



Shift of phytoplankton and microbial communities cause seasonal dynamics of odor compounds in *Oncorhynchus mykiss* cultured in a freshwater reservoir

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ABSTRACT

The presence of the odorous compounds geosmin (GSM) and 2-methylisoborneol (MIB) can cause off-flavor in aquacultural products and result in large economic losses. Here, we investigated the effects of various factors on the abundance of microorganisms that mediate the production of off-flavor compounds as well as the environmental conditions that promote the growth of these microorganisms. We monitored seasonal variation in water quality, odorous compounds, and phytoplankton and microbial communities in a cold freshwater reservoir with *Oncorhynchus mykiss*. All water quality parameters significantly differed among seasons with the exception of salinity and total suspended solids. The concentrations of GSM and MIB in fish varied among seasons, and the highest values were observed in the summer. Pearson correlation analysis revealed that Cyanophyta and Bacillariophyta in water were significantly related to both GSM and MIB concentrations in fish, and three genera in Cyanophyta, including *Aphanizomenon*, *Oscillatoria*, and *Anabaena*, were positively correlated with GSM and MIB concentrations ($P < 0.05$). Principal component analysis indicated that microbial communities were distinct among the four seasons. However, no significant correlations of both the order Myxococcales and phylum Actinobacteria with odorous compounds were detected ($P > 0.05$). Temperature, soluble reactive phosphorus (SRP), and total phosphorus (TP) were significantly positively correlated with the phyla and genera that were significantly positively correlated with GSM and MIB concentrations ($P < 0.05$). Various water quality parameters (SRP and TP) could be managed to reduce the presence of GSM and MIB-induced off-flavor in this reservoir as well as other cold freshwater systems used for aquaculture.

1. Introduction

The earthy-musty off-flavor in aquaculture products induces major economic losses because products with these properties cannot be marketed and the depuration of off-flavor compounds is time-consuming (Petersen et al., 2011; Schram et al., 2021). Geosmin (GSM) and 2-methylisoborneol (MIB) often cause off-flavor in aquaculture products. Lipophilic compounds, such as GSM, can accumulate in the fatty tissues of aquatic products, and human senses are highly sensitive to these compounds. Even at nanomolar concentrations (4–10 ng L⁻¹), GSM can cause off-flavor (Lukassen et al., 2017; Lukassen et al., 2019). The

earthy-musty off-flavor can be derived from many different sources; it is thus difficult to completely eliminate all flavors by conventional water treatment processes, such as coagulation, sedimentation, and sand filtration (Tucker and van der Ploeg, 1999), and the removal rate of GSM and MIB by conventional water treatment processes is often <20% (Li et al., 2019). The primary solution involves depurating the tainted fish for several days in clean water prior to sale. During depuration, the fish are not fed, and they lose weight quickly, which decreases meat quality (Burr et al., 2012). Aside from these effects, the depuration process increases the time it takes for fish to enter the market, requires additional labor and energy, and increases water consumption (Kropp et al., 2022).

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Therefore, depuration increases consumption, delays sales, and costs as much as \$0.25/kg USD per fish (Lukassen et al., 2017). The depuration process is not always effective for removing off-flavored compounds from fish (Schram et al., 2021). Many studies have focused on the development of methods to minimize the production of off-flavor compounds in aquaculture production systems (Lukassen et al., 2022).

The primary sources of GSM and MIB are the secondary metabolites of several microorganisms, such as Actinomycetes, Cyanobacteria, Myxobacteria, and Proteobacteria (Lukassen et al., 2017; Lindholm-Lehto and Vielma, 2019). Actinomycetes, especially *Streptomyces*, are mainly associated with off-flavor compounds in indoor recirculating aquaculture systems (RASs), and off-flavor in outdoor aquatic systems is mainly attributed to Cyanobacteria (Podduturi et al., 2020). Another study has reported that *Planktothrix* is the main source of GSM, and *Streptomyces* (Actinobacteria) is likely responsible for the production of MIB in a eutrophic reservoir (Clerc and Druschel, 2019). The composition of phytoplankton and microbiota are likely the main factors determining the presence of GSM- and MIB-producing organisms and the potential for GSM and MIB production (Lukassen et al., 2022; Wu et al., 2022).

Water quality management is critically important in aquaculture, and previous studies have suggested that environmental factors affect the growth of Cyanobacteria, microbial structure, and the production of GSM/MIB in water (Barros et al., 2019; Abd El-Hack et al., 2022). When water is enriched with nitrogen and temperatures are cooler MIB- and GSM-producing bacteria are more abundant in eutrophic reservoirs (Clerc and Druschel, 2019). High nutrient concentrations (total concentrations of soluble reactive phosphorus, ammonium, and nitrate) and low ratios of dissolved inorganic nitrogen to soluble reactive phosphorus (DIN:SRP ratios) are optimal conditions for GSM production by freshwater biofilms (Espinosa et al., 2021). Hyung-Seok et al. (2017) investigated the effects of environmental factors on the cyanobacterial production of odorous compounds (i.e., GSM and MIB). A lower incidence of GSM has been observed in environments with high light intensity and phosphorus stress (Hyung-Seok et al., 2017). In addition, seasonal variation in odorous compounds has been observed in reservoirs (Wu et al., 2022). For example, in the water intake surface layer of Yanghe Reservoir in north China, the concentrations of odorous compounds are highest in the summer; in the water of Eagle Creek Reservoir, which is located in central Indiana, the concentrations of odorous compounds are highest in the spring (Li et al., 2010; Clerc and Druschel, 2019).

Identifying the microorganisms involved in off-flavor compound production and understanding the environmental conditions that promote their growth could provide important insights that would aid the management of water quality in aquatic systems to reduce the levels of GSM and MIB (Lukassen et al., 2022). Rainbow trout (*Oncorhynchus mykiss*) is an economically important cold-water species that is cultured worldwide. In China, this species is mainly cultured in plateau regions with natural reservoir systems; Liujiaxia reservoir is one such system (Han et al., 2020). Off-flavor problems have been reported in rainbow trout cultured in freshwater (Schrader and Summerfelt, 2010). In this study, we characterized seasonal changes in odorous compounds (GSM and MIB) in rainbow trout cultured in a freshwater reservoir. We also analyzed changes in water quality, microbial communities, and phytoplankton communities. Our aims were to 1) detect seasonal variation in off-flavor compounds in fish, water quality, phytoplankton and microbial communities; 2) identify the microorganisms involved in the production of off-flavor compounds; and 3) determine the environmental conditions most suitable for the growth of these microorganisms. Our findings provide new insights that could be used to optimize the management of water quality in aquaculture systems to reduce the incidence of GSM and MIB-induced off-flavor.

2. Materials and methods

2.1. Sampling collection

Liujiaxia Reservoir (Gansu province, China, 35°54'11"N, 103°18'51"E) is located at northwestern China. The water temperature varies greatly across seasons. Although the highest water temperature lasted from June to August, temperature was always <20 °C (Du et al., 2021). Due to the low average temperature, cold-water fish (such as rainbow trout) aquaculture is growing in the area. The fish cage that served as the focus of this project was 40 m in perimeter downstream of the reservoir. The average depth of the study area is around 50 m.

Rainbow trout were collected from the cage in four seasons (April in the spring, July in the summer, October in the autumn during 2018, and January in the winter 2019). Six fish were randomly collected from the cage in each season. All experiments were conducted according to the Guidelines of Animal Research and Ethics Committees of the Ocean University of China. Briefly, ethyl 3-aminobenzoate methanesulfonic acid (MS-222, 0.26 g/L) was used to anesthetize the fish. Then, the fish were sacrificed by decapitation quickly with scissors cutting the spinal column to minimize suffering of the animals. The fish was immediately dissected and the fillets were put on dry ice and transported to the laboratory of the Ocean University of China (Qingdao, China) where they were stored at -20 °C until analysis.

The water temperature, dissolved oxygen (DO), pH, and salinity were measured on site using a YSI-6920 multi-parameter system (Yellow Springs Instruments, USA) at a depth ~1 m below the water surface. Ample water samples were collected from the top layer of the cage using a hydrophore. Three replicates were collected, stored on ice, and immediately transported to the laboratory of the Ocean University of China (Qingdao, China) for analysis. A 3 L water sample was filtered through a 0.22 µm glass fiber filter that was stored in liquid nitrogen and immediately transported to the laboratory of Ocean University of China (Qingdao, China) for analysis. Three replicates were collected for the filters in each season. All of the filters were stored in a freezer at -80 °C prior to microbial analysis.

2.2. Sample analysis

2.2.1. GSM and MIB analysis

For each sample, 20 g fillet were collected, finely chopped, and then the GSM and MIB in fish fillets were extracted using a microwave distillation device as described earlier (Han et al., 2021). Nitrogen flow rate for the device was 70 mL/min. The nitrogen gas takes out the vaporized off-flavor compounds. The compounds were further concentrated in a condenser and the fraction was collected in a collection tube. The 10 mL fraction was put in a 20 mL headspace bottle (Agilent, USA) with 3 g NaCl before immediately sealing with a PTFE-coated silicone rubber cap.

Similar to Schrader et al. (2013) and Wu et al. (2022), the headspace solid phase microextraction (HS-SPME) method coupled with gas chromatography-mass spectrometry (GC-MS) was used to quantify levels of GSM and MIB. Briefly, the 50/30 µm DVB/CAR/PDMS microextraction fiber (Supelco, Bellefonte, PA) was exposed to the headspace of the bottle. The headspace bottle was placed on a heated magnetic agitator (IKA® RCT BASIC heated magnetic agitator, IKA, Germany) where the sample was heated at 65 °C for 45 min. After extraction, the fiber was manually removed from the vial and inserted into the injection port of the GC-MS instrument (Agilent 7890-5975C) and desorbed at 250 °C for 15 min in splitless mode. The gradient temperature program of the GC-MS was set as follows: initial temperature of 40 °C for 2 min; increased to 250 °C at a rate of 8 °C/min and held at 250 °C for 10 min. Helium (He) was used as the carrier gas (0.8 mL/min) of the GC-MS. A HP-5MS capillary column (30 mm × 0.25 mm × 0.25 µm; Agilent, USA) was used. The molecular ion base peaks were monitored at the ratio of molecular mass to ionic charge (m/z) of 112 for GSM and 95 for MIB. Original standard solutions

of GSM and MIB were purchased from Macklin Biochemical Co., Ltd. (Shanghai, China) and were prepared at 10, 50, 100, 200, 500, 1000 ng L⁻¹ in deionized water. The standards were run before each group of samples analyzed (Schrader et al., 2013). All standard curves had good linearity and the R-squared exceeded 0.999. The limit of detection (LOD) and the limit of quantification (LOQ) were determined based on the signal-to-noise ratio (S/N) with S/N = 3 for LOD and S/N = 10 for LOQ. LODs were 4.38 ng/L (GSM) and 5.42 ng/L (MIB), and LOQs were 10.87 ng/L (GSM) and 16.20 ng/L (MIB). The repeatability of solutions (RSD) and spike recovery of the matrix of GSM and MIB at different concentrations (0.5, 2, 5, 10, and 20 µg/kg) were also investigated (N = 5). The average recovery rate of MIB and GSM ranged from 63.16% to 69.53% and 41.75% to 48.80%, respectively. The RSDs were <10%, indicating good reproducibility of the SPME.

2.2.2. Water quality analysis

Total ammonia nitrogen (TAN) was detected by the indophenol blue spectrophotometric method, nitrite nitrogen (NO₂⁻-N) was detected by the ethylenediamine dihydrochloride spectrophotometric method, nitrate nitrogen (NO₃⁻-N) was analyzed by the ultraviolet spectrophotometric method, and soluble reactive phosphorus (SRP) was detected by the molybdenum blue method. Alkaline potassium persulfate digestion ultraviolet spectrophotometry method was applied to analyze total nitrogen (TN) and total phosphorus (TP). All of the above methods and the analysis of chlorophyll-*a* (Chl-*a*), and total suspended solids (TSS) were performed as described by Clesceri et al. (1995). Water samples, which were filtered through Whatman GF/F filter membranes (pore size 0.7 µm, diameter 25 mm), were used to measure dissolved organic carbon (DOC) using a total organic carbon analyzer (Multi N/C 2100S, AnalyticJena, Germany) (Zhang et al., 2016). The ratio of dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) was calculated.

2.2.3. Microbial analysis

The total genomic DNA in the filter was extracted using E.Z.N.A.® Water DNA Kit (OMEGA, USA) according to the manufacturer's protocol. The concentration and quality of extracted DNA were monitored using a spectrophotometer (ND-2000, Nanodrop, USA), after which the integrity of DNA was determined by agarose gel electrophoresis. The DNA was then diluted to 1 ng/µL using sterile water. The V4–V5 region of the 16S rRNA gene was amplified using primers of 515F (5'-GTGCCAGCMGCCGCGG-3')/ 907R (5'-CCGTCAATTCMTTTRAGTTT-3'). The amplification products were purified using a PCR fragment purification kit (Takara, Japan) and the purification was checked using a 1.8% agarose gel electrophoresis. Finally, these products were sequenced on an IonS5™XL platform (ThermoFisher). The sequencing data were deposited in the NCBI Sequence Read Archive Database and the accession number is PRJNA887957.

The raw data were merged using FLASH (v. 1.2.7). Raw tags were further filtered using Cutadapt (V1.9.1, <http://cutadapt.readthedocs.io/en/stable/>). Chimera sequences were identified and removed on the website <https://github.com/torognes/vsearch/>. Those clean tags were clustered into operational taxonomic units (OTUs) at a 97% sequence similarity level using UPARSE (Uparse v7.0.1001, <http://www.drive5.com/uparse/>). The OTUs sequences were made taxonomic annotations against the SILVA132 (Quast et al., 2013; Glöckner et al., 2017).

2.2.4. Statistical analysis

The Qiime software (Version 1.9.1) was used to calculate the indices of bacterial operational taxonomic units (OTUs), Chao1, abundance-based Coverage Estimator (ACE), and Shannon and Simpson Diversity indices. Kolmogorov-Smirnov and Levene's tests were conducted to check the normality of data and homogeneity of variances, respectively. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (variances were homogeneous) or Tamhane's T2 multiple comparison test (variances were inhomogeneous) was applied to

compare the differences of water quality, concentrations of GSM and MIB, alpha diversity, and beta diversity over four seasons. A $P < 0.05$ was set to denote significant differences. All the statistics were conducted using SPSS software (version 19.0, IBM SPSS Statistics, Armonk, NY, USA). To study the relationships among water quality, GSM and MIB concentrations, and biological variables, multivariate analyses were conducted by R software (version 3.6.3). Principal component analysis (PCA) was performed with the "vegan" package. Pearson correlation analysis, which was performed to determine the correlation between GSM and MIB concentrations and the abundance of phytoplankton and microbial genera, was performed using the "stats" package. Correlation heatmaps were created with the "corrplot" package.

3. Results

3.1. Water quality and off-flavor compounds

All of the water quality parameters were significantly different among seasons except salinity and TSS (Table 1). The temperature was significantly higher in the summer followed by autumn, spring, and winter ($P < 0.05$). The average DO was significantly higher in the spring, followed by winter, summer, and autumn ($P < 0.05$). DO was 36.73% lower in the summer compared with spring. The pH in different sampling seasons followed: summer>winter>spring>autumn. As for the nutrients, TAN, NO₂-N, and TN were lowest in the spring and highest in the autumn, while the NO₃-N was higher in the spring and winter than in the summer and autumn. TAN concentration in the summer was approximately three times more than that in the spring, and NO₃-N concentration in the summer decreased by 68.33% compared with spring. SRP and TP were significantly higher in the summer than in the other three seasons ($P < 0.05$). The DIN:SRP was lowest in the summer, and was reduced by 49.52% from spring to summer. The Chl-*a* in the winter was significantly lower than in the other three seasons ($P < 0.05$). The DOC was highest in the winter and significantly lowest in the summer ($P < 0.05$).

The concentrations of GSM and MIB in fish varied in the same trend among different sampling seasons (Fig. 1). The highest values were observed in the summer and were significantly higher than that in the other three seasons ($P < 0.05$). In the summer, the average GSM and MIB concentrations were 482.03 and 726.71 ng/kg, respectively. Lower values were observed both in the autumn and winter, and there was no significant difference for both compounds between the two seasons ($P > 0.05$). The concentrations of MIB was generally higher than GSM in all the seasons except winter.

3.2. Phytoplankton community

Seven phytoplankton phyla, including Bacillariophyta (5 orders and 14 genera), Chlorophyta (4 orders and 12 genera), Pyrrophyta (2 orders and 4 genera), Cyanophyta (3 orders and 4 genera), Euglenophyta (1 order and 3 genera), Xanthophyta (1 order and 2 genera), and Chrysophyta (1 order and 3 genera), were identified (Table S1). If the abundance of a genus accounted for >10% of the total abundance of the phytoplankton community, this genus would be defined as the dominant genus. Eight dominant genera, including *Synedra*, *Selenastrum*, *Phacus*, *Oscillatoria*, *Coscinodiscus*, *Anabaena*, *Gonyaulax*, and *Microcystis*, were identified. Only *Synedra* was identified as the dominant genus in the winter (Table S2).

Cyanophyta and Bacillariophyta have been shown to produce odorous compounds in previous studies (Wu et al., 2021). Pearson correlation analysis indicated that Cyanophyta and Bacillariophyta were positively correlated with both GSM and MIB ($P < 0.05$) (Fig. 4). The Cyanophyta had the highest abundance in the summer and the lowest abundance in the winter ($P < 0.05$). Three subsidiary genera of Cyanophyta, including *Aphanizomenon*, *Oscillatoria*, and *Anabaena*, were identified to be positively correlated with GSM and MIB concentrations.

Table 1

Comparison of water quality in the Liujiaxia Reservoir system in different periods and results of one-way ANOVA followed by Tukey's multiple comparison test (variances were homogeneous) or Tamhane's T2 multiple comparison test (variances were inhomogeneous).

	Temperature (°C)	DO (mg/L)	Salinity (ppt)	pH	TAN (mg/L)	NO ₂ -N (mg/L)	NO ₃ -N (mg/L)	TN (mg/L)	SRP (mg/L)	TP (mg/L)	DIN: SRP	Chl- <i>a</i> (µg/L)	TSS (g/L)	DOC (mg/L)
Spring	12.9 ^c	11.00 ^a	0.20 ^a	8.23 ^b	0.05 ± 0.00 ^b	0.01 ± 0.00 ^b	0.17 ± 0.01 ^a	1.51 ± 0.08 ^d	0.02 ± 0.01 ^b	0.02 ± 0.01 ^b	13.59 ± 4.18 ^{ab}	1.07 ± 0.17 ^a	0.01 ± 0.00 ^a	5.65 ± 0.53 ^b
Summer	20.3 ^a	6.96 ^c	0.20 ^a	8.47 ^a	0.15 ± 0.00 ^a	0.02 ± 0.00 ^{ab}	0.05 ± 0.01 ^b	5.44 ± 0.22 ^b	0.03 ± 0.01 ^a	0.17 ± 0.04 ^a	6.86 ± 1.48 ^b	1.26 ± 0.18 ^a	0.02 ± 0.01 ^a	4.89 ± 0.29 ^c
Autumn	13.1 ^b	6.46 ^d	0.20 ^a	7.77 ^c	0.18 ± 0.01 ^a	0.05 ± 0.02 ^a	0.06 ± 0.01 ^b	6.77 ± 0.16 ^a	0.01 ± 0.00 ^b	0.07 ± 0.03 ^b	24.26 ± 2.43 ^{ab}	1.17 ± 0.08 ^a	0.01 ± 0.00 ^a	5.94 ± 0.23 ^{ab}
Winter	4.6 ^d	8.21 ^b	0.20 ^a	8.33 ^b	0.14 ± 0.06 ^a	0.04 ± 0.01 ^a	0.19 ± 0.01 ^a	2.89 ± 0.91 ^c	0.01 ± 0.01 ^b	0.05 ± 0.03 ^b	80.44 ± 70.19 ^a	0.09 ± 0.01 ^b	0.01 ± 0.01 ^a	6.51 ± 0.28 ^a

Mean ± standard deviation of 3 replicates are shown; different lower-case letters represent significant difference of water quality factors in different periods ($P < 0.05$). DO, dissolved oxygen; TAN, total ammonia nitrogen; NO₂-N, nitrite; NO₃-N, nitrate; TN, total nitrogen; SRP, soluble reactive phosphate; DIN, dissolved inorganic nitrogen; TP, total phosphate; Chl-*a*, Chlorophyll *a*; TSS, total suspended solid; DOC, dissolved organic carbon.

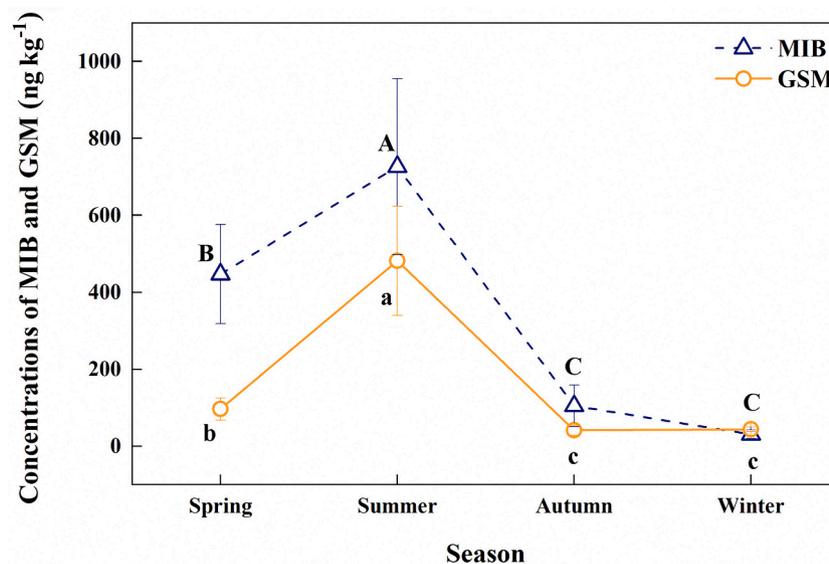


Fig. 1. Mean ± standard deviation of concentrations of 2-methylisoborneol (MIB) and geosmin (GSM) in *Oncorhynchus mykiss* collected from the Liujiaxia Reservoir system over four seasons. Different letters represent significant differences between sampling times ($P < 0.05$).

Aphanizomenon had significantly higher abundance in the summer and was positively related to both MIB and GSM ($P < 0.05$). The abundance of *Oscillatoria* in the spring and summer was significantly higher than in the other two seasons ($P < 0.05$), and *Oscillatoria* was also positively related to both MIB and GSM ($P < 0.05$). *Anabaena* had the highest abundance in the summer, followed by autumn, winter, and spring ($P < 0.05$). This genus was significantly related to the odorous compound GSM, but not MIB. The Bacillariophyta had the highest abundance in the summer and 13 subsidiary genera were identified. Six genera (*Navicula*, *Nitzschia*, *Cyclotella*, *Coscinodiscus*, *Skeletonema*, and *Triceratium*) were selected to have significant relationships with the odorous compounds. All of the six genera in Bacillariophyta had higher abundance in the summer than in the other three seasons. *Navicula* and *Cyclotella* were the only taxa significantly related to GSM ($P < 0.05$), while *Nitzschia*, *Coscinodiscus*, *Skeletonema*, and *Triceratium* were significantly related to both the two odorous compounds ($P < 0.05$).

3.3. Microbial community structure

For the bacterial community, temporal differences were found in OTUs, Shannon, Simpson, Chao1, and ACE indices (Table S3). The OTUs

were significantly higher in the autumn, followed by summer, winter, and spring. Both Shannon and Simpson indices were the lowest in the spring, while the Chao1 and ACE values were the highest in the autumn. All indices in the autumn were significantly higher than those in the spring ($P < 0.05$).

The first ten most abundant phyla were shown in Fig. 2a. The dominant phyla were Proteobacteria (36.28%), Cyanobacteria (21.69%), Actinobacteria (17.00%), and Bacteroidetes (15.62%). One-way ANOVA was performed for the top ten phyla between different sampling seasons, and all the phyla, except Gracilibacteria, showed significant differences among seasons ($P < 0.05$) (Table S4). The highest abundance of Bacteroidetes, Actinobacteria, Verrucomicrobia, and Chloroflexi were observed in the winter and the lowest values were observed in the spring, while Proteobacteria and Cyanobacteria showed the opposite trends. The taxonomic bacterial composition was dominated by unidentified *Cyanobacteria*, the average occurrences percentages of which was 14.90% (Fig. 2b).

Principal component analysis (PCA) was conducted to determine temporal variations in OTUs over four seasons, and the PCA analysis showed that the four sampling seasons had distinctively different microbial community distributions (Fig. 3).

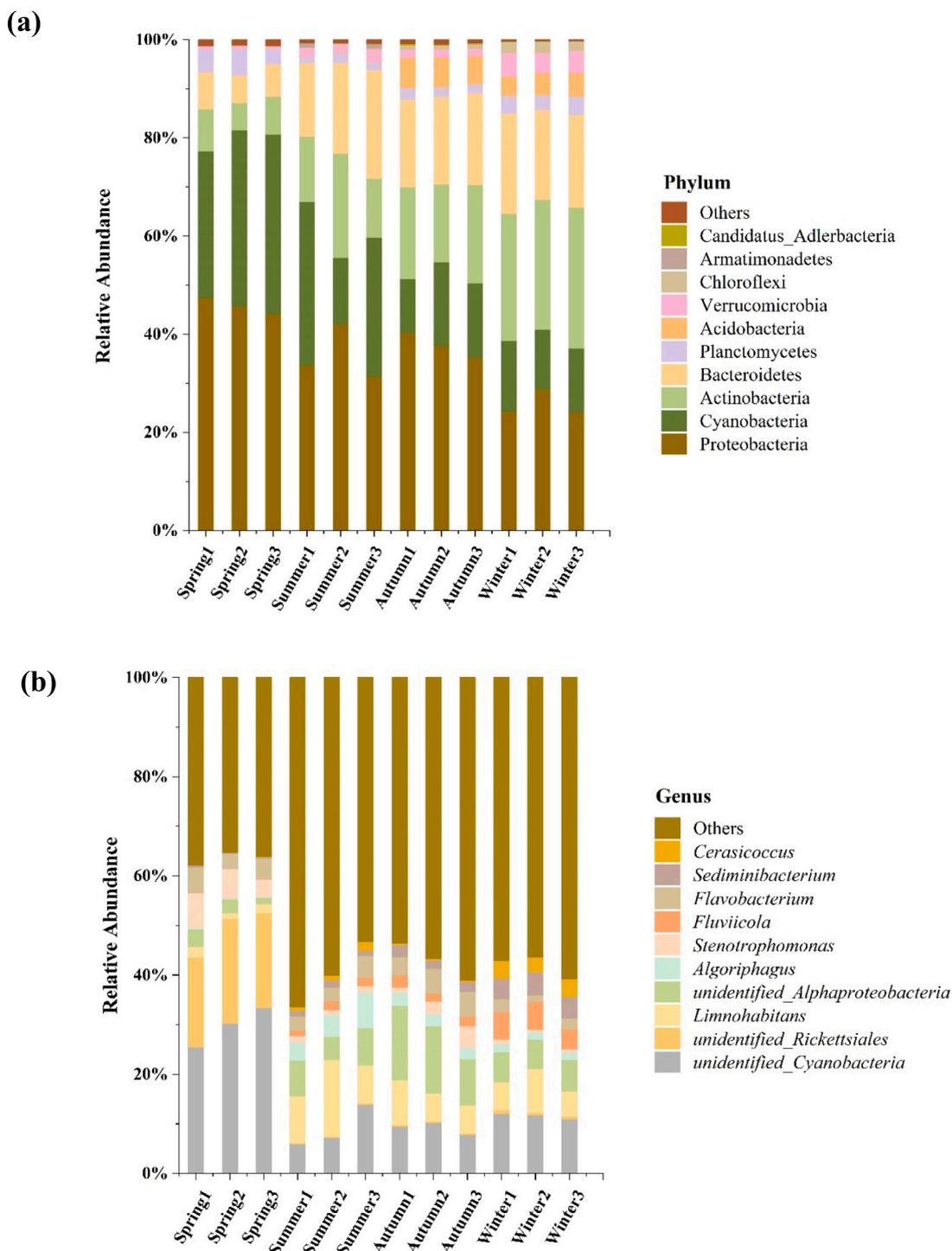


Fig. 2. Relative abundance of microbial communities at (a) phylum level and (b) genus level of the Liujiaxia Reservoir in four seasons.

The GSM and MIB were mainly produced by Actinobacteria (mainly *Streptomyces*), Myxobacteria, and Cyanobacteria (Abd El-Hack et al., 2022). As Cyanobacteria have already been discussed, the correlations of Myxobacteria and Actinobacteria with the odorous compounds were further investigated (Fig. 5). No significant correlations of both the order Myxococcales and phylum Actinobacteria with the odorous compounds were detected ($P > 0.05$). The genera in the order Myxococcales and phylum Actinobacteria with total abundance above 0.1% of the total bacteria community were further selected. Eight genera in the phylum

Actinobacteria were selected to investigate its relationship with the odorous compounds. Three genera have significantly positive correlations with the odorous compounds (Fig. 5). *Candidatus_Planktophila* was only significantly correlated with GSM ($P < 0.05$), while the *Candidatus_Aquiluna* and *Microbacterium* were significantly correlated with both the two odorous compounds ($P < 0.05$).

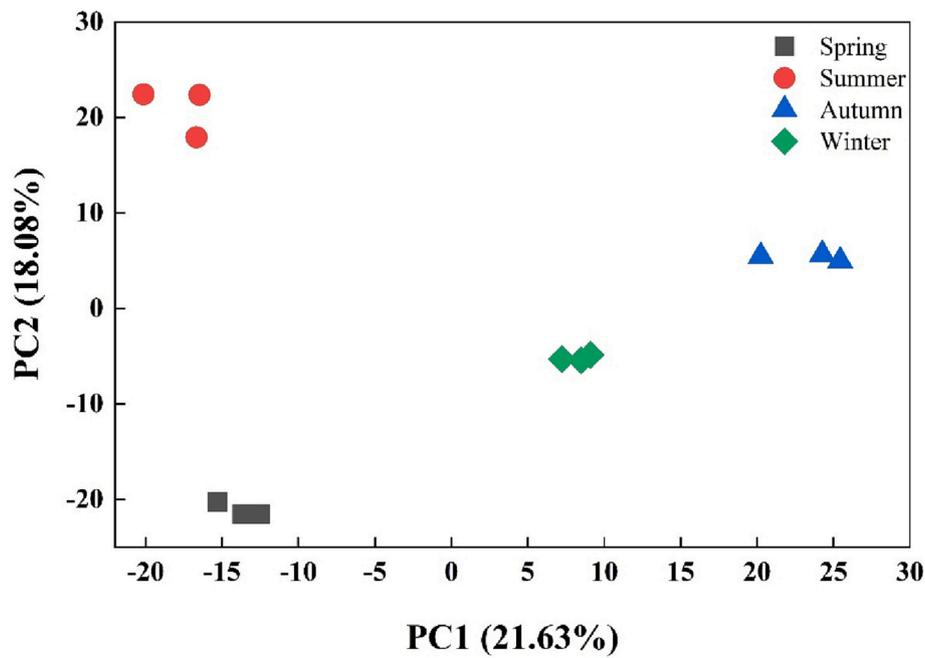


Fig. 3. Principal component analysis (PCA) based on the OTU sequences in four months.

3.4. Relationship of phytoplankton and microbial community with the water quality parameters

Pearson correlation analysis was further adopted to analyze the relationship between environmental factors and the phyla and genera significantly correlated with the odorous compounds (Fig. 6). Temperature, SRP, and TP were the water quality parameters having the most significantly positive correlations with the phyla and genera ($P < 0.05$). The temperature had significant positive correlations with all the phyla and genera except *Cyclotella*, *Skeletonema*, *Candidatus Planktophilia*, and *Microbacterium* ($P < 0.05$). SRP was significantly positively correlated with all the phyla and genera except *Aphanizomenon*, Bacillariophyta, *Skeletonema*, and *Microbacterium* ($P < 0.05$). Total phosphorus was significantly positively correlated with all the phyla and genera except *Oscillatoria*, *Cyclotella*, and *Microbacterium* ($P < 0.05$). DOC was significantly negatively correlated with all the phyla and genera except *Cyclotella*, *Candidatus Planktophilia*, and *Microbacterium* ($P < 0.05$).

4. Discussion

4.1. Concentration of GSM and MIB in *O. mykiss*

The average MIB concentration in rainbow trout was 307.3 ng/kg, and the highest value observed was 727 ng/kg. The average and highest GSM concentrations in rainbow trout were 156.3 ng/kg and 482 ng/kg, respectively. These values are consistent with concentrations reported in previous studies ($< 1 \mu\text{g}/\text{kg}$) (Abd El-Hack et al., 2022). Humans are highly sensitive to GSM and MIB, and the threshold data are typically derived from sensory evaluations, which vary among test subjects. The sensory threshold for GSM in *O. mykiss* is 0.9 $\mu\text{g}/\text{kg}$, and 0.7 $\mu\text{g}/\text{kg}$ for MIB (Robertson et al., 2005). However, other studies have reported that the human detection threshold for GSM in *O. mykiss* is 0.25, and the threshold for MIB is 0.55 $\mu\text{g}/\text{kg}$ (Petersen et al., 2011; Abd El-Hack et al., 2022). Based on the above data, the concentration of MIB in the summer slightly exceeded the detection threshold. These fish can be depurated to reduce the GSM and MIB concentrations to levels below their detection thresholds.

Seasonal changes in both GSM and MIB were observed in fish, and concentrations of both compounds were highest in the summer. Similar

seasonal changes have been observed in a water supply reservoir, where the concentrations of both compounds increased from spring to late summer and not detectable in the winter (Westerhoff et al., 2005). Li et al. (2010) reported the odorous compounds are highest in the summer due to algal blooms. However, the outbreak seasons of odorous compounds vary among studies. For example, outbreaks of both MIB and GSM were observed to be more intense in the spring than at any other time in the year in a eutrophic reservoir (Clerc and Druschel, 2019). In a subtropical drinking water reservoir, the MIB concentration was high in the spring; however, after a heavy rain in the late spring, the concentration of MIB decreased sharply. This indicates that seasonal changes in odorous compounds are complex and affected by various hydrometeorological processes (Wu et al., 2022).

The average concentration of MIB was higher than GSM in both spring and summer when the concentrations of odorous compounds were higher in fish. Previous studies have shown that the geographic distributions of these two compounds are uneven. Although the average concentration of GSM is higher than MIB in the seven drinking water reservoirs investigated, some reservoirs have high MIB and low GSM concentrations (Wu et al., 2021). The MIB concentrations are consistently higher than GSM concentrations in water supply reservoirs in Arizona (Westerhoff et al., 2005). The off-flavor of red sea bream (*Pagrus major*) reared in RASs with low salinity has been shown to be caused by MIB (Kawaguchi et al., 2019). On the other hand, the GSM was detected in a much wider range than MIB in the catfish farms in Alabama, USA (McCrummen et al., 2018). These two odorous compounds are produced by different microorganisms (Abd El-Hack et al., 2022). A wide range of Cyanobacteria, such as *Aphanizomenon*, *Anabaena*, and *Lyngbya*, can produce GSM. Over 40 species of Cyanobacteria, including *Pseudanabaena*, *Planktothrix*, *Oscillatoria*, and *Lyngbya*, can produce MIB (Devi et al., 2021).

4.2. GSM and MIB-producing phytoplankton and bacterial communities

Phytoplankton succession and biomass are related to odorous compound production (Wu et al., 2022). Previous studies have shown that Cyanobacteria (i.e., Cyanophyta) are some of the most important producers of odorous compounds (Olsen et al., 2016; Abd El-Hack et al., 2022). The abundances of three genera (*Aphanizomenon*, *Oscillatoria*,

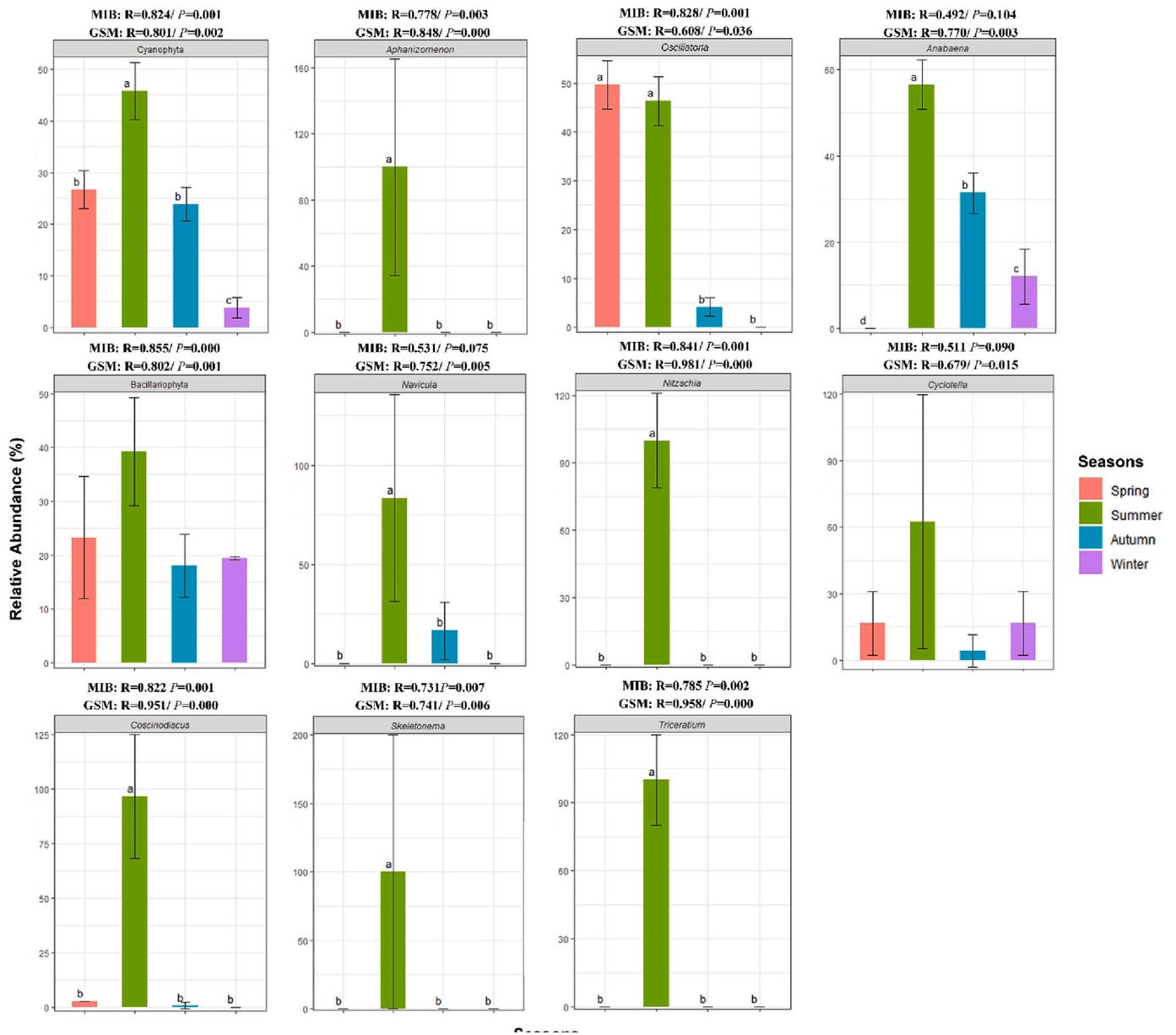


Fig. 4. Relative abundance of phytoplankton genera that were significantly correlated with concentrations of 2-methylisoborneol (MIB) and geosmin (GSM) in fish. Different letters represent significant differences between sampling times ($P < 0.05$).

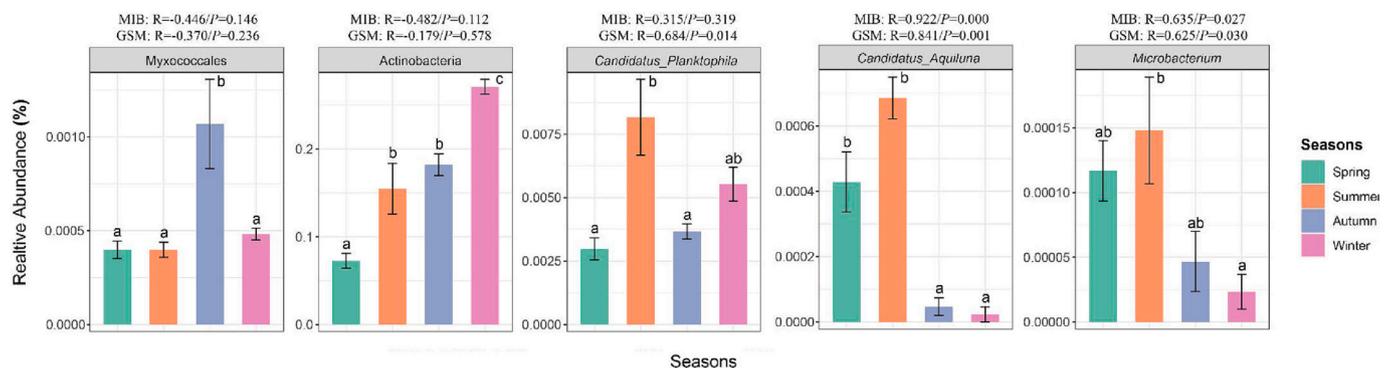


Fig. 5. Relative abundance of microbial genera that were significantly correlated with concentrations of 2-methylisoborneol (MIB) and geosmin (GSM) in fish. Different letters represent significant differences between sampling times ($P < 0.05$).

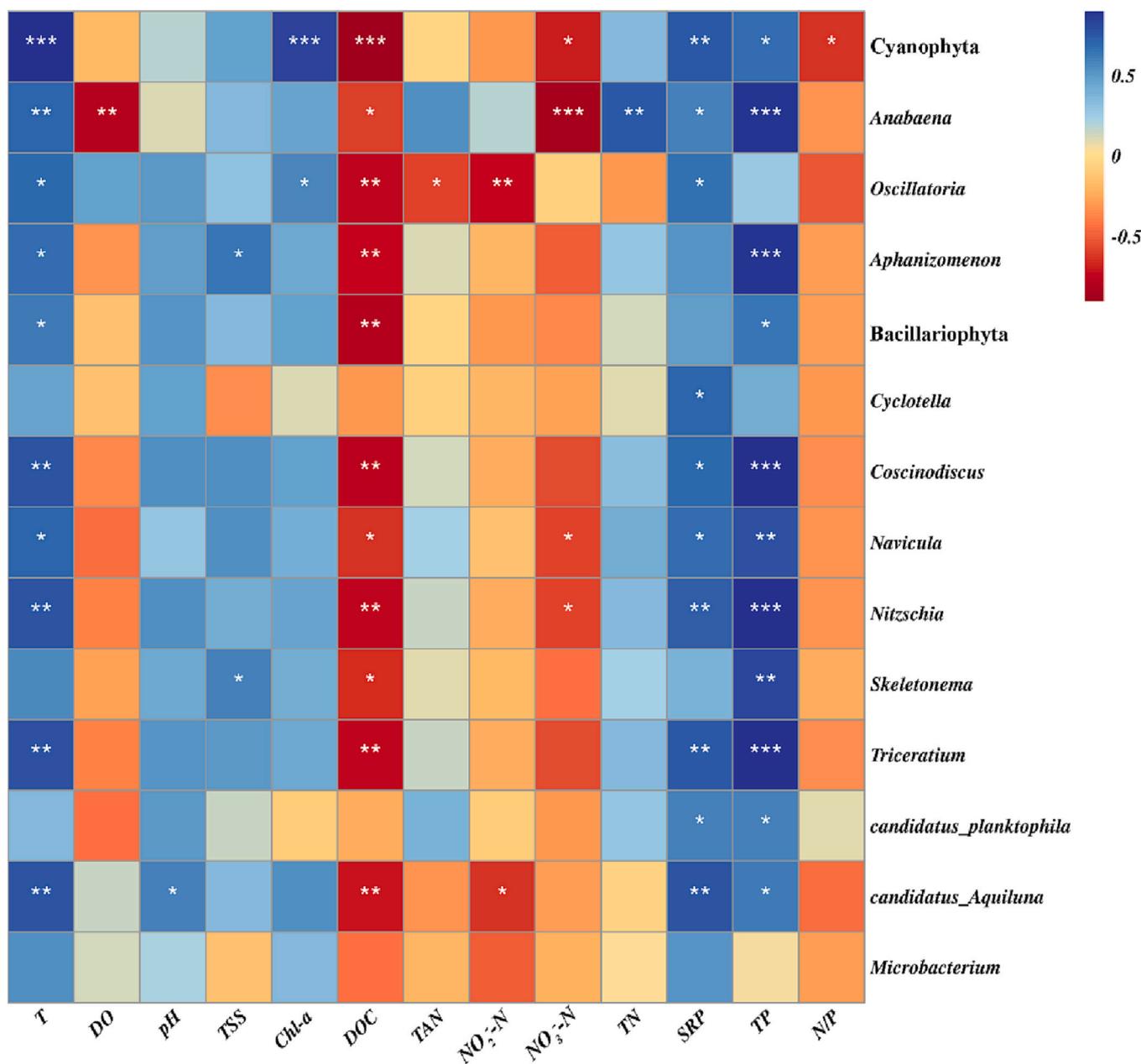


Fig. 6. Correlations between environmental factors and the abundance of phytoplankton genera and microbial genera, *, ** and *** represent $P \leq 0.05$, 0.01 and 0.001, respectively; T, temperature ($^{\circ}\text{C}$); DO, dissolved oxygen (mg/L); TSS, total suspended solid (g/L); Chl-a, Chlorophyll a ($\mu\text{g/L}$); DOC, dissolved organic carbon (mg/L); TAN, total ammonia nitrogen (mg/L); $\text{NO}_2\text{-N}$, nitrite (mg/L); $\text{NO}_3\text{-N}$, nitrate (mg/L); TN, total nitrogen (mg/L); SRP, soluble reactive phosphate (mg/L); TP, total phosphate (mg/L); N/P: DIN/SRP; DIN, dissolved inorganic nitrogen (mg/L).

and *Anabaena*) in the phylum Cyanophyta were significantly related to the odorous compounds in our study. All three genera have been demonstrated to be producers of GSM or MIB (Devi et al., 2021). MIB is mainly derived from *Aphanizomenon* in a subtropical drinking water reservoir (Wu et al., 2022). Many records of *Oscillatoria* strains, which can produce both GSM and MIB, are also obtained (Tsuchiya and Matsumoto, 1999).

Besides Cyanophyta, the production of the odorous compounds by Bacillariophyta has also been reported in previous studies (Wu et al., 2021). A strong correlation between MIB and diatom biovolume has been observed in a field limnocorral experiment in a drinking water reservoir. The diatoms in the system can produce odorous compounds or serve as a substrate for the growth of certain diatoms to produce odorous compounds (Olsen et al., 2016). For example, *Navicula*, *Nitzschia*, and *Cyclotella* can produce odorous compounds (Xu et al., 2010), and

Cyclotella is one of the main sources of GSM in a subtropical drinking water reservoir (Wu et al., 2022).

The microbial communities in the water samples varied among the four seasons, and this might be related to differences in water quality conditions among seasons. As our study was mainly related to off-flavor compounds, we focused on the main bacterial communities that are potential odor producers. Actinobacteria and Myxobacteria are producers of odorous compounds (Abd El-Hack et al., 2022). However, no significant correlations of the abundances of the order Myxococcales and phylum Actinobacteria with odorous compounds were detected in this study. The genus *Streptomyces*, which accounts for a large fraction of Actinobacteria, is usually the main contributor to the characteristic odor in aquatic environments (Abd El-Hack et al., 2022). In our study, the abundance of *Streptomyces* in the reservoir was low, and it was not significantly correlated with the concentrations of both GSM and MIB.

The other three genera of Actinobacteria (*Candidatus Planktophila*, *Candidatus Aquiluna*, and *Microbacterium*) were significantly correlated with both the odorous compounds. However, these genera have not been shown to directly produce GSM and MIB in previous studies.

Our findings demonstrated that phytoplankton (Cyanophyta and Bacillariophyta) were the main producers of odorous compounds in the fish, but not bacteria (mainly Myxococcales and Actinobacteria). This can be explained by the fact that Cyanobacteria are the causes of most off-flavor problems in eutrophic environments and outdoor aquaculture facilities (Buley et al., 2021; Wu et al., 2021; Abd El-Hack et al., 2022; Wu et al., 2022), and Myxococcales and Actinobacteria are producers of GSM and MIB in indoor RASs (Lindholm-Lehto and Vielma, 2019). Cyanobacteria usually thrive in nutrient-rich environments (Abd El-Hack et al., 2022). Large amounts of feed with high nitrogen and phosphorus concentrations are used to promote fish growth, but the utilization rate of fish on feed is not high. The release of uneaten feed and feces into the system increases nutrient levels in aquaculture systems, which might favor the growth of Cyanobacteria (Lukassen et al., 2022).

4.3. Effect of environmental factors on the production of GSM and MIB

In our study, the temperature was significantly correlated with the abundances of phyla and genera that were significantly positively correlated with GSM and MIB. Previous studies have shown that temperature is a major factor affecting the production of MIB and GSM in three reservoirs in Arizona (Westerhoff et al., 2005). Three GSM-producing strains of *Anabaena* sp. show relatively higher growth when the water temperature exceeds 20 °C (Hyung-Seok et al., 2017). The concentration of MIB might exceed the odor threshold concentration when the water temperature in a subtropical drinking water reservoir exceeds 12 °C (Wu et al., 2022). In our study, the temperature was highest in the summer (20.3 °C), which might enhance the production of odorous compounds. In addition, the effect of temperature on the production of odorous compounds is a complex process. Combined effects of temperature and other environmental factors, such as light, have been observed. Some GSM producers have been shown to be significantly more sensitive to high light intensity (>100 μmol/m²/s) than to low temperature (<20 °C). However, some MIB producers can adapt to a relatively wide range of light intensities but are sensitive to low temperatures (Hyung-Seok et al., 2017).

Nutrients, such as nitrogen and phosphorus, in water can affect the production of GSM and MIB (Olsen et al., 2016; Abd El-Hack et al., 2022). Both SRP and TP affected the occurrence of odorous compounds in this study. These findings are consistent with the results of previous studies. The content of off-flavor compounds in *O. mykiss* filets is related to the dietary phosphorus level and metabolic phosphorus waste outputs into the RAS (Sarker et al., 2014). Phosphorus levels might affect GSM production in a large number of full-scale RAS (Lukassen et al., 2022). The concentration of odorous compounds in fish was higher in the summer in our study when SRP and TP levels were higher. However, the opposite patterns were observed in a laboratory experiment (Zhang et al., 2017). The lowest GSM concentration and highest Chl *a* content were observed when the SRP concentration was highest (5.44 mg/L). A low SRP concentration can inhibit Chl *a* synthesis and promote the use of farnesyl diphosphate in GSM synthesis in the GSM-producing Cyanobacteria *Lyngbya kuetzingii* UTEX (Zhang et al., 2017). The SRP concentration was low in the reservoir in our study during the entire year, and this might explain why our findings are inconsistent with the results of previous studies.

Aside from phosphorus, the ratio of nitrogen-to-phosphorus (N:P) is also associated with GSM and MIB production. Both N:P and TP have been shown to be positively associated with cyanobacterial taxa in reservoirs in a semi-arid region of Brazil (Barros et al., 2019). In our study, DIN:SRP was lowest in the summer, when the concentrations of odorous compounds in fish were highest. This is consistent with the

results of previous studies. High nutrient concentrations and a low DIN:SRP ratio have been shown to promote the production of GSM by freshwater biofilms (Espinosa et al., 2021). With the exception of the significant correlation between the abundance of *Anabaena* and TN, no significant positive correlations of TN, TAN, NO₂-N, and NO₃-N with the abundances of phyla or genera that were significantly correlated with the concentration of odorous compounds were observed in this study. High nitrate levels also do not have adverse or positive effects on GSM-related off-flavor in a RAS with *O. mykiss* (Schrader et al., 2013). Aside from temperature, phosphorus, nitrogen, and other environmental factors, such as oxygen levels can also affect the occurrence of odorous compounds (Abd El-Hack et al., 2022; Lukassen et al., 2022). However, these factors were not significantly correlated with the production of the odorous compounds examined in our study.

5. Conclusions

Previous studies have demonstrated that Cyanobacteria, Actinobacteria (mainly *Streptomyces*), and Myxobacteria are the main producers of geosmin (GSM) and 2-methylisoborneol (MIB). However, the odorous compounds in the present study were mainly produced by Cyanobacteria but not Actinobacteria (mainly *Streptomyces*) or Myxobacteria. Several water quality parameters, including temperature, soluble reactive phosphorus, and total phosphorus, were mainly positively correlated to the level of GSM and MIB-producing bacteria in the reservoir. Thus, to minimize odorous compound production, these factors should be managed, where feasible. As an outdoor culturing system, it is not easy to control the temperature of the system. However, the phosphorus levels of the system in the summer could be reduced by reducing the amount of uneaten feed or changing to a diet with lower phosphorus content to reduce the production of odorous compounds in the system.

CRediT authorship contribution statement

Shuting Li: Methodology, Formal analysis, Writing – original draft, Writing – review & editing. **Fayi Wei:** Investigation, Formal analysis. **Xuan Dong:** Validation, Methodology. **Miaojun Pan:** Formal analysis, Software. **Li Li:** Conceptualization, Validation, Writing – original draft, Writing – review & editing. **Xiangli Tian:** Methodology, Writing – review & editing. **Qinfeng Gao:** Supervision, Resources, Writing – review & editing. **Alan E. Wilson:** Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2023.739422>.

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