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Microcystis aeruginosa/Pseudomonas pseudoalcaligenes interaction effects on off-flavors in algae/bacteria co-culture system under different temperatures

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ABSTRACT

We conducted an experiment to study the interaction effects of *Microcystis aeruginosa* and *Pseudomonas pseudoalcaligenes* on off-flavors in an algae/bacteria co-culture system at three temperatures (24, 28 and 32°C). Gas chromatography–mass spectrometry was applied to measure off-flavor compounds dimethyl sulfide (DMS), dimethyl trisulfide (DMTS), 2-methylisoborneol, geosmin (GEO) and β-cyclocitral. During the lag phase of co-cultured *M. aeruginosa* (first 15 days), *P. pseudoalcaligenes* significantly increased the production of DMS, DMTS and β-cyclocitral at all three temperatures. In the exponential phase of co-cultured *M. aeruginosa* (after 15 days), *M. aeruginosa* became the main factor on off-flavors in the co-culture system, and β-cyclocitral turned to the highest off-flavor compound. These results also indicated that DMS, DMTS and β-cyclocitral were the main off-flavor compounds in our *M. aeruginosa*/*P. pseudoalcaligenes* co-culture system. Univariate analysis was applied to investigate the effects of *M. aeruginosa* and *P. pseudoalcaligenes* on the production of off-flavors. The results demonstrated that both *M. aeruginosa* and *P. pseudoalcaligenes* could increase the production of DMS and DMTS, while β-cyclocitral was mainly determined by *M. aeruginosa*. Our results also provide some insights into understanding the relationship between cyanobacteria and heterotrophic bacteria.

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Introduction

In the past decades, increasing eutrophication has led to frequent outbreaks of cyanobacterial blooms (mainly *Microcystis aeruginosa*) in many lakes around the world. Growth and decay of these blooms caused off-flavor episode, and off-flavor episodes are becoming a worldwide problem in

aquatic environments, especially in eutrophic lakes and reservoirs in China (Yu et al., 2009). Since 2005, episodes of strong earthy-musty off-flavors have occurred every year, which lead to off-flavor contamination of drinking water and aquaculture systems in the Xionghu Reservoir (Hubei, China) (Zhang et al., 2010a). It also has triggered a serious drinking water pollution incident when a dense cyanobacterial bloom

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occurred in Taihu Lake during the summer in 2007 (Chen et al., 2010; Yang et al., 2008). These nuisance cases have resulted in large economic losses to the aquaculture industry, negative esthetic impacts to many tourist sites and large increases in the water treatment costs (Li et al., 2007).

Off-flavors in water supplies usually originate from volatile organic compounds (VOCs) including 2-methylisoborneol (MIB) (Rosen et al., 1968), geosmin (GEO) (Guttman and van Rijn, 2009), dimethyl sulfide (DMS) (Giger and Schaffner, 1981), methyl thiols, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS) (Zhang et al., 2010b), β -cyclocitral (Ozaki et al., 2008), ethanethiol (Slater and Blok, 1983), 2-isopropyl-3-methoxypyrazine (Buttery and Ling, 1973) and β -ionone (Höckelmann and Jüttner, 2005). Eutrophic lakes were complicated ecosystems and studies about off-flavors should be discussed in regard to various influencing factors. In the plankton, cyanobacteria and heterotrophic bacteria are the dominant organisms, and their metabolism largely controls energy flow and nutrient cycling in aquatic ecosystems (Cole et al., 1982). Off-flavor episodes were related to physiological change of cyanobacteria and heterotrophic bacteria. Cyanobacteria blooms affect both the nature and the magnitude of their metabolite-related impacts on water quality. In more eutrophic systems, cyanobacteria are frequent sources of VOCs, such as terpenoids, thiols, and pigment derivatives (Watson et al., 2008). Meanwhile, phototrophic non-sulfur bacteria could produce methylated sulfur compounds, such as DMS and DMDS (McCarthy et al., 1993). Several works have been carried out to study the off-flavors from single aspects, such as sources and dynamics or the effects of temperature and light on the production of off-flavors (Klausen et al., 2005; Zhang et al., 2009). However, there are few studies which pay attention to the interactions between cyanobacteria and heterotrophic bacteria on the off-flavors.

Pseudomonas pseudoalcaligenes belonged to *Pseudomonas* species, which had been demonstrated to play a pivotal role of DMTS in cyanobacterial bloom of Taihu Lake in 2007. Detected DMTS at concentrations of 11,399 and 1768 ng/L in drinking-water intake and the water agglomerate, respectively-high enough to account for the odors (Yang et al., 2008). Thus we selected *P. pseudoalcaligenes* and *M. aeruginosa* in co-culture system to investigate the effects of *M. aeruginosa* and *P. pseudoalcaligenes* on the production of off-flavor compounds, such as DMS, DMTS, MIB, GEO and β -cyclocitral. Our results also provide new data for understanding the mutual interactions between *M. aeruginosa* and *P. pseudoalcaligenes* in co-culture systems.

1. Materials and methods

1.1. Strains, cultures and experimental design

P. pseudoalcaligenes was isolated in our laboratory from a sampling site in Gonghu Bay in Taihu Lake (Fig. 1) which was the site of the Wuxi odor accident in 2007 (Yang et al., 2008). Water samples were collected in the August of 2008. At the time of sampling, a large part of the lake surface was covered with dense cyanobacterial blooms, consisting mainly of *M. aeruginosa* (Niu et al., 2011).

The unicellular toxic *M. aeruginosa* used in the experiment was obtained from the Culture Collections of Freshwater Algae at the Institute of Hydrobiology (Wuhan, China). In order to eliminate the effects from contaminating bacteria, *M. aeruginosa* was purified using a streak plate on BG-11 agar medium (Stanier et al., 1971) before the experiment was carried out, and then grown in batch culture in BG-11 medium at $(25 \pm 1)^\circ\text{C}$ illuminated in a 12 hr:12 hr of light:dark cycle at irradiance of $50 \mu\text{E}/(\text{m}^2\text{-sec})$.

The axenic cultures were transferred weekly to fresh medium and maintained in exponential phase. Regular inspection using 4',6-diamidino-2-phenylindole (DAPI) staining, in conjunction with epifluorescence microscope, showed that cultures were axenic at the beginning of the experiment and that the biomass of bacteria in the axenic cultures never exceeded 1% of *Microcystis* biomass during the experiment. Each step of the isolation procedure was carried out under sterile conditions.

A co-culture system was designed as independent axenic *M. aeruginosa* and *P. pseudoalcaligenes* (control), and co-cultured axenic *M. aeruginosa* with *P. pseudoalcaligenes* (treatment) groups. The initial concentrations of *M. aeruginosa* and *P. pseudoalcaligenes* were 1×10^5 and 1×10^7 cells/mL, respectively. Each treatment was prepared in triplicate and the experiment lasted 33 days. The batch cultures were maintained at 24, 28 and 32°C and at irradiance of approximately $20 \mu\text{E}/(\text{m}^2\text{-sec})$. The cultures were harvested every third day to monitor changes in growth and off-flavor compounds. Samples for gas chromatography-mass spectrometry (GC-MS) analysis were filtered and stored at -20°C prior to analysis.

1.2. *Microcystis* cell count and Chlorophyll a determination

After staining with Lugol's solution, *Microcystis* cells were enumerated in a hemocytometer using an Olympus BX50 microscope at $600\times$ magnification (Olympus, Tokyo, Japan). Chlorophyll a (Chl-a) concentrations in *M. aeruginosa* cells were measured after extraction overnight in 80% acetone at 4°C in the dark. The supernatant was collected by centrifugation and then analyzed at 665 nm using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan) with an 80% acetone blank. The concentrations of Chl-a were calculated according to Wellburn (MacGregor et al., 2001).

1.3. Bacterial abundance

Samples for determination of bacterial abundance were preserved with 4% (V/V) formaldehyde. The fixed sample of 0.5–2 mL was stained with $1 \mu\text{g}/\text{mL}$ DAPI for 10–15 min (Porter and Feig, 1980). Afterwards, the sample was gently filtered onto a $0.22 \mu\text{m}$ pore size black polycarbonate filter (Whatman, Maidstone, UK). Total bacterial cell numbers were counted using epifluorescence microscope (Zeiss Axioskop 20, Oberkochen, Germany). A minimum of 10 replicates were counted for each sample.

1.4. Determination of off-flavors

The dissolved off-flavors in water were analyzed by GC-MS according to previous studies (Chen et al., 2010). The instrumentation used for analysis the off-flavor compounds

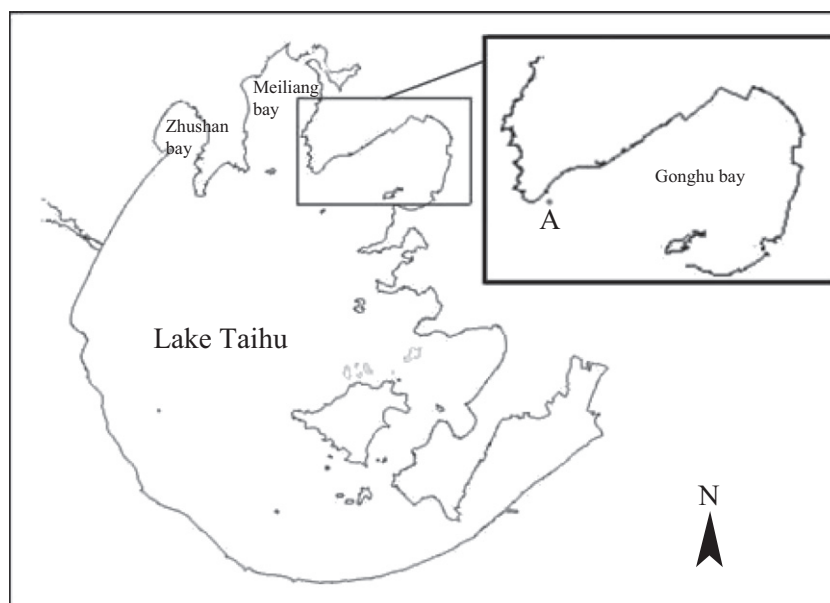


Fig. 1 – Location of the sampling site (A) in Gonghu Bay of Lake Taihu.

consisted of a GC (2010 GC, Shimadzu, Kyoto, Japan), MS (QP2010 plus, Shimadzu, Kyoto, Japan), purge and trap concentrator (O.I. Analytical 4660, State of Texas, USA), and an auto sampler (O.I. Analytical 4551, State of Texas, USA). A capillary column (30 m × 0.25 mm × 0.25 mm, Rxi@-17) was used to separate the off-flavors. The GC injector temperature was set at 270°C with a split ratio of 10:1. The oven temperature program was as follows: initially held at 40°C for 2 min, 100°C (30°C/min), 135°C (5°C/min), 250°C (30°C/min), and remained at 250°C for 5 min. The carrier gas flow rate was 3 mL/min. The selective ion monitoring mode was used at m/z 62 and 47 for DMS, m/z 79 and 126 for DMTS, m/z 95, 108 and 135 for MIB, m/z 137, 152 and 124 for β -cyclocitral, m/z 112, 125 and 149 for GEO.

1.5. Statistical analysis

Concentrations of off-flavors were compared using analysis of variance (ANOVA) followed by Tukey post-hoc tests to identify significant differences among various treatments. Univariate analysis was conducted using SPSS software (version 19.0). The statistical significance level (alpha level) was set at 0.05.

2. Results and discussion

2.1. Growth curves of *M. aeruginosa* and *P. pseudoalcaligenes*

Growth curves of *M. aeruginosa* and *P. pseudoalcaligenes* were depicted in Fig. 2. The growth characteristics of *M. aeruginosa* showed pronounced differences among 24°C and the other two temperatures. The lag phase of axenic *M. aeruginosa* (control) (day 0 to day 12) at 24°C (Fig. 2a) lasted longer than that at 28°C (day 0 to day 9) (Fig. 2b) and 32°C (day 0 to day 6) (Fig. 2c). The co-cultured *M. aeruginosa* (treatment) did not show obvious growth at 24°C. The cell density of the co-cultured *M. aeruginosa*

(treatment) at 28 and 32°C increased rapidly after the lag phase. Cell count results demonstrated that *P. pseudoalcaligenes* with algicidal activities could inhibit the growth of *Microcystis* obviously at three temperatures, even decomposed *Microcystis* completely at 24°C in our experiment.

The abundance of *P. pseudoalcaligenes* decreased at three temperatures simultaneously (Fig. 2), and there was also an obvious decline point on day 15, which coincided with the dramatic increase of *M. aeruginosa* in co-culture system. Pearson correlation analysis showed that the growth rates of *M. aeruginosa* were negatively correlated to *P. pseudoalcaligenes* (Pearson correlation = -0.221, $p = 0.01$), i.e., higher concentration of *M. aeruginosa* inhibited *P. pseudoalcaligenes*, and in turn, high concentration of *P. pseudoalcaligenes* resulted in a decrease in the *M. aeruginosa* growth rate. Influence of *M. aeruginosa* on bacterial abundance was very limited during the lag phase, but was strongly increased during the exponential phase. This result differs from previous studies (Shen et al., 2011), showing that cell densities of heterotrophic bacteria co-cultured with *M. aeruginosa* continuously grow throughout the whole experiment period. It was doubted that different species of heterotrophic bacteria would account for the diverse results, although further studies were required to make more clear explanation in the future.

2.2. Results of off-flavors

DMS, DMTS and β -cyclocitral were the main off-flavors in our experiment (Fig. 3). DMS, DMDS and DMTS belonged to VOSCs (volatile organic sulfur compounds), which can originate from the decomposition of algal blooms. It seems that the heterotrophic bacteria played an important role in decomposition of the cyanobacteria, whose organic sulfur reserves were then transformed into VOSCs (Zhang et al., 2010b). Yang et al. (2008) considered *Pseudomonas* species as the main

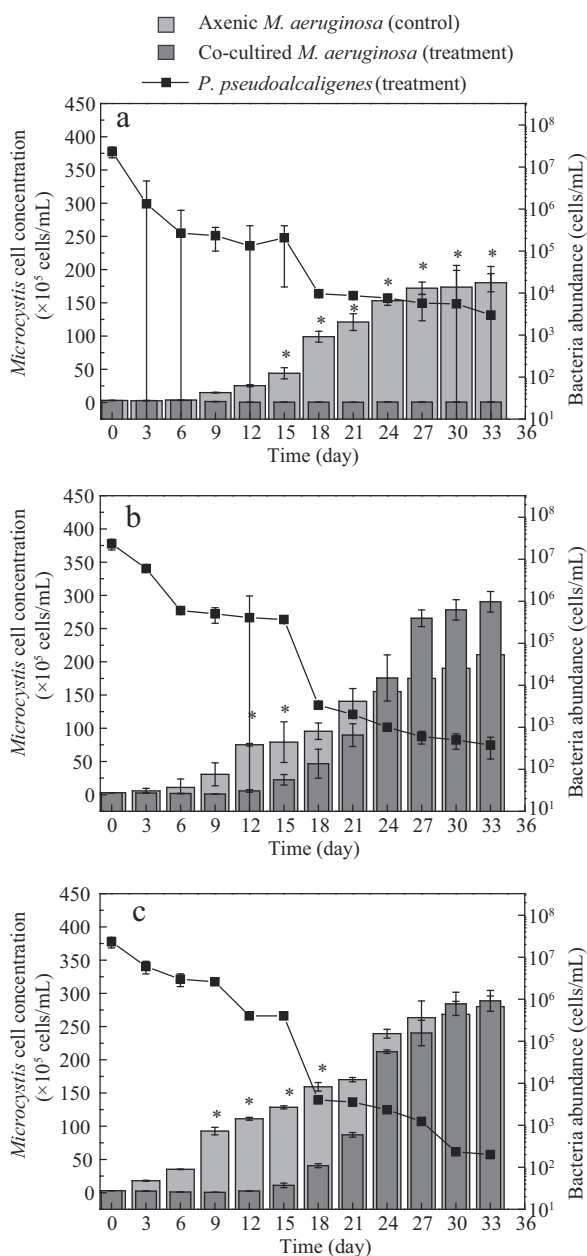


Fig. 2 – Influence of *P. pseudoalcaligenes* on the cell concentration of *M. aeruginosa* at different temperatures axenic *M. aeruginosa* (control) co-cultured *M. aeruginosa* (treatment). (a) 24°C, (b) 28°C, (c) 32°C. Columns represent means and error bars represent SD. * $p < 0.05$ indicates significant differences between axenic and non-axenic *M. aeruginosa*.

cause of off-flavors in the crisis of Taihu Lake in 2007. In our study, both independent *P. pseudoalcaligenes* and *M. aeruginosa* could produce DMS and DMTS, and the concentrations of DMS and DMTS in co-culture system (treatment) were obviously higher than those in independent *P. pseudoalcaligenes* and *M. aeruginosa* (control) at all three temperatures ($p < 0.05$) (Fig. 3). With univariate analysis, both *M. aeruginosa* and

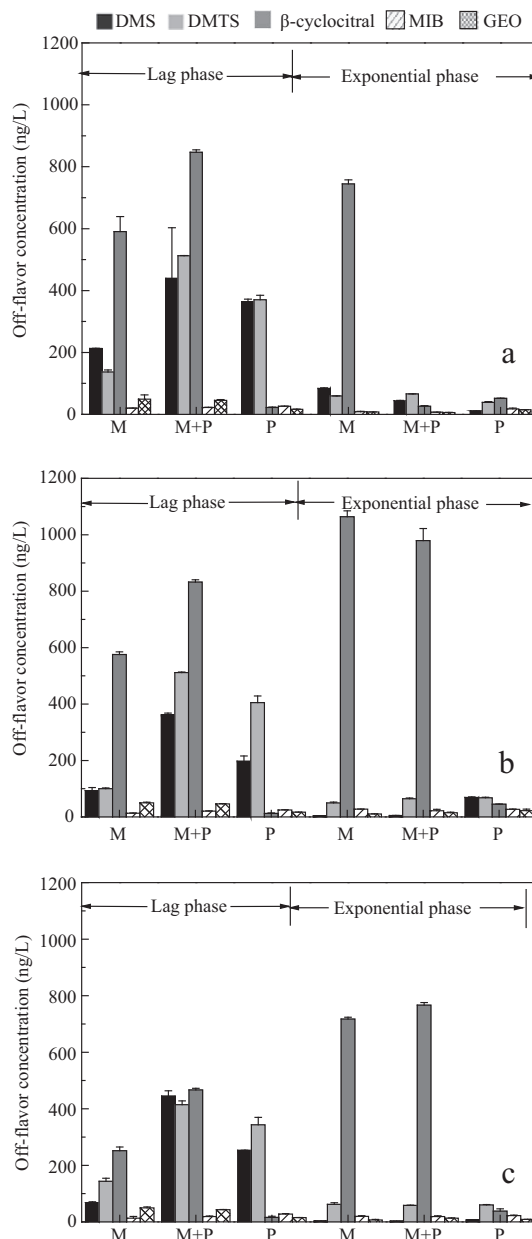


Fig. 3 – Interaction effects of *M. aeruginosa* and *P. pseudoalcaligenes* on off-flavors concentrations in different groups at 24 (a), 28 (b) and 32°C (c). M: *M. aeruginosa*, P: *P. pseudoalcaligenes*. Results are expressed as mean \pm standard deviation (SD).

P. pseudoalcaligenes affected DMS and DMTS ($p < 0.01$) (Table 1). Our results indicated that off-flavors of *P. pseudoalcaligenes* and the decomposition of *M. aeruginosa* aggravated the production of DMS and DMTS, which can generally in agreement with the variation of off-flavors results and the previous studies (Zhang et al., 2010b). Considering three different temperatures, the concentrations of DMS and DMTS of co-culture system (treatment) during lag phase among 24, 28 and 32°C have no significant differences. During the exponential phase, *M. aeruginosa* grew rapidly, resulting in a severe decrease of *P. pseudoalcaligenes*, and

Table 1 – Significant differences of off-flavor concentrations between *P. pseudoalcaligenes* and *M. aeruginosa*. DMS: dimethyl sulfide, DMTS: dimethyl trisulfide, MIB: 2-methylisoborneol, GEO: geosmin.

Temp.	Test groups	Off-flavors				
		DMS	DMTS	MIB	GEO	β -cyclocitral
24°C	<i>P. pseudoalcaligenes</i> effect	F = 123.351 p = 0.000**	F = 8.365 p = 0.010**	F = 0.173 p = 0.684	F = 0.026 p = 0.875	F = 2.024 p = 0.048*
	<i>M. aeruginosa</i> effect	F = 29.580 p = 0.000**	F = 4.238 p = 0.001**	F = 0.756 p = 0.768	F = 0.613 p = -0.906	F = 2.746 p = 0.013*
	Interaction effect	F = 110.924 p = 0.000**	F = 06.920 p = 0.001**	F = 0.867 p = 0.527	F = 0.017 p = 1.000	F = 1.052 p = 0.035*
28°C	<i>P. pseudoalcaligenes</i> effect	F = 2.694 p = 0.029*	F = 7.855 p = 0.011**	F = 0.399 p = 0.534	F = 0.006 p = 0.939	F = 0.191 p = 0.667
	<i>M. aeruginosa</i> effect	F = 4.429 p = 0.042*	F = 7.163 p = 0.000**	F = 1.207 p = 0.327	F = 0.768 p = 0.774	F = 1.469 p = 0.042*
	Interaction effect	F = 3.049 p = 0.016*	F = 15.531 p = 0.000**	F = 0.265 p = 0.927	F = 0.047 p = 0.999	F = 0.861 p = 0.523
32°C	<i>P. pseudoalcaligenes</i> effect	F = 5.026 p = 0.015*	F = 4.483 p = 0.043*	F = 0.308 p = 0.589	F = 0.005 p = 0.942	F = 0.841 p = 0.385
	<i>M. aeruginosa</i> effect	F = 4.090 p = 0.013*	F = 3.370 p = 0.028*	F = 0.868 p = 0.659	F = 0.381 p = 0.992	F = 5.158 p = 0.003**
	Interaction effect	F = 7.016 p = 0.009**	F = 8.601 p = 0.005**	F = 0.353 p = 0.837	F = 0.013 p = 1.000	F = 0.637 p = 0.646

* Correlation is significant at the 0.05 level (2-tailed).

** Correlation is significant at the 0.01 level (2-tailed).

the concentrations of DMS and DMTS decreased (Fig. 3). These phenomena might account for that the death and decay of cyanobacteria blooms is often associated with high concentrations of VOSCs.

β -Cyclocitral is considered to be the main off-flavor from cyanobacteria (Harada et al., 2009; Li et al., 2007; Yang et al., 2008), which is released during the growth of *Microcystis* and the disruption of cell integrity (Zhang et al., 2010b). It looks likely that the production of β -cyclocitral is related to metabolism of *M. aeruginosa* and bacterium (*P. pseudoalcaligenes* in our study) is not the origin of β -cyclocitral. During the lag phase, β -cyclocitral concentration in co-culture system (treatment) was higher than that in axenic *M. aeruginosa* (control) at three temperatures ($p < 0.05$) (Fig. 3). We considered *P. pseudoalcaligenes* decomposed *M. aeruginosa* to increase the concentration of β -cyclocitral. β -Cyclocitral concentration was reduced obviously in co-culture system (treatment) during the exponential phase of *M. aeruginosa* at 24°C ($F = 347.95$, $p = 0.000$) (Fig. 3a). It was related with the growth of *M. aeruginosa* which was restrained by *P. pseudoalcaligenes*. However, *Microcystis* cells were not inhibited and decomposed obviously by low concentrations of *P. pseudoalcaligenes* at 28 and 32°C according to the growth curves of *M. aeruginosa* and *P. pseudoalcaligenes* (Figs. 2b and c), and β -cyclocitral concentrations in co-culture system had no differences with axenic *M. aeruginosa* groups (Figs. 3b and c). When comparing the temperatures, β -cyclocitral at 24°C were lower than that at 28°C ($F = 132.79$, $p = 0.000$) and 32°C ($F = 72.51$, $p = 0.000$) in axenic *M. aeruginosa* (control). This could also be explained by the metabolism of *M. aeruginosa* at optimal temperatures (28°C) would be better than that at lower temperature (24°C). Meanwhile, β -cyclocitral would volatilize faster at higher temperature (32°C), which was reported as a short-lived compound in aqueous environments due to rapid volatilization (Cole, 1982). The interaction analysis indicated that β -cyclocitral was mainly affected by *M. aeruginosa* at 28 and

32°C, and also influenced by *P. pseudoalcaligenes* at 24°C ($p < 0.05$) (Table 1), which generated the same conclusion with above results.

MIB and GEO are two earthy-muddy-musty smelling terpenoids, which account for the global majority of drinking water odor outbreaks, and are widespread in many eutrophic lakes (Zhang et al., 2010a). They are synthesized by a selected number of planktonic bloom-forming and benthic-littoral cyanobacteria as well as by some actinomycetes, myxobacteria, and fungi and soil microbiota (Watson et al., 2008). In treated drinking water supplies, *Microcystis* rarely produce either of the more resilient taste and odor compounds GEO and MIB (Watson et al., 2008). During our experiment, the average concentration of MIB and GEO was much lower than other off-flavors ($p < 0.05$) (Fig. 3), indicating that MIB and GEO were not the main off-flavors of *M. aeruginosa*. Nevertheless, MIB production showed significant differences among different temperatures ($p < 0.01$). Chen et al. reported seasonal variations of MIB concentrations of Taihu Lake, but the variation in seasonality of MIB was related to the complexity in the production and there was no temperature records (Chen et al., 2010). The interaction analysis showed that MIB and GEO were not influenced by both *P. pseudoalcaligenes* and *M. aeruginosa* and their interaction effects ($p < 0.05$) (Table 1). It seemed that MIB was produced by other different organisms with different physiological status.

3. Conclusions

To date, few studies have addressed the interaction effects of *M. aeruginosa* and *P. pseudoalcaligenes* on the production of off-flavors in heterotrophic bacteria/bloom-forming *M. aeruginosa* co-culture system. Our study indicates that *P. pseudoalcaligenes* significantly increased DMS, DMTS and β -cyclocitral during

the lag phase of *M. aeruginosa*. β -Cyclocitral was mainly affected by *M. aeruginosa* during exponential phase of *M. aeruginosa*. MIB and GEO were not the main off-flavors of *M. aeruginosa*. *M. aeruginosa*, *P. pseudoaeruginosa* and interaction effects on the production of off-flavors in cyanobacteria/bacteria co-culture system is rather complex, and needs more systematic studies in the future.

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