Nutrient enrichment and vertical mixing mediate 2-methylisoborneol and geosmin concentrations in a drinking water reservoir

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ABSTRACT

Few ecosystem-level studies have experimentally determined the physicochemical and biological factors that mediate concentrations of off-flavor compounds in drinking water reservoirs. Consequently, the watershed-scale mechanisms determining production of these compounds are still poorly understood. In a recent study, the addition of both nitrogen and phosphorus significantly increased 2-methylisoborneol (MIB). Not surprisingly, MIB was correlated with cyanobacterial abundance (a well-known producer of off-flavor compounds); however, MIB was most strongly correlated with diatom abundance. To empirically test for differences in the production of two important off-flavor compounds, specifically MIB and geosmin, by either cyanobacteria or diatoms, we conducted a fully factorial experiment that manipulated two factors that typically promote cyanobacteria (nitrogen and phosphorus fertilization) or diatoms (vertical mixing of the water column). As predicted, fertilization promoted cyanobacteria, and vertical mixing favored diatoms. Interestingly, the production of geosmin was rapid and consistent with an increase in cyanobacteria while MIB production increased later in the experiment when cyanobacterial biovolume tended to decline and diatom biovolume increased. Based on our current and previous studies, MIB and geosmin production is associated with cyanobacteria, but the direct or indirect influence of diatoms on production should not be ignored.

Key words | cyanobacteria, diatoms, eutrophication, field experiment, MIB, nitrogen, phosphorus

INTRODUCTION

Geosmin and 2-methylisoborneol (MIB) are organic compounds, collectively referred to as off-flavors, that create earthy and musty tastes and odors in freshwater ecosystems (Graham et al. 2010). These compounds are problematic for drinking water and aquaculture facilities since methods of removal are expensive and potentially ineffective or dangerous for human consumption (Srinivasan & Sorial 2011). Geosmin and MIB were initially thought to be produced primarily by actinomycetes, a gram-positive microbe commonly found in soils (Gerber 1968). However, subsequent studies have shown that both compounds can be produced by isolated, axenic colonies of cyanobacteria (primarily Oscillatoria/Planktothrix spp. and Lyngbya spp.) (Tabachek & Yurkowski 1976). These same cyanobacterial taxa have also come under particular scrutiny for their ability to produce toxic secondary metabolites. Consequently, cyanobacteria are regularly considered the primary source of these off-flavors and are the focus of many studies on MIB and geosmin outbreaks (Jüttner & Watson 2007). Some studies have indicated that diatoms, particularly Synedra spp., can contribute to off-flavor production, but prior evidence has been mostly correlative (Schrader et al. 2011; Olsen et al. 2016). Other studies have suggested that diatoms do not directly produce off-flavor compounds but can provide substrate and carbon resources for actinomycete attachment and growth, thus enabling actinomycetes to produce MIB and geosmin (Sugiura et al. 1998).
Prior efforts associated with off-flavor management have generally focused on engineering approaches to remove these compounds. However, ultimate environmental factors that mediate off-flavor outbreaks in nature are still relatively unexplored (Srinivasan & Sorial 2011). Past laboratory-based studies have manipulated variables such as nutrients, light intensity, and temperature using cyanobacterial isolates capable of producing off-flavors (Saadoun et al. 2001). Additionally, several observational studies have successfully correlated water quality parameters, such as algal biovolume, water chemistry, temperature, and weather events, to off-flavor concentrations within and among waterbodies (Izaguirre et al. 1982; Dzialowski et al. 2009). However, correlation does not equal causation, and these variables can be difficult to manipulate in situ. A recent field experiment conducted by Olsen et al. (2016) that manipulated nitrogen concentration and nitrogen-to-phosphorus ratios (TN:TP) in limnocorals in a drinking water reservoir found that both nitrogen and phosphorus were co-limiting in the production of MIB. As with previous observational studies, Olsen et al. (2016) found a strong correlation between MIB and cyanobacteria ($R^2 = 0.48$). However, in this experiment, the correlation between diatoms and MIB was statistically stronger ($R^2 = 0.65$).

Given the expected seasonal shifts in phytoplankton species composition associated with changing dominant limnological processes (Sommer et al. 1986), predicting off-flavor concentrations based on phytoplankton species composition could aid water resource management (Mazumder et al. 1990). However, few models are available that forecast off-flavor concentrations. For example, Qi et al. (2012) found that cyanobacterial biomass was the best predictor during warmer months, whereas the diatom, Synedra, was a better predictor of off-flavors during cooler months in Lake Taihu, China. The primary driver of seasonal differences involves changes in temperature and light, which influences water chemistry and water movement in lakes. Summer stratification and high temperatures tend to favor cyanobacterial blooms (Paerl & Huisman 2008) but generally exclude diatoms, which rely on lake mixing for movement to the surface given that they lack flagella or buoyancy-regulation vacuoles (Sommer et al. 1986). Other studies that have investigated off-flavor events have focused on geosmin (Saadoun et al. 2001; Billica et al. 2010) since biosynthetic pathways are better known for this compound than for MIB (Jüttner & Watson 2007). Past studies that have addressed both MIB and geosmin tended to either focus on removal methods (Geldenhuys et al. 1996) or on monitoring corresponding environmental factors during off-flavor outbreaks (Dzialowski et al. 2009). Few studies have hypothesized why one compound should be favored over the other (Izaguirre & Taylor 2007).

After complaints of a high occurrence of off-flavor compounds in a local drinking water reservoir during the summer and fall of 2013, Olsen et al. (2016) empirically showed that MIB increased under elevated nitrogen and phosphorus concentrations but was generally unaffected by additions of either phosphorus or nitrogen alone. The increase in MIB concentrations was primarily correlated with cyanobacterial and diatom biovolume. Here, we continue to investigate differences in off-flavor production between these two phytoplankton taxa by manipulating nutrients and vertical mixing in a fully factorial mesocosm-field experiment during the summer of 2014 when the reservoir was fully stratified. Treatments were chosen based on the conditions that are expected to favor cyanobacteria (fertilization) or diatoms (vertical mixing).

### METHODS

Nutrients (nitrogen and phosphorus) and mixing were manipulated in a fully-factorial design throughout a 28-day field experiment in 3,800 L limnocorals (diameter = 1.18 m, depth = 3.5 m) composed of clear polyethylene. Enclosures were open to the atmosphere, sealed at the bottom, and suspended by floating PVC frames. The experiment was conducted during the summer stratification period (24 July 2014 through 21 August 2014) to also quantify the effects of stratified versus artificially mixed conditions. The reservoir is polymictic and mesotrophic (total nitrogen (TN) = 300 μg/L; total phosphorus (TP) = 30 μg/L, TN:TP = 10:1 (by mass)) with a maximum depth of 8 m. Throughout the experiment, the lake was fully stratified with a temperature difference ranging from 7.9 °C to 6.1 °C over 7.3 m. The thermocline was at ~4.3 m. Sixteen enclosures were filled by pumping lake water through a 75 μm sieve to remove large-bodied zooplankton and small fish. Four enclosures were
randomly sampled on 24 July 2014 (day 0) before treatments were established for baseline data. Off-flavors in the reservoir measured at the start of the experiment were low (MIB = 8 ng/L and geosmin = 12 ng/L) but near human detection (i.e., MIB = 10 ng/L and geosmin = 30 ng/L; Persson 1980; Korth et al. 1992). One of four treatments was randomly assigned to each enclosure in a 2×2 fully factorial design with four replicates per treatment. The four treatments included, (treatment A) control (ambient nutrients; no mixing), (treatment B) mixing only (ambient nutrients), (treatment C) nutrient addition only (no mixing), and (treatment D) mixing and nutrient addition. Each replicate of the two mixed treatments (treatments B and D) was thoroughly vertically mixed using the circular frame of a zooplankton net wrapped in clear polyethylene to create a solid disk (diameter = 0.55 m). Holes were punctured in the polyethylene to aid in sinking. The mixing device was connected to a rope at three places equally spaced around the disk that was used to pull the device through the water column. Nutrient and mixing treatments were applied on 26 July 2014 (day 2). Mixing was conducted every other day generally before noon by slowly dropping the disk to the bottom of relevant enclosures and quickly pulling the device through the water column. This process was repeated three times per enclosure per mixing event. The mixing device was not used for the other two treatments. Nutrients were added (as NaNO₃ and NaH₂PO₄) once on day 2 to relevant enclosures (treatments C and D) and were not applied again throughout the remainder of the 28-day experiment. Target TN (1,000 μg/L) and TP (100 μg/L) concentrations in the nutrient treatments were chosen based on the results of Olsen et al. (2016), which showed that MIB increased significantly under these nutrient conditions.

Enclosures were sampled weekly throughout the duration of the experiment from the surface to 2 m using a rigid, depth integrated tube sampler after mixing, when applicable. Depth profiles measuring temperature (°C) were taken in each enclosure using a Hydrolab multisonde. Samples were brought to the laboratory and processed for nutrients (TN and TP), phytoplankton biovolume and composition, and MIB and geosmin concentrations. Nutrient concentrations were determined by spectrophotometry using ultraviolet (TN) and colorimetric (TP) standard methods (Olsen et al. 2016). Phytoplankton samples were preserved in 1% Lugol’s solution and were identified to genus and enumerated using inverted microscopy (Olsen et al. 2016). For each phytoplankton sample, 25 fields were counted at two magnifications (100× and 400×); cells were measured using Nikon Image to calculate average algal cell biovolume for each taxa. Phytoplankton were counted for the pre-treatment date (24 July 2014, day 0), the first date after treatments were applied (31 July 2014, day 7), and the two final sampling dates (15 August 2014, day 22; 21 August 2014, day 28). Whole-water off-flavor samples were stored in glass vials sealed with parafilm and analyzed for MIB and geosmin through solid phase micro-extraction using gas chromatography–mass spectrometry (Standard Methods 6040-D; APHA 2012).

One enclosure from treatment D was damaged during the study and not included in the analysis. Nutrient data for 15 August 2015 are not presented due to sample contamination with phosphorus. All data were log-transformed to meet normality and homogeneity assumptions. Repeated measures analysis of variance (RM-ANOVA) was used to examine the effects of nutrients, mixing, and their interaction on stratification (measured as the mean difference in dissolved oxygen (DO) concentrations (mg/L) measured below the surface and near the bottom of the enclosures on the same sampling day), total phytoplankton biovolume, and MIB and geosmin concentrations for the dates that phytoplankton were counted excluding the pre-treatment samples. Post-hoc testing on phytoplankton biovolume revealed two unique subsets among treatments (first subset: treatments A and B, second subset: treatments C and D). To explicitly test whether off-flavor patterns tracked effects on phytoplankton biovolume, we used a linear contrast to compare concentrations of the two subsets for both MIB and geosmin. Analyses were focused on phytoplankton that have previously been shown to correlate with MIB and geosmin concentrations including cyanobacteria and diatoms (Olsen et al. 2016). A mixed model was used to determine the relationship between the biovolume of specific phytoplankton taxa and MIB or geosmin concentrations (response variables = MIB or geosmin, predictors = the two algal taxa, random effect = time) across all sampling dates (except the initial sampling
date). Pearson’s correlation coefficient compared temporal patterns in MIB and geosmin concentrations over time.

RESULTS AND DISCUSSION

Stratification patterns in the lake and enclosures

Although our study lake is polymictic, it fully stratifies each summer. Stratification can be temporally disrupted by heavy winds during storm events. During this study, the lake stratified at 4.3 m. The enclosures were 3.5 m deep. Given that the enclosures were shallower than the lake (and within the mixing zone depth of the lake) and two of the four treatments involved artificial mixing, all of the enclosures did not strongly stratify during the entire experiment. To estimate stratification in each enclosure on each sampling day, the absolute difference in DO concentration (mg/L) near the surface and bottom was calculated. As expected, DO concentration differences varied over time and across treatments during the entire experiment (mean difference (±1 standard error) = 1.56 ± 0.21 mg/L; difference range = 0.03 to 6.92 mg/L). Interestingly, fertilization was more important for stratification in the enclosures than mixing (nutrients only: RM-ANOVA F1,11 = 51, P < 0.001; mixing: RM-ANOVA F1,11 = 0.001, P < 0.984; mixing and nutrients: RM-ANOVA F1,11 = 1.07, P = 0.323) likely as a result of increased photosynthesis by phytoplankton near the water surface (Figure 1(a)). However, the effect of fertilization waned by day 22 of the experiment. The mixed only treatment consistently maintained the lowest DO difference throughout the experiment (mean difference (±1 standard error) = 0.34 ± 0.07 mg/L; difference range = 0.04 to 0.82 mg/L).

Treatment effects on phytoplankton composition and abundance

Certain phytoplankton taxa have been shown to influence off-flavor dynamics (Jüttner & Watson 2007; Dzialowski et al. 2009; Olsen et al. 2016). In this experiment, two treatments were employed to examine the contribution of specific phytoplankton taxa (cyanobacteria and diatoms) to two common off-flavors. Total phytoplankton biovolume was significantly affected by fertilization (treatments C and D; repeated measures (RM)-ANOVA F1,11 = 34.26, P < 0.0001), but was not affected by mixing (RM-ANOVA F1,11 = 0.16, P = 0.70) or the interaction of fertilization and mixing (RM-ANOVA F1,11 = 0.51, P = 0.49) (Figure 1(a)). Total phytoplankton biovolume was nearly an order of magnitude higher in the two treatments that received a nutrient addition (nutrients only, C; mixing and nutrients, D) when compared to the control (A) and mixing only (B) treatments (Tukey’s post hoc tests: α = 0.05).

Figure 1 | Dynamics of (a) phytoplankton biovolume (mm³/L), (b) 2-methylisoborneol (MIB) (ng/L), and (c) geosmin (ng/L) over the 28-day experiment. Data points represent means ± one standard error. White circles indicate the control treatment, grey circles represent the mixing only treatment, black circles represent the nutrients only treatment, and black and grey circles indicate the mixing and nutrients treatment.
As expected for the targeted phytoplankton taxa, fertilization promoted cyanobacteria and vertical mixing stimulated diatoms (but at a smaller scale). Cyanobacteria initially increased after the treatments were established, but the greatest cyanobacterial concentration was found in the nutrient-only treatment (treatment C) (Figure 2(a)). Nutrient addition resulted in a significant increase in cyanobacterial biovolume (RM-ANOVA $F_{1,12} = 20.75, P = 0.001$) with no significant effect of mixing (RM-ANOVA $F_{1,12} = 0.16, P = 0.70$) or their interaction (RM-ANOVA $F_{1,12} = 2.79, P = 0.123$) (Figure 2(a)). Mean cyanobacterial biovolume was highest in the nutrient only (C) treatment; however, post hoc testing revealed that this difference was only statistically significant when comparing the nutrient only treatment to the control (A) ($P = 0.003$) or mixing only (B) ($P = 0.032$) treatments but did not differ from the mixing and nutrient addition treatment (D) ($P = 0.79$).

In general, diatom biovolume was significantly lower than cyanobacterial biovolume in both nutrient addition treatments (Figure 2(a) and 2(b)). In fact, diatom biovolume was lowest in the nutrient only treatment (C). Mixing significantly increased diatom biovolume (RM-ANOVA $F_{1,12} = 18.64, P = 0.001$), which was not affected by nutrient addition (RM-ANOVA $F_{1,12} = 1.90, P = 0.19$) or the interaction of fertilization and mixing (RM-ANOVA $F_{1,12} = 2.74, P = 0.13$).

In terms of relative phytoplankton composition, fertilization also increased the cyanobacterial relative biovolume (RM-ANOVA: nutrients only $- F_{1,11} = 13.14, P < 0.004$; mixing and nutrients $- F_{1,11} = 8.45, P < 0.014$; Figure 3). In contrast the relative biovolume of diatoms decreased in the nutrient only (C) treatment (nutrients only $- F_{1,11} = 8.27, P < 0.004$; mixing and nutrients $- F_{1,11} = 4.75, P < 0.052$). Relative diatom abundance consistently increased in mixing only (B) treatment and was highest on day 22 in the control (A) and mixing and nutrient addition (D) treatments. Cyanobacteria continued to dominate treatments where nutrients were added (C and D), but were less dominant in enclosures with mixing and nutrient addition (D) due to relative diatom biovolume. It was surprising to see an increase in diatoms in the control enclosures that were not mixed or were given additional nutrients although the total biovolume of diatoms was relatively small compared to the fertilized treatments (Figure 2).

**Ecological contributions to off-flavor compounds**

Mixing (RM-ANOVA $F_{1,11} = 6.69, P = 0.025$) or adding nutrients (RM-ANOVA $F_{1,11} = 40.76, P < 0.0001$) significantly increased MIB (Figure 1(b)) over time. Although the interaction between fertilization and mixing showed a marginally significant effect on MIB (RM-ANOVA $F_{1,11} = 4.51, P = 0.057$; Figure 1(b)), MIB in the mixing and nutrient addition treatment (D) was over two times higher than in the nutrient only treatment (C) ($P = 0.036$) and approximately five times higher than in the control (A) ($P < 0.0001$) or mixing only (B) treatments ($P = 0.001$). In general, geosmin was elevated in the nutrient only treatment (RM-ANOVA $F_{1,11} = 39.33, P < 0.0001$), with no statistically significant effects observed due to mixing (RM-ANOVA $F_{1,11} = 0.084, P = 0.77$; Figure 1(c)) or the interaction between mixing and fertilization (RM-ANOVA $F_{1,11} = 1.70, P = 0.22$).

![Figure 2](image-url) | Dynamics of (a) cyanobacterial biovolume (mm$^3$/L) and (b) diatom biovolume (mm$^3$/L) over the 28-day experiment. Data points represent means ± one standard error. White circles indicate the control treatment, grey circles represent the mixing only treatment, black circles represent the nutrients only treatment, and black and grey circles indicate the mixing and nutrients treatment.
Cyanobacteria were the only phytoplankton taxon that had a positive effect on MIB ($P = 0.0008$) or geosmin ($P = 0.0012$) over time. Fertilization and vertical mixing (D) caused the greatest increase in MIB by the end of the experiment (Figure 1(b)). There was also a slight increase in MIB for the nutrient-only treatment (C), although not statistically different from the mixing only treatment (B) (Figure 1(b)).

Diatoms only increased within the last two dates of the experiment in the same treatment where MIB demonstrated an increase (D), but were present in relatively low numbers (Figure 2(b)), which may have affected the ability to detect a significant relationship between diatoms and MIB.

Geosmin was markedly higher in both fertilized treatments without (C) and with mixing (D) immediately after treatments were applied and decreased to less than 20 ng/L in all treatments by the end of the experiment. As with MIB, cyanobacteria was the only phytoplankton group that correlated with geosmin over time. Although geosmin decreased throughout the duration of the experiment, cyanobacterial biovolume did not exhibit similar patterns, and even increased in the nutrient only treatment on the second to last sampling date (day 22).

After an initial increase post-fertilization, phosphorus levels slightly decreased in all treatments (Figure 4(a)). Nitrogen generally exhibited a similar pattern as phosphorus, but increased considerably in the fertilized only treatment (C, Figure 4(b)). As *Cylindrospermopsis* spp. was the dominant phytoplankton (>99% of algal biovolume) in this treatment, it is feasible to consider that nitrogen fixation mediated the change in nitrogen concentration (Chislock *et al.* 2014), made even more evident by an increase in cyanobacterial biovolume during the previous week (Figure 2(a)). However, we 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 over time**

MIB and geosmin dynamics were not consistent throughout this experiment and few studies have addressed the relationship between these two compounds. Although geosmin and MIB concentrations were poorly correlated throughout the duration of the experiment ($R^2 = 0.025$, $F_{59} = 1.49$, $P = 0.227$), the off-flavors tended to show an inverse relationship over time (Figure 1(b) and 1(c)). The highest geosmin concentrations were found in the two fertilized treatments (C, D) soon after treatments were established. Geosmin concentrations decreased in all enclosures over time so that by the end of the experiment all treatments were near or below concentrations observed at the start of the experiment (~10 ng/L; Figure 1(c)). MIB did not increase in any treatments until the third week of the experiment (Figure 1(b)). The highest MIB concentrations were observed at the end of the experiment in enclosures that were mixed and received nutrients (treatment D). In the nutrients and mixing treatment (D), geosmin and MIB concentrations were significantly negatively correlated throughout the experiment ($R^2 = 0.48$, $F_{1,10} = 8.59$, $P = 0.018$). No other significant correlations were observed for the other three treatments.
Because many studies have not conducted manipulative field experiments on MIB and geosmin across multiple weeks, similar inverse relationships are rarely described in the literature. One possible explanation for the discrepancies between MIB and geosmin production may be related to other off-flavor producing organisms, such as actinomycetes, which were not measured in this study. 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 through actinomycetes. Given that actinomycetes are important decomposers, added nutrients would indirectly influence actinomycetes through increased producer and consumer detritus. Few, if any studies, have looked at the effect of mixing on actinomycetes, but Allgaier & Grossart (2006) found that actinomycete biomass peaked during the spring and fall, corresponding to expected seasonal mixing events for a dimictic lake. Additionally, actinomycetes have been found to use diatoms and cyanobacteria as substrate (Sugiura et al. 1994), which may explain the opposing patterns found between the two off-flavor compounds during this study. These results suggest that lake management strategies may need to include a combination of approaches that minimize year-round nutrient inputs and consider stratification patterns to control the production of both off-flavor compounds.

CONCLUSIONS

This study empirically examined the influence of two important phytoplankton taxa, cyanobacteria and diatoms, associated with the production of two common off-flavors, MIB and geosmin, in a drinking water reservoir. Treatments were applied using a complete factorial design to cause conditions known to favor one taxon over the other (lake mixing favors diatoms; fertilization favors cyanobacteria). Increases in MIB and geosmin for relevant treatments did not occur concurrently throughout the experiment. The initial fertilization with phosphorus and nitrogen quickly promoted geosmin regardless of mixing and was positively correlated with cyanobacterial biovolume only. Geosmin concentrations were initially high after treatments were established, but fell below 20 ng/L in all treatments by the end of the experiment. MIB exhibited the opposite pattern with concentrations remaining low initially and reaching their greatest concentrations at the end of the experiment. MIB was promoted by fertilization and mixing and correlated with cyanobacteria. Diatom increases near the end of the experiment in the mixed and fertilized treatment were concurrent with an increase in MIB, but did not statistically affect either off-flavor compound. Although fertilization tends to promote both off-flavor compounds, only MIB was significantly affected when mixing of the water column occurred. Management solutions to repeated off-flavor events will require a reduction in eutrophication from the surrounding watershed but should also consider the potential effect of lake-wide mixing on off-flavor dynamics.

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