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Allelopathic Effects of *Myriophyllum aquaticum* on Two Cyanobacteria of *Anabaena flos-aquae* and *Microcystis aeruginosa*

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Abstract Allelopathy has been proposed as a sustainable means to control undesired algal growth and to reduce blooms threatening freshwater systems worldwide. In this study, the allelopathic effects of *Myriophyllum aquaticum* and its exudate on two typical bloom-forming cyanobacteria, *Microcystis aeruginosa* and *Anabaena flos-aquae*, were investigated under laboratory conditions. The growth of the cyanobacteria was strongly inhibited by live *M. aquaticum* while the primary addition of *M. Aquaticum* exudates had a significant inhibitory effect on the growth of *M. aeruginosa* but not *A. flos-aquae*. The results suggested that the persistent release of allelochemicals from live *M. aquaticum* was needed to effectively constrain the growth of *A. flos-aquae*. Analysis of cyanobacterial physiological indexes indicated that *M. aquaticum* produced an inhibitory effect on SOD enzyme activity of *A. flos-aquae*, while it affected membrane lipid peroxidation in *M. aeruginosa*. The results show the potential of *M. aquaticum* to mitigate cyanobacterial blooms in coexistence systems.

Keywords Allelopathy · *Myriophyllum aquaticum* · Cyanobacteria · Allelochemicals · Membrane lipid peroxidation

Algal blooms in freshwater systems have become one of the foremost concerns in freshwater conservation and management worldwide (Chiang et al. 2004). For example, in summer, China's two well-known lakes (Lake Taihu and Lake Dianchi) suffered from severe algal blooms, which were mainly composed of *Microcystis aeruginosa, A. flos-aquae*, and *Aphanizomenon flos-aquae* (Li et al. 2007; Ye et al. 2009). Algal blooms can have negative impacts on freshwater systems as they release toxins that are lethal to animals and humans. Moreover, under nutrient-rich conditions, epiphytic algae and other phytoplankton grow rapidly and can cover the surface of plants and water body. Limited light together with oxygen depletion creates unfavourable conditions for various biotic components of the system (Chislock and Doster 2013; Gross 2003; Kirk 2011). In due course, this reduces biological diversity and the ecological quality of the water body (Chislock and Doster 2013). The alternative state, dominated by macrophytes, is a situation where clear water and dense stands of submerged vegetation prevail (Chislock and Doster 2013).

Allelopathy is defined as any direct or indirect effect of organism on another through the release of chemical substances (known as allelochemicals) into the environment (Chiang et al. 2004; Nakai et al. 1999). Multiple macrophytes have been reported to have strong allelopathic activity against various cyanobacteria (Jin et al. 2003; Takamura et al. 2003). Allelopathic activity in fresh water, particularly of macrophytes towards phytoplankton, has been proposed as a measure to prevent or reduce undesired algal blooms (Dhote and Dixit 2009; Liu et al. 2007; Nakai et al.

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1999). This approach could minimise the use of expensive chemical herbicides and support an alternative, sustainable method of aquatic weed management (Willis 2007). However, demonstrating the existence of allelopathy in a natural setting is challenging (Gross 2003; Hilt and Gross 2008). For example, it is difficult to distinguish allelopathy from other direct and indirect impacts of macrophytes on algal growth (e.g. shading, competition, existence of zooplankton in macrophyte beds.) (Mulderij et al. 2005). In addition, a suitable macrophyte species must be considered in any study of allelopathy in aquatic systems. We and other groups have demonstrated the potential of *Myriophyllum aquaticum* for application in phytoremediation as it effectively removed nutrients and organic matter from eutrophic waters (Li and Recknagel 2002; Liu et al. 2016).

The aim of our study was to examine the potential allelopathic activity of *M. aquaticum* on the growth of two typical bloom-forming cyanobacterial species, *M. aeruginosa* and *A. flos-aquae*, by conducting laboratory experiments. The growth of cyanobacteria in two culture systems was compared to detect allelopathic effects. Superoxide dismutase (SOD), soluble carbohydrates (SC), chlorophyll a (Chl-a), and other parameters were determined to explore the effects and mechanisms of *M. aquaticum* on algal growth and physiology.

Materials and Methods

M. aeruginosa and *A. flos-aquae* were obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB-Collection). Cells were axenically cultivated in culture medium (FACHB-Collection 2015) at 25 ± 2°C in a tissue culture room with a 12-h photoperiod, and shaken three times every day. Cells in the exponential growth phase were used in all tests.

Fresh *M. aquaticum* used in this study was hand-collected from a novel constructed drainage ditch (Liu et al. 2013). The plants were gently washed free from debris and aquatic invertebrates present using distilled water, and were then grown separately in 500 mL of culture medium in sterilised 1-L Erlenmeyer flasks at 25 ± 2°C with a 12-h photoperiod. The plants were allowed to adapt to the laboratory environment for 7 days before use.

A mixed culture system was employed in the coexistence culture (co-culture) experiment. *M. aquaticum* was washed with sterile water three times and added to sterilised 1-L Erlenmeyer flasks containing 500 mL of culture medium inoculated with *M. aeruginosa* or *A. flos-aquae* at Chl-a contents of 0.04 and 0.28 mg L^{-1}, respectively. The *M. aquaticum* biomass density was 20 g fresh weight (gfw) L^{-1}. Cyanobacteria grown in fresh culture medium were used as the control. Treatments and controls were prepared in triplicate. During the incubation period of 9 days, 25-mL samples were taken for assay on days 0, 1, 3, 5, 7, and 9, and 50 mL of a 2× stock solution of culture medium was added to each flask on day 3 to prevent nutrient depletion in culture medium.

Culture medium of *M. aquaticum* was added to cyanobacterial cultures in the exudate culture (e-culture) experiment. *M. aquaticum* at an initial concentration of 20 gfw L^{-1} was cultivated for 7 days, after which the plants were removed from the cultures. Two hundred-fifty millilitres of plant-free culture medium was enriched with 250 mL of fresh culture medium. *A. flos-aquae* and *M. aeruginosa* were inoculated into this medium at Chl-a contents of 0.11 and 0.48 mg L^{-1}, respectively. The incubation, sample, and nutrient replenishment methods were the same as those in above co-culture experiment.

The algal cells were collected by centrifugation at 4,000 rpm for 10 min and transferred into a 10-mL tube with 5 mL of 95% ethanol for dark extraction for 2 h. Then, the mixture was centrifuged; the supernatant was obtained and made up to 10 mL with 95% ethanol. Using 95% ethanol as the control, the optical density (OD) was determined at 665 and 649 nm and the Chl-a content ($C_{Chl\text{-}a}$, mg L^{-1}) was calculated by using Eq. (1).

$$C_{Chl\text{-}a} = \frac{(13.95 \times A_{665} - 6.88 \times A_{649}) \times V_1}{V_2} \quad (1)$$

where, V_1 (mL) is the final volume of the extract and V_2 (mL) is the volume of the algal liquid.

The specific growth rate μ (day^{-1}) was determined on the basis of the Chl-a content using Eq. (2).

$$\mu = \frac{\ln(\frac{x_t}{x_0})}{t} \quad (2)$$

where x_t (mg L^{-1}) denotes the Chl-a content on day t and x_0 (mg L^{-1}) denotes the Chl-a content on day 0.

Photochemical reduction of nitro blue tetrazolium (NBT) was determined to measure SOD activity (Hao et al. 2004). The SC content was determined using the anthrone-sulphuric acid colorimetric method (Li 2007).

The experimental data were analyzed using Excel. The Chl-a, SOD, and SC contents were compared by Tukey's multiple comparison post hoc test of one way analysis of variance (ANOVA) at 5% level of significance with SPSS v.13.0.

Results and Discussion

After three days of co-culture, the growth of *A. flos-aquae* was significantly inhibited as compared to the control for

days 5, 7 and 9. In the e-culture, no significant differences were found in the growth of *A. flos-aquae* compared to the control (Fig. 1a). In contrast, the growth of *M. aeruginosa* was significantly inhibited from day 3 onwards in both culture systems when compared with the control (Fig. 1b). All of the treatments were assessed under controlled laboratory conditions, without shading, and nutrients were added on day 3 to avoid nutrient depletion in the culture. Thus, the growth inhibition of the cyanobacteria was mostly caused by allelochemicals released by *M. aquaticum*. The results indicated that the *M. aquaticum* exhibited different inhibitory effects on *A. flos-aquae* under different culture conditions. The limited effect of the exudate on *A. flos-aquae* under e-culture condition may be attributed to the degradation or metabolism of the allelochemicals by the cyanobacteria over time when the live materials were no longer present to secrete additional allelochemicals. This suggests that these chemicals must be added continuously for prolonged algicidal activity. This result was consistent with previous findings (Nakai et al. 1999). Allelopathic inhibitions were found for *M. aeruginosa* under different culture conditions in this study, suggesting that *M. aeruginosa* might have lower tolerance to allelochemicals released by *M. aquaticum* or degrade or metabolize the allelochemicals at a slower rate compared with *A. flos-aquae*.

Details of the specific growth rates of *A. flos-aquae* and *M. aeruginosa* in the presence of *M. aquaticum* are shown in Table 1. When studying allelopathic interactions between macrophytes and microalgae, the effects of allelochemicals in each phase of the microalgal life cycle (the lag, exponential, stationary, declining, and death phases) need to be considered, especially for the growth phases (Mulderij et al. 2005). The initial decline in cyanobacterial growth rates may have been due to their adjustment to the new growth conditions. After the initial decline, the growth rate of the cyanobacteria in the control conditions increased strongly. The presence of allelochemicals slightly suppressed exponential growth of *A. flos-aquae* in e-culture and markedly decreased the exponential growth rate in all other treatments. The growth of *A. flos-aquae* under control condition showed a discernible decline after day 7 as expected in a normal life cycle, while in e-culture treatment, the growth rate started to decrease at day 3, suggesting that the presence of *M. aquaticum* mostly affects *A. flos-aquae* in the exponential growth phase. Allelochemicals will reduce algal density; however, some growth will still occur (Mulderij et al. 2005). In contrast to *A. flos-aquae*, the growth rate of *M. aeruginosa* steadily increased after day 3 in the control group. This may have been due to the lower initial algal density and resultant longer lag phase or growth differences between both species. The presence of exudate

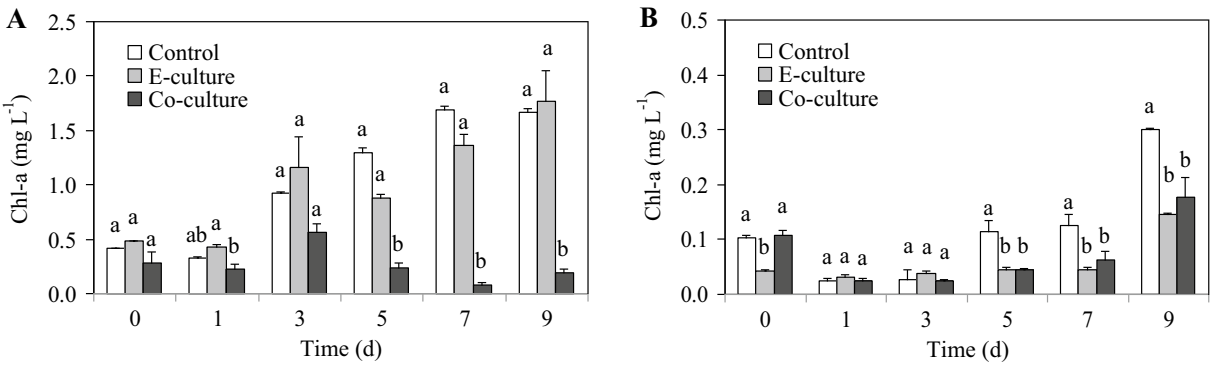

Fig. 1 Effects of *M. aquaticum* on *A. flos-aquae* (**a**) and *M. aeruginosa* (**b**) Chl-a contents. *Error bars* represent the standard deviation (n = 3), and different letters on *error bars* indicate significant differences at $p < 0.05$ (*error bars* and letters in Figs. 2, 3 share the same meaning)

Table 1 Effects of *M. aquaticum* on the specific growth rates of *A. flos-aquae* and *M. aeruginosa*

Time (days)	Specific growth rate of *A. flos-aquae* (day^{-1})			Specific growth rate of *M. Aeruginosa* (day^{-1})		
	Control	Co-culture	E-culture	Control	Co-culture	E-culture
1	−0.23 ± 0.04	−0.63 ± 0.35	0.04 ± 0.03	−1.35 ± 0.18	−1.26 ± 0.26	−1.46 ± 0.12
3	0.27 ± 0.07	0.10 ± 0.06	0.34 ± 0.12	−0.68 ± 0.26	−0.35 ± 0.07	−0.49 ± 0.04
5	0.23 ± 0.06	−0.11 ± 0.06	0.15 ± 0.02	0.04 ± 0.03	−0.18 ± 0.03	−0.17 ± 0.02
7	0.20 ± 0.04	−0.24 ± 0.05	0.17 ± 0.08	0.05 ± 0.04	−0.13 ± 0.08	−0.07 ± 0.05
9	0.16 ± 0.09	−0.09 ± 0.03	0.16 ± 0.02	0.12 ± 0.04	0.04 ± 0.02	0.06 ± 0.03

resulted in a longer lag phase in both co-culture and e-culture as compared to the control group and the growth rate increased only after day 7. The effect of allelochemicals in lengthening the lag phase of microalgal growth results in almost no growth, allowing macrophytes to become competitively dominant (Pakdel et al. 2013).

In both e-culture and co-culture, the SOD activity of *A. flos-aquae* increased before day 3 and did not differ from the control. However, the SOD activity of *A. flos-aquae* was significantly decreased when compared with the control after day 3 (Fig. 2a). Benzenetriol can increase intracellular SOD activity in *M. aeruginosa* (Shao et al. 2009). As a type of metallic enzyme, SOD is extensively present in organisms and converts superoxide anions to H_2O_2 and O_2 by catalytic disproportionate reaction (Yu et al. 2013). SOD is the only enzyme that uses free radicals as a substrate and plays a important role in the maintenance of a dynamic balance in metabolism within the organism (Yu et al. 2013). The phenomenon of inhibition of phenolic acids on algae occurs as a result of increase or decrease in the activity of SOD (Hua 2008). SOD enzyme activity could better reflect the effects of environmental stress on algal growth. The fact that the SOD enzyme activity in *A. flos-aquae* decreased dramatically after 3 days in comparison with the control indicated that *M. aquaticum* had a lethal effect on *A. flos-aquae*. In contrast, in *M. aeruginosa*, SOD activity showed a similar pattern in both culture systems as well as the control, indicating that *M. aquaticum* did not inhibit the SOD enzyme in *M. aeruginosa* (Fig. 2b).

Figure 3 shows the changes in SC content in *A. flos-aquae* and *M. aeruginosa* in the presence of *M. aquaticum*. The SC content of *A. flos-aquae* under control condition increased slowly with the increase in the algal growth rate. *M. aquaticum* had no effect on the SC content of *A. flos-aquae* in each of the treatment groups; no significant difference in comparison to the control group was noted. In contrast, the SC content of *M. aeruginosa* showed no obvious changes over time in the treatment groups while in the control, it increased obviously after day 3 and showed a significant difference as compared to the control for days 5 and 9, indicating that *M. aquaticum* affected the normal metabolic processes of *M. Aeruginosa* to some extent. SC is one of the main mechanisms for osmotic adjustment in algae and has a protective effect on lipid membranes(Yu et al. 2013). *M. aeruginosa* could alleviate membrane lipid peroxidation by osmotic adjustment in the initial stage (Yu et al. 2013).

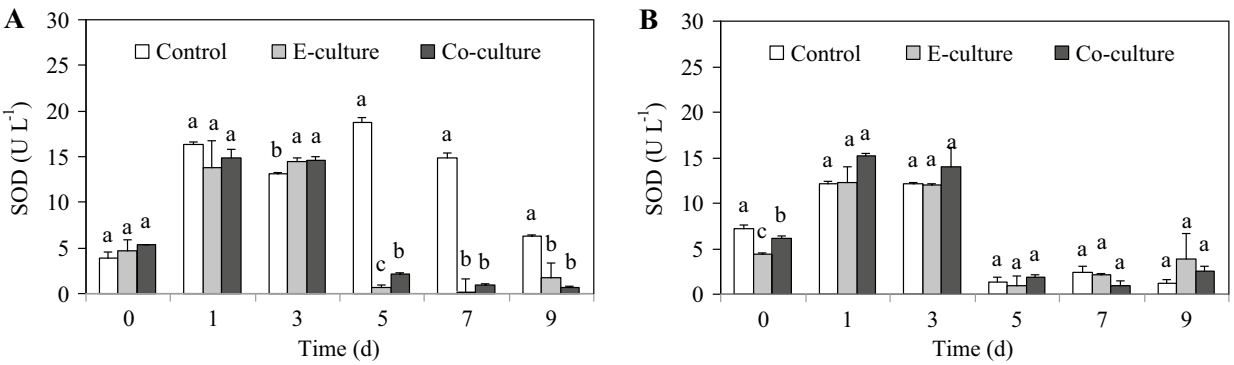

Fig. 2 Effects of *M. aquaticum* on SOD contents of *A. flos-aquae* (**a**) and *M. aeruginosa* (**b**)

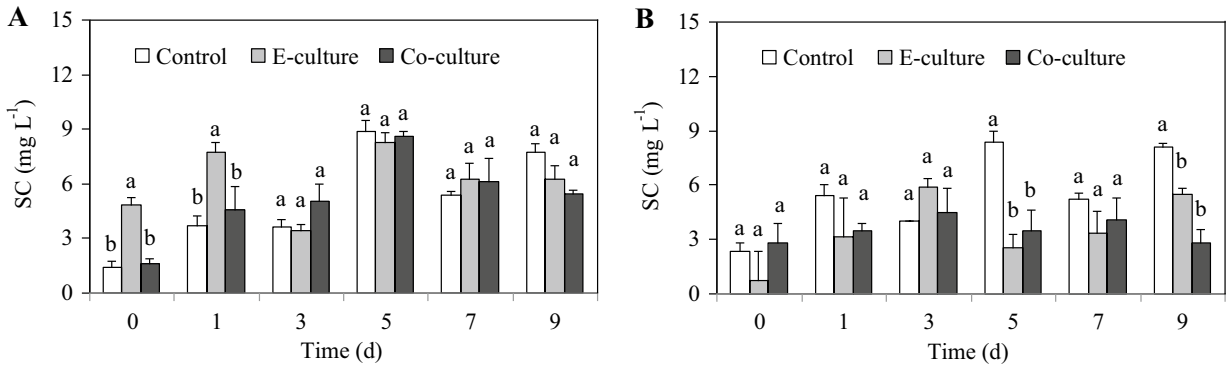

Fig. 3 Effects of *M. aquaticum* on SC contents of *A. flos-aquae* (**a**) and *M. aeruginosa* (**b**)

SC could reflect algal adjustment capacity to environmental stress. Thereafter, cell growth and metabolism were inhibited, and the SC content was lower than that in the control after 3 days; therefore, after long-term exposure to stress, algae did not have the capacity to sustain the stress, eventually resulting in death of the algal cells.

The effect of allelopathy as a phytoplankton control mechanism is dependent on the macrophyte and phytoplankton species involved (Mulderij et al. 2002; Pakdel et al. 2013). Phytoplankton species exhibit variable sensitivities and responses to allelopathy in different phases (Pakdel et al. 2013). This may further explain the different allelopathic effects and mechanisms of *M. aquaticum* on two Phytoplankton species in our study, particularly in the growth phases, and suggests that other potential allelopathic mechanisms may exist in the culture systems.

In conclusion, the growth of *M. aeruginosa* was inhibited by allelochemicals released by *M. aquaticum* in both co-culture and e-culture. In contrast, the growth of *A. flos-aquae* was strongly inhibited only in co-culture. These findings suggest that continuous addition of allelochemicals would be essential for prolonged algicidal activity. Determination of SOD and SC indicated that *M. aquaticum* has an inhibitory effect on SOD enzyme activity of *A. flos-aquae*, while it affected membrane lipid peroxidation in *M. aeruginosa*. Our findings indicate the potential of *M. aquaticum* to mitigate cyanobacterial blooms in coexistence systems. Further works are needed to study other enzyme activity of *A. flos-aquae* (e.g. catalase) and other potential allelopathic mechanism of *M. aquaticum*. Further investigation of the allelopathic effects of *M. aquaticum* in natural conditions is also needed because the dominant microalgal species and the environmental conditions in the sampled lake and water bodies at large might differ from those in our laboratory tests. Furthermore, analysis of the chemical composition of the allelochemicals would benefit assessing the potential of *M. aquaticum* as a biological agent for the control of nuisance algal blooms.

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