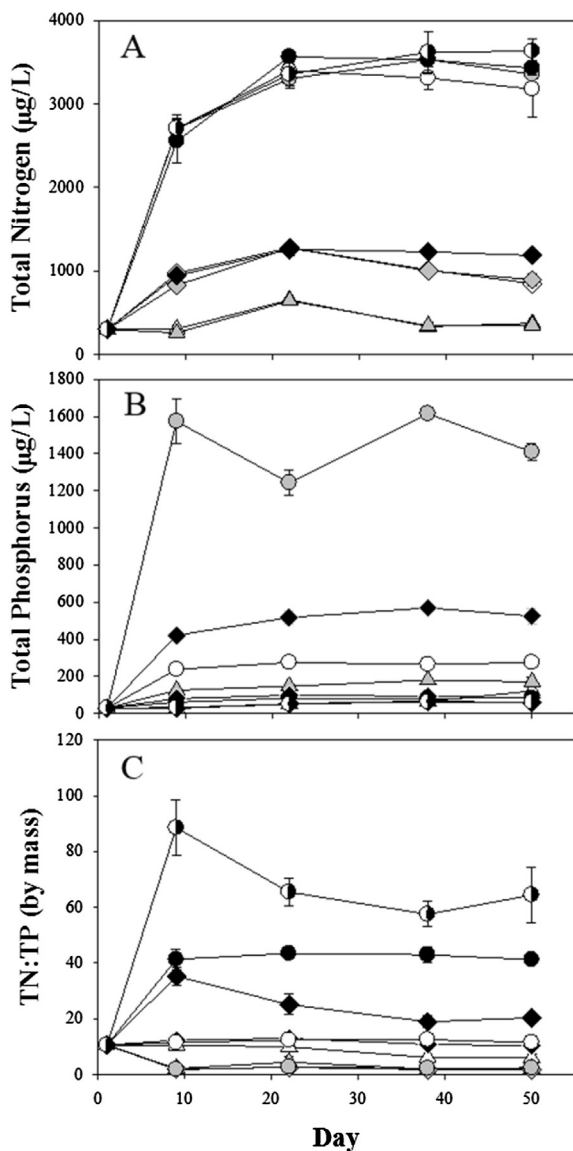


Fig. 1. (A) Total nitrogen ( $\mu\text{g/L}$ ), (B) total phosphorus ( $\mu\text{g/L}$ ), and (C) total nitrogen (TN)-to- total phosphorus (TP) (by mass) over the seven-week experiment. Data represent means  $\pm$  one standard error. Refer to Table 1 for symbol legends.

sampling of all enclosures was conducted on days 9, 22, 38, and 50. Depth-integrated samples (2.0 m) depth were collected for nutrients (TN, TP), phytoplankton biovolume and species composition, and MIB using a rigid tube sampler. Temperature was taken at 0.5 m, 1 m and 2 m with a Hydrolab sonde 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 to 400 $\times$ ) 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 cyanophytes (cyanobacteria), bacillariophytes (diatoms), chlorophytes (green algae), and cryptophytes (cryptomonas). Phytoplankton were counted for the pre-treatment date (28 October 2013), mid-experiment date (18 November 2013), and the final sampling date (16 December 2013). MIB samples were stored at 4 $^{\circ}$  C in parafilm-sealed 20 ml glass vials without air bubbles and analyzed by solid-phase microextraction and gas chromatography/mass spectrometry (APHA, 2012).

Three enclosures were damaged during the study and not included in any statistical analyses. MIB was not analyzed on day 38 due to a malfunction with the GC/MS and samples were taken on day 43 for MIB only. All data except for measured TN, TP and TN:TP were log-transformed to meet normality and constant variance assumptions. The effects of continuous data (values derived from actual nutrient concentrations in each limnocorral) for TN, TP and TN:TP, and their interaction on total phytoplankton biovolume were tested using a mixed model (main factors = TN level, TP level, and TN:TP, random effect = time) across all dates excluding pre-treatment samples. Similarly, the effects of continuous TN, TP, and TN:TP on MIB over time were tested using a mixed model. Continuous data were not sufficient in providing information regarding specific treatment differences, but did indicate the significance of nutrient addition on MIB and phytoplankton biovolume. Categorical tests were used to better understand these differences.

The effects of projected TN levels (300, 1000, and 3000), TN:TP (2, 10, 33, and 90), and their interaction on total phytoplankton biovolume were tested using repeated measures analysis of variance (RM-ANOVA main factors = TN level and TN:TP, repeated measure = time) across all dates excluding pre-treatment samples. Tukey's post-hoc tests for total phytoplankton biovolume identified two unique subsets on the basis of categorical nutrient treatments (group 1: no nutrients added or only N or P added; group 2: both TN and TP added). To explicitly test whether off-flavor patterns tracked phytoplankton patterns, we used a linear contrast to compare concentrations for these two subsets. 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). Pre-treatment phytoplankton counts were excluded from the model to focus on the effects of the nutrient treatments. Linear regression was used to determine the relationship between MIB and any phytoplankton taxa that was significant in the mixed effects linear regression. Absolute differences in these relevant phytoplankton taxa over two dates (days 22 and 50) were calculated and tested for treatment differences using an analysis of variance (ANOVA main factors = TN level and TN:TP). Relative abundances for the four phytoplankton taxa were calculated. A repeated measures analysis of variance was conducted using TN, TN:TP, and their interaction as predictors and each phytoplankton taxa showing significance in the mixed effects linear regression model as the response.

### 3. Results

Throughout the experiment, TN and TP concentrations were maintained near target levels (Fig. 1A and B). TN:TP decreased slightly over time in the high TN:TP treatments (90:1 and 33:1) since small differences in TN or TP can affect TN:TP. However, TN:TP maintained the same rank order over time (Fig. 1C). Average temperatures ranged from 18.6 °C on 28 October 2013 to 9.7 °C on 16 December 2013.

The mixed model used to assess the relationships between total phytoplankton biovolume and continuous values of TN, TP and TN:TP indicated significance for TN ( $T_{59} < 0.0001$ ) and TN:TP ( $T_{59} = 0.0009$ ). Results from the mixed model also showed that the relationships between MIB and continuous values of TN, TP and TN:TP were similar given that TN ( $T_{59} = 0.0058$ ) and TN:TP ( $T_{59} = 0.0438$ ) showed statistically significant effects.

Total phosphorus was not included in categorical tests as it showed no significant effects in continuous models. Categorical TN, TN:TP, and their interaction had significant effects on total phytoplankton biovolume over time (TN-level  $F_{2,24} = 79.82$ ,  $P < 0.0001$ ; TN:TP  $F_{3,24} = 24.19$ ,  $P = 0.0001$ ; TN-level  $\times$  TN:TP  $F_{3,24} = 15.25$ ,  $P < 0.0001$ ) (Fig. 2A). Post-hoc testing using data from the final two sampling dates revealed two unique subsets showing 360% higher phytoplankton biovolume in treatments where both TN and TP were added (Medium TN, 10:1 TN:TP; Medium TN, 2:1 TN:TP; High TN, 10:1 TN:TP; High TN, 2:1 TN:TP; and High TN, 33:1 TN:TP) compared to treatments where only TN, only TP, or neither nutrient was added (Low TN, 10:1 TN:TP; Low TN, 2:1 TN:TP; Medium TN, 33:1 TN:TP; and High TN, 90:1 TN:TP) (Tukey's test:  $F_{8,23} = 28.62$ ,  $P < 0.0001$ ). The contrast explicitly comparing MIB for these two

groups revealed that MIB was significantly higher in treatments where both nutrients were added compared to treatments that received only one nutrient or neither nutrients ( $T_{127} = 8.03$ ,  $P < 0.0001$ ) (Fig. 2B).

The mixed effects linear regression evaluating relationships between phytoplankton taxa and MIB showed a significant relationship between MIB and diatoms, ( $T_{59} = 6.13$ ,  $P < 0.00001$ ) (Fig. 3A) and MIB and cyanobacteria ( $T_{59} = 3.96$ ,  $P = 0.0002$ ) (Fig. 3B). Statistical contrasts were run to determine differences between treatment subsets that had been previously identified by post-hoc testing for these two taxa. Both cyanobacterial ( $T_{61} = 3.55$ ,  $P < 0.0001$ ) and diatom ( $T_{62} = 5.83$ ,  $P < 0.0001$ ) biovolume significantly increased (cyanobacteria = 65% increase, diatoms = 450% increase) when both nutrients were added. Even though both taxa split into the two unique subsets, overall cyanobacterial biovolume decreased over time (i.e., cyanobacteria taxa died; Fig. 4A). Diatom biovolume increased over time in treatments with TN and TP, but decreased or remained relatively constant in treatments with no nutrient additions or only TN or TP added (Fig. 4B) as seen with MIB (Fig. 2B).

The relationship between MIB and diatom biovolume ( $R^2 = 0.65$ ,  $F_{1,31} = 55.88$ ,  $P < 0.0001$ ) or cyanobacterial biovolume were highly significant ( $R^2 = 0.48$ ,  $F_{1,29} = 25.66$ ,  $P < 0.0001$ ). Absolute differences were calculated for cyanobacterial and diatom biovolume, but univariate analysis of variances for each taxa showed that

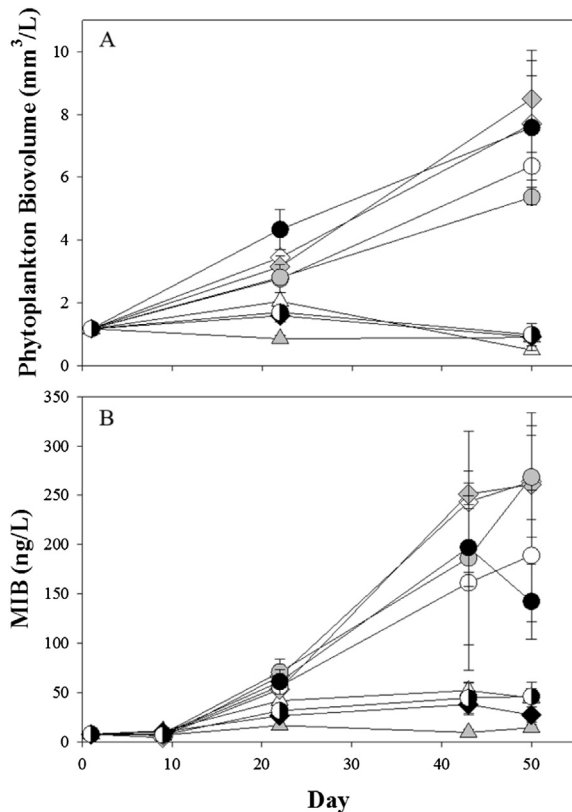


Fig. 2. (A) Total phytoplankton biovolume ( $\text{mm}^3/\text{L}$ ) and (B) 2-methylisoborneol (MIB) concentrations ( $\text{ng}/\text{L}$ ) throughout the seven-week experiment. Data represent means  $\pm$  one standard error. Refer to Table 1 for symbol legends.

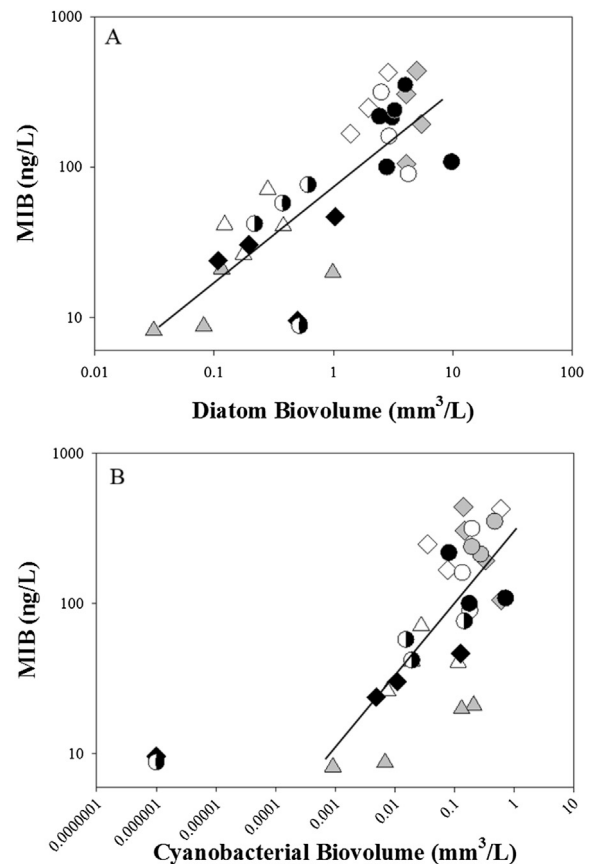
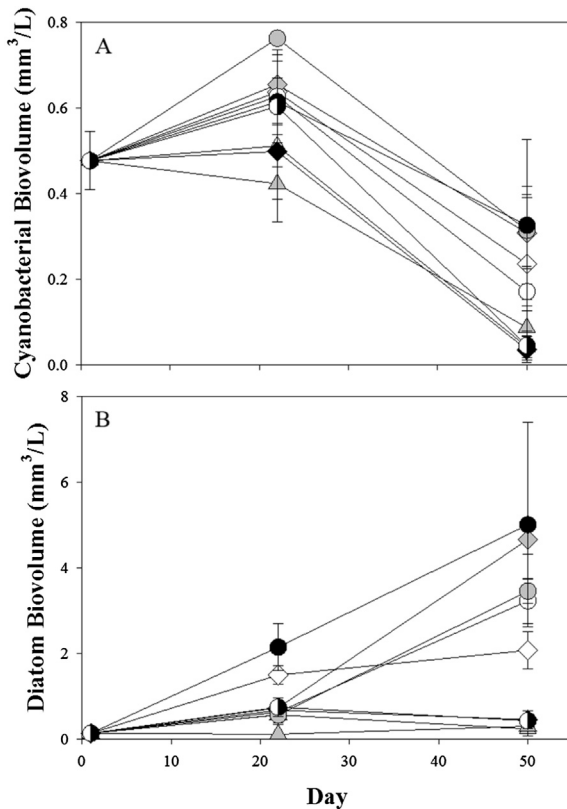


Fig. 3. The relationship between (A) log 2-methylisoborneol (MIB) ( $\text{ng}/\text{L}$ ) and log diatom biovolume ( $\text{mm}^3/\text{L}$ ) ( $R^2 = 0.65$ ,  $F_{1,31} = 55.88$ ,  $P < 0.0001$ ) (B) log 2-methylisoborneol (MIB) ( $\text{ng}/\text{L}$ ) and log cyanobacterial biovolume ( $\text{mm}^3/\text{L}$ ) and log MIB ( $\text{ng}/\text{L}$ ) ( $R^2 = 0.48$ ,  $F_{1,29} = 25.66$ ,  $P < 0.0001$ ) on the last day of the seven-week experiment (16 December 2013). Outliers include enclosures where cyanobacteria were not present but not included in the regression. Refer to Table 1 for symbol legends.



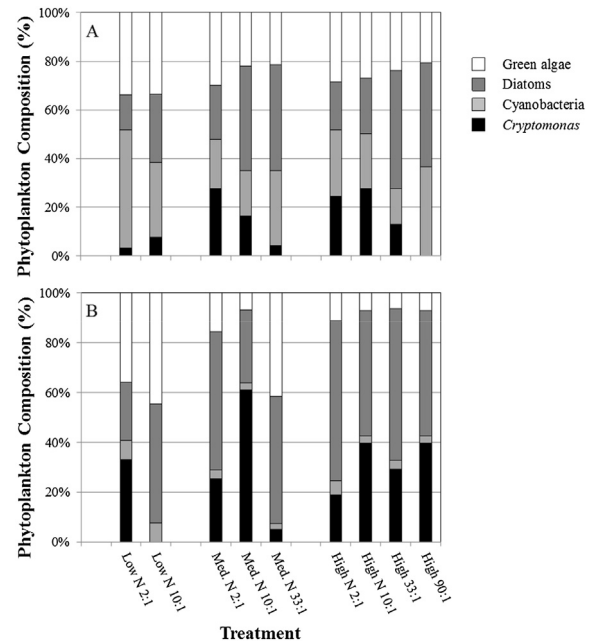


**Fig. 4.** (A) Cyanobacterial biovolume (mm<sup>3</sup>/L), and (B) diatom biovolume (mm<sup>3</sup>/L) over the seven-week experiment. Data represent means  $\pm$  one standard error. Refer to Table 1 for symbol legends.

treatments mediated these differences in diatom biovolume only (TN-level  $F_{2,31} = 9.09$ ,  $P < 0.001$ ; TN:TP  $F_{3,31} = 5.20$ ,  $P = 0.007$ ; TN-level  $\times$  TN:TP  $F_{3,31} = 3.48$ ,  $P < 0.032$ ). Repeated measures analysis of variance using diatom or cyanobacterial relative abundance as a response and TN, TN:TP and their interaction showed only that TN and relative abundance of cyanobacterial biovolume were significantly related (TN-level  $F_{2,23} = 7.019$ ;  $P = 0.004$ ). No other factors significantly contributed to the relative abundance of cyanobacterial or diatom biovolume (Fig. 5A and B).

#### 4. Discussion

Nutrient limitation is often a significant driver of phytoplankton competition and dominance in aquatic systems. It is commonly accepted that nitrogen-fixing cyanobacteria will dominate under low TN:TP (Schindler, 1977). As the experiment progressed, we expected to see this paradigm reflected with cyanobacteria dominating algal species composition in low TN:TP enclosures. Despite being composed of ~25–50% cyanobacteria in all treatments mid-experiment, percent composition of cyanobacteria decreased to less than 10% by the end of experiment across all treatments (Fig. 4A). Specifically, cyanobacterial species observed included *Oscillatoria* spp., *Anabaena* spp., and *Dactylococcopsis* spp. Relatively small differences were observed across treatments regarding cyanobacterial composition (Fig. 5A and B) even though overall cyanobacterial abundance decreased throughout the experiment (Fig. 4A). *Oscillatoria* spp. increased from 50 to 58% of the cyanobacterial composition, whereas *Anabaena* spp. increased from 22 to 29% and *Dactylococcopsis* spp. composition declined from 27 to 12%. Both *Anabaena* and *Oscillatoria* have been previously implicated in



**Fig. 5.** Phytoplankton relative abundance (% biovolume) for the middle (day 22) (A) and end (day 50) (B) of the experiment for each treatment. Low represents the ambient (300  $\mu\text{g/L}$ ) total nitrogen treatment, Medium (Med.) represents the 1000  $\mu\text{g/L}$  total nitrogen treatments, and High indicates the 3000  $\mu\text{g/L}$  total nitrogen treatment. 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 gray), cyanobacteria (light gray), and the flagellate, *Cryptomonas* (black).

the production of off-flavors (Blevins et al., 1995; Izaguirre and Taylor, 2007). Nutrient manipulations did not affect the rate of decrease over time. Since the experiment was conducted during November and December, the decrease in cyanobacteria may be attributed to colder temperatures, higher rates of thermal-mediated mixing (Visser et al., 1996), and reduced daylight. Percent composition of diatoms did not differ by treatment, but, unlike cyanobacteria, demonstrated an increase over the course of the experiment in all treatments. These patterns fall in line with what has been predicted and shown through seasonal manipulations and phytoplankton succession models (Sommer et al., 1986; Visser et al., 1996), but 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).

Although TN, TN:TP and their interaction were significantly related to MIB concentration, the greatest predictor of MIB appeared to be the addition of both N and P. We cannot adequately compare our findings to those of Smith (1983) given seasonal differences and the lack of cyanobacterial dominance in any of the enclosures. However, the treatments that produced the highest concentrations of MIB consisted of low TN:TP (<29) with the exception of the low TN treatments. Because MIB generally grouped into treatments that had either been fertilized with both TN and TP or those that had been fertilized with one or no nutrients, it is difficult to determine whether specific nutrient levels or individual TN:TP ratios truly had an effect. However, it is clear that MIB concentrations increased when both TN and TP were added to the system, which is valuable information for water resource managers given the potential ramifications of eutrophication on drinking water as well as the huge economic costs associated with removing taste and odor compounds during drinking water production.

As the experiment progressed, cyanobacteria represented the

least abundant taxa, but still contributed significantly to MIB. A possible mechanism contributing to the observed differences in MIB across treatments, especially toward the final date, may be cyanobacterial decay as lake temperatures cooled. After isolating *Fischerella muscicola*, Wu and Jüttner (1988) found another common off-flavor compound, geosmin, at higher concentrations within the intracellular fraction, causing a period of high off-flavor occurrence during bloom die-off. Nonetheless, MIB and geosmin do not always follow similar patterns (Ma et al., 2013), and studies looking at factors affecting geosmin may not be a good predictor of MIB. Another issue with this potential mechanism is that, unlike MIB concentration, there was no apparent difference in cyanobacterial decomposition among treatments. However, it is unknown how nitrogen and phosphorus affect the dissolved/intracellular ratio of MIB or geosmin in cyanobacteria, but composition and abundance may not predict off-flavor events if intracellular ratios vary with nutrient conditions. In general, MIB seems to be less prevalent than geosmin in the literature, which has resulted in fewer studies looking at MIB, specifically.

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 (Izaguirre and Taylor, 1998; Sugiura et al., 1998, 2004; Xu et al., 2010; Schrader et al., 2011). 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, including this one, 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 an important component of the increases in MIB. Several studies have examined environmental variables contributing to actinomycete growth and off-flavor production, although most of these studies have been focused solely on geosmin and a consensus regarding these conditions has not been reached in the literature. In general, actinomycete biomass tends to increase under elevated nutrients (both TN and TP) (Wood et al., 1985). Although geosmin production by actinomycetes has been observed at both high and low TN (Blevins et al., 1995; Schrader et al., 2013), this off-flavor compound has only been detected in systems with high TP (Schrader and Blevins, 2001). Despite reported patterns for TN, TP, actinomycete biomass, and geosmin concentrations, a strong correlation across waterbodies that vary in nutrient concentrations has yet to be determined (Schrader and Blevins, 2001; Schrader et al., 2013). 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 geosmin can be best produced at 30° C, but will still occur at 15° C. However, Aoyama et al. (1993) reported lower optimal temperatures for geosmin production (15° C), but higher temperatures for actinomycete growth (30° C), although results varied across strains. 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 geosmin production at 35° C under pH 9. However, conditions which favor actinomycete production of MIB may differ from those that favor geosmin.

Prior evidence has showed that actinomycetes and off-flavor production are absent under anaerobic conditions (Wood et al., 1985). Given the time of year and the increased mixing 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 due to stratification. However, with the discrepancies in the literature regarding optimal conditions for actinomycete growth, the apparent lack of relationship between actinomycete biomass and off-flavor production, 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. However, integrating ecological principles into lake management techniques should be considered for reducing future off-flavor events which could minimize or eliminate the need for additional water treatment processes.

## 5. Conclusions

In summary, using a seven-week, field limnocorral experiment in a drinking water reservoir where we tested the effects of different combinations of nitrogen and phosphorus additions on MIB dynamics, we found large increases (>700%) in MIB when both nitrogen and phosphorus were added. In contrast, additions of only nitrogen or phosphorus did not significantly affect MIB concentrations relative to the ambient treatment. Although cyanobacteria were expected to correlate with MIB based on past studies, we found a stronger correlation between MIB and diatom biovolume. Moreover, diatoms generally increased in absolute and relative abundance during the experiment, while cyanobacteria waned in all treatments. It is important for water resource management to understand the connection between species interactions and eutrophication regarding MIB production. Our data suggest that creating conditions to manage and reduce nitrogen and phosphorus is critical for minimizing off-flavor events in drinking water reservoirs.

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