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Volatile organic compounds released from *Microcystis flos-aquae* under nitrogen sources and their toxic effects on *Chlorella vulgaris*

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ABSTRACT

Eutrophication promotes massive growth of cyanobacteria and algal blooms, which can poison other algae and reduce biodiversity. To investigate the differences in multiple nitrogen (N) sources in eutrophicated water on the emission of volatile organic compounds (VOCs) from cyanobacteria, and their toxic effects on other algal growth, we analyzed VOCs emitted from *Microcystis flos-aquae* with different types and concentrations of nitrogen, and determined the effects under Normal-N and Non-N conditions on *Chlorella vulgaris*. *M. flos-aquae* released 27, 22, 20, 27, 19, 25 and 17 compounds, respectively, with $NaNO_3$, $NaNO_2$, NH_4Cl, urea, Ser, Lys and Arg as the sole N source. With the reduction in N amount, the emission of VOCs was increased markedly, and the most VOCs were found under Non-N condition. *C. vulgaris* cell propagation, photosynthetic pigment and Fv/Fm declined significantly following exposure to *M. flos-aquae* VOCs under Non-N condition, but not under Normal-N condition. When *C. vulgaris* cells were treated with two terpenoids, eucalyptol and limonene, the inhibitory effects were enhanced with increasing concentrations. Therefore, multiple N sources in eutrophicated water induce different VOC emissions from cyanobacteria, and reduction in N can cause nutrient competition, which can result in emissions of more VOCs. Those VOCs released from *M. flos-aquae* cells under Non-N for nutrient competition can inhibit other algal growth. Among those VOCs, eucalyptol and limonene are the major toxic agents.

1. Introduction

Eutrophication is one of the most widespread environmental problems of inland waters, and becomes more serious with the increasing inputs of nutrients, mainly nitrogen (N) and phosphorus (P), caused by human activities (Cloern, 2001). With the inputs of tremendous volume of urban, agricultural, breeding and industrial wastewater, multiform N nutrients are found in eutrophicated water bodies, mainly including inorganic and organic N (Aslan and Kapdan, 2006; Li et al., 2010; Boelee et al., 2011; Abdel-Raouf et al., 2012). Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) are the most common ionic forms of dissolved inorganic N, and urea is the most common organic form (Howarth, 1988; Rabalais, 2002; Glibert et al., 2004). Meanwhile, other organic N is also found, such as amino acids, polypeptides and proteins (Rabalais, 2002; Glibert et al., 2004).

Eutrophication promotes the excessive algal growth and even blooms, especially cyanobacteria (Azevedo et al., 2002). In recent decades, occurrences of cyanobacteria blooms have increased around the world, and *Microcystis* is the uppermost cyanobacterial genus responsible for water blooms (Azevedo et al., 2002; Hudnell and Dortch, 2008). It is well known that many cyanobacteria can produce toxins such as microcystin, hepatotoxins, neurotoxins, neosaxitoxins and anatoxin-a (Codd, 2000; Frangópulos et al., 2004). Most species of *Microcystis*, such as *M. flos-aquae*, *M. aeruginosa*, *M. ichthyoblabe*, and *M. viridis*, are reported to produce a family of nearly 80 microcystins (Briand et al., 2009). Those algal toxins can cause a series of water quality and ecological problems, such as poisoning other algae (Li and Li, 2012; Sanna et al., 2004), aquatic plants (Pflugmacher, 2002), zooplankton (Abrantes et al., 2006) and fishes (Guzmán-Guillén et al., 2013), and even hazarding human health (Hoeger et al., 2007).

Besides toxins, cyanobacteria also release a wide spectrum of volatile organic compounds (VOCs) which can pollute water bodies and destroy their quality via resulting in unpleasant odor. However, there are limited reports about cyanobacteria VOCs compared to

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toxins. Walsh et al. (1998) found that *M. aeruginosa* released abundant VOCs, including aliphatic hydrocarbons (C15-C21), naphthalene, terpenoids, β-cyclocitral and β-ionone. Huang et al. (2007) analyzed the VOCs from 3 cyanobacteria (*Anabaena*, *Microcystis* and *Oscillatoria*) and found two main compounds, geosmin and 2-methylisoborneol, that cause the unpleasant, earthy-musty odor in source waters (Fujise et al., 2010). Moreover, other VOCs generated by lipoxygenase and carotene oxygenase reactions and fermentation has also been found in lake and river waters (Jüttner, 1995).

It has been reported that *M. aeruginosa* cells produce microcystin with alanine (Ala), leucine (Leu) and arginine (Arg) as sole N source, but not with glutamic acid (Glu), aspartic acid (Asp) and lysine (Lys) (Dai et al., 2009). Under limiting nutrient condition, a remarkable increase in the production of algal toxins was found (Frangópulos et al., 2004; Wang et al., 2012), e.g., 2.9-fold increases in *Trichormus doliolum* with limiting P supply (von Elert and Jüttner, 1997). Meanwhile, the emission amount of alcohols (2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol) and β-cyclocitral was markedly raised from *M. aeruginosa* cells, after the cells were kept for 35 days with the exhaustion of nitrate N nutrient (Hasegawa et al., 2012). Those results indicate that different nutrients and limiting nutrient stresses may affect the secondary metabolism in algae.

In terrestrial ecosystems, the VOCs from higher plants are involved in a broad array of ecological functions, such as communication between plants (Weir et al., 2004; Baldwin et al., 2006), inhibiting seed germination and seedling growth of other plants (Zuo et al., 2011; Zhang et al., 2012), defense against herbivores and pathogens (Rapisarda et al., 2012; Bee Park et al., 2013; Zhang et al., 2014). In aquatic ecosystems, geosmin, β-cyclocitral, α-ionones, β-ionones and geranylacetone are the main compounds of the VOCs from cyanobacteria and algae in freshwater lakes, which have inhibitory effects on the growth of green alga *Chlorella pyrenoidosa* (Ikawa et al., 2001). When *Chlamydomonas reinhardtii* cells were stressed by acetic acid, NaCl and Na_2CO_3, the cells released abundance of VOCs, including alkanes, alkenes, terpenoids, alcohols, aldehydes, ketones and esters, and those VOCs can transfer stress information to other normal cells, leading to a reduction in cell growth and photosynthetic ability, as well as an increase in reactive oxygen species (ROS) level and antioxidant enzyme activity (Zuo et al., 2012a, 2012b). Green alga *Ulothrix fimbriata* releases abundant VOCs which can act as a clue for its predator (Fink et al., 2006). Those results suggest that algal VOCs can also function as information messengers and affect the growth of other algae and predators.

In eutrophicated water bodies, complicated and multiple N nutrients promote massive growth of cyanobacteria, which causes water pollution, poisons other algae and reduces biodiversity. Besides the toxic effects of algal toxins (Li and Li, 2012; Sanna et al., 2004), we hypothesized that cyanobacteria VOCs might also play important roles in the toxic event, and N nutrient conditions may affect emissions of the VOCs. To test the hypothesis, we investigated the VOC emissions from *M. flos-aquae*, a typical species of *Microcystis* for cyanobacteria blooms, under the conditions with different types and amount of N nutrients, and determined the effects of VOCs and their 2 main compounds on the growth of *Chlorella vulgaris*.

2. Material and methods

2.1. Cell cultures

M. flos-aquae FACHB-1028 was provided by Freshwater Algae Culture Collection at the Institute of Hydrobiology, China, grown in liquid BG11 medium (Rippka et al., 1979), and kept at a light (16 h)/dark (8 h) regime at 23 °C, with an illumination of 50 μmol m^{-2} s^{-1}. *C. vulgaris* was also kept under the same condition in the same medium. Cell cultures in mid-logarithmic phase were used for the experiments. The density of cell cultures was determined by using the blood cell counting plate, with each value being the means of 6 repeats.

2.2. Supplementation of N sources

M. flos-aquae cells were harvested by centrifugation at 5000 g for 5 min, washed twice with BG11−N (BG11 minus N) medium, and resuspended in the same medium to give a final density of 5×10^6 cells per ml. When treating with different N sources, $NaNO_3$, $NaNO_2$, NH_4Cl, urea, serine (Ser), Lys and Arg were individually supplied to the cells as N source, and the N element concentration was the same as in the normal medium ($NaNO_3$ as N source, 17.6 mM N). For treatment with different N concentration, the cells in BG11−N medium were added into 0, 8.8, 17.6 and 35.2 mM $NaNO_3$, for Non-N, Low-N, normal N (Normal-N) and High-N treatment, respectively. After 24 h, the VOCs were collected and analyzed.

2.3. Treatment of C. vulgaris cells with VOCs

The VOCs from *M. flos-aquae* under Normal-N and Non-N conditions were got through *C. vulgaris* culture, respectively, according to our previous method (Zuo et al., 2012a). The volumes of *M. flos-aquae* and *C. vulgaris* were the same (1 L) with the cell densities of approximately 1×10^7 and 4×10^6 cells per ml, respectively. *C. vulgaris* cells without exposure to *M. flos-aquae* VOCs were the control. Each treatment and control had 3 replicates. After 24 h, the cell density, photosynthetic pigment content and photosynthetic capacity were determined.

2.4. Treatment of C. vulgaris cells with eucalyptol and limonene

Eucalyptol and limonene were the 2 main terpenoids in the VOCs from *M. flos-aquae*, and they were used to treat *C. vulgaris* cells. 500 mM solutions of eucalyptol and limonene were prepared using ethanol, and added separately into *C. vulgaris* culture (6×10^6 cell per ml) to obtain final concentrations of 0.4, 0.8, 1.6 and 4 mM. β-cyclocitral is also a main terpenoid in cyanobacteria VOCs, which can cause *Nitzschia palea* rupture at 5–10% (Chang et al., 2011) and inhibit K^+ channels in snail neurons at 3–5 mM (Zeraatpisheh and Vatanparast, 2015). The concentration of the 2 compounds in this study were set based on above studies, and these concentrations are much lower than the concentration (2–5 mg ml^{-1}, about 66–165 mM) of β-cyclocitral and other compounds from cyanobacteria VOCs that inhibit *C. pyrenoidosa* cell growth (Ikawa et al., 2001). *C. vulgaris* culture added with same amount of ethanol without the above compounds was the control. There were 3 replicates for each treatment and control. After 24 h, the cell density, photosynthetic pigment levels and photosynthetic capacity were measured.

2.5. Collection and analysis of VOCs

According to our previous method, *M. flos-aquae* VOCs were collected using the dynamic headspace air-circulation method (Zuo et al., 2012a). In each treatment, three conical flasks of cell cultures (each conical flask contained 250 ml of cell suspensions, approximately 1×10^7 cells per ml) were analyzed, and each of them was regarded as a repeat. The chemical composition of VOCs was analyzed with the thermal-desorption system/gas chromatography/mass spectrum (TDS/GC/MS), and the qualitative and quantitative analyses of the GC/MS data were obtained from NIST/EPA/NIH Mass Spectral Library (NIST 08) (National Institute of Standards and Technology, Gaithersburg, USA). The peak area per 10^7 cells was used to indicate the content of the compounds (Zuo et al., 2012a).

2.6. Determination of the content of photosynthetic pigments

C. vulgaris cells in 3 ml culture medium collected by centrifugation were resuspended in 3 ml of 80% acetone, and the pigment contents were determined as described by Lichtenthaler and Welburn (1983)