

Comparing effects of berberine on the growth and photosynthetic activities of *Microcystis aeruginosa* and *Chlorella pyrenoidosa*

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ABSTRACT

Berberine is a potent algicidal allelochemical of *Microcystis aeruginosa*. To optimize its application in the control of *Microcystis* blooms, the effects of berberine on the growth and photosynthetic activities of *M. aeruginosa* and a non-target green alga, *Chlorella pyrenoidosa*, were compared. The results showed that the algicidal activity of berberine on *M. aeruginosa* was light dependent. Berberine had no algicidal effects on *C. pyrenoidosa* with or without light exposure. Under light-dark conditions, berberine significantly decreased the chlorophyll fluorescence parameters in *M. aeruginosa* while no significant berberine-induced changes were observed under constant darkness. Significant reductions of photosystem II (PSII) and whole chain electron transport activities in *M. aeruginosa* exposed to berberine suggested that PSII was the important target site attacked by berberine. Contrary to *M. aeruginosa*, no berberine-induced inhibition in photosynthesis activities were observed in *C. pyrenoidosa*. The differences in photosynthetic apparatuses of these two algae might be responsible for their different sensitivities to berberine.

Key words | algicidal activity, berberine, *Chlorella pyrenoidosa*, *Microcystis aeruginosa*, photosynthetic activity

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INTRODUCTION

Cyanobacterial blooms and their adverse effect on aquatic ecosystems and human health have been reported worldwide, and create an urgent need for their monitoring and control (Codd *et al.* 2005; O'Neil *et al.* 2012; Ma *et al.* 2016). Cyanobacteria produce toxic metabolites, which are usually classified as dermatotoxins, neurotoxins, and hepatotoxins according to the toxic effects on animals. Microcystins are hepatotoxins to which special attention has been given, not only due to their ability to cause acute poisoning but also due to their cancer promoting potential through the chronic exposure of humans (de Figueiredo *et al.* 2004). Controlling cyanobacterial blooms has become an urgent issue worldwide because of its increasing occurrence (Ni *et al.* 2012; Lu *et al.* 2014; Lei *et al.* 2018; Page *et al.* 2018).

Among physical, chemical, and biological measures proposed, allelopathy has been considered as one of the most promising biological algal control technologies for its higher environmental safety (Shao *et al.* 2013; Yang *et al.* 2013). Allelopathic effects of exudates of one aquatic

organism on the other one are rather common, but only a fraction of compounds responsible for these effects can potentially be used in controlling growth; some allelochemicals are likely to change a variety of physiological processes of cyanobacteria, such as photosynthesis, respiration, gene expression and enzyme function (Dziga *et al.* 2007; Shao *et al.* 2009; Zhu *et al.* 2010; Huang *et al.* 2015). It has been reported that the photosystem of phytoplankton is probably an important target of some allelochemicals (Zhu *et al.* 2010).

Microcystis aeruginosa is responsible for approx. 70–75% of freshwater blooms. (Azevedo *et al.* 2002; Wu *et al.* 2007; Steffen *et al.* 2014). In a previous study, it was found that golden thread (*Coptis chinensis*) could significantly inhibit *M. aeruginosa* growth and berberine was the main bactericidal chemical of golden thread (Zhang *et al.* 2010). A subsequent study indicated that berberine had no sterilizing effects on *M. aeruginosa* without light exposure (Zhang *et al.* 2013). Based on its light-dependent sterilizing effects on *M. aeruginosa*, we speculated that

berberine might be a photosynthesis-inhibiting algicidal allelochemical. However, the target sites in the photosystem attacked by berberine remained unknown.

Application of algicides, such as metals, photosensitizers, and herbicides, has been developed as the 'acute' treatment for the control of cyanobacterial blooms (Costas & Lopez-Rodas 2006; Jančula & Maršálek 2011). Cylindrospermopsin has been shown to control the growth of *M. aeruginosa*, and even inhibit microcystin production by intact cells (Rzymiski et al. 2014). However, cylindrospermopsin itself is a toxin so one cannot rely on this compound in environmental management. Although some algicides are effective within a short period after application, their usage is potentially dangerous because of the threat to environmental safety and lack of selectivity (Jančula & Maršálek 2011; Walker 2017; Zhang et al. 2019). Most algicides do not selectively target harmful cyanobacteria in aquatic environments, and thus non-harmful algae may also be eliminated or negatively affected simultaneously.

It has been found that various algal species show different tolerance to some photosynthesis-inhibiting herbicides (Peterson et al. 1997; Fairchild et al. 1998). Pollution by a photosynthesis-inhibiting herbicide in aquatic environments could affect the competitive battle between susceptible algae and tolerant ones, thereby inducing changes in algal community structure (Lürling & Roessink 2006). As berberine might be a photosynthesis-inhibiting algicidal allelochemical, comparing the sensitivities of *M. aeruginosa* and non-target algae to berberine will benefit the application strategy of berberine in cyanobacterial bloom control.

In this study, to reveal whether or not berberine can be used selectively to target *M. aeruginosa*, we compared the effects of berberine on the growth of the cyanobacteria *M. aeruginosa* and the non-harmful green alga *Chlorella pyrenoidosa* with and without light exposure. In addition, changes in photosynthetic activities were also investigated to reveal the target sites of *M. aeruginosa* for berberine attack, and elucidate the algicidal mechanism involved. The results provide insights for the optimal application of berberine in cyanobacterial blooms control.

MATERIALS AND METHODS

Algal cultures

M. aeruginosa (FACHB-905), a microcystin producing strain, and *C. pyrenoidosa* were provided by the Institute

of Hydrobiology, Chinese Academy of Sciences. Algae were cultured in sterilized BG11 medium containing (g/L): NaNO₃, 1.5; K₂HPO₄, 0.04; MgSO₄·7H₂O, 0.075; CaCl₂·2H₂O, 0.036; citric acid, 0.006; iron (III) ammonium citrate, 0.006; Na₂-EDTA, 0.001; and Na₂CO₃, 0.02; and 1 mL of trace element solution (mg/L): H₃BO₃, 61; MnSO₄·H₂O, 169; ZnSO₄·7H₂O, 287; CuSO₄·5H₂O, 2.5; and (NH₄)₆Mo₇O₂₄·4H₂O, 12.5; at pH 7.4 (Rippka et al. 1979), under light-dark conditions (12:12 LD cycle) with a light density of 40 μmol photons/m²/s at 25 °C. *M. aeruginosa* and *C. pyrenoidosa* from the exponential growth phase were used for the subsequent experiment.

Berberine treatment

Berberine stock solution (10%, w/v) prepared by dissolving hydrochloride berberine (Northeast General Pharmaceutical Factory, China) in heated distilled water was added to the algal cultures of *M. aeruginosa* (3.99 × 10⁶ ind/ml) and *C. pyrenoidosa* (4.04 × 10⁶ ind/ml), respectively. The final concentration of berberine was 4 mg/L, and algal cultures without berberine addition were used as the controls. All treatments were done in triplicate. The algae were cultured in sterilized BG11 medium at 25 °C under light-dark conditions (40 μmol photons/m²/s, 12:12 LD cycle) or constant dark conditions for 9 days. Samples were removed from the cultures at 24-hour intervals to observe the changes in cell densities and chlorophyll fluorescence parameters. Moreover, under light-dark conditions, the chlorophyll fluorescence parameters of *M. aeruginosa* were measured at 2-hour intervals in the first 10 hours of light exposure.

Measurement of algal cell density and chlorophyll fluorescence parameters

The algal densities were examined with a haemocytometer (Improved Neubauer Counting Chamber) under a microscope. The chlorophyll fluorescence parameters of *M. aeruginosa* and *C. pyrenoidosa* were measured using a PHYTO-PAM phytoplankton analyzer (Walz, Germany). The maximum relative photosynthetic electron transport rate (ETR) values could be read directly, and the maximum PSII quantum yield (*Fv/Fm*) could be calculated according to the following formula (Campbell et al. 1998):

$$Fv/Fm = (Fm - Fo)/Fm$$

where F_0 was the minimum fluorescence yield in the dark-adapted state, and F_m was the maximum PSII fluorescence yield achieved by illuminating algae under a light intensity of 3,500 $\mu\text{mol photons/m}^2/\text{s}$ for 0.7 seconds.

Measurement of dark respiration, photosynthetic O_2 evolution and electron transport activities

The 50% inhibition concentration (EC_{50}) of berberine was determined based on the cell densities of *M. aeruginosa* exposed to berberine for 24 hours: the 24 h- EC_{50} value of berberine on *M. aeruginosa* was 0.47 mg/L. As the dosage of berberine used in the current study had no inhibitory effects on the growth of *C. pyrenoidosa* and therefore the 24 h- EC_{50} value could not be determined, both *M. aeruginosa* and *C. pyrenoidosa* were treated with 0 (control) and 0.47 mg/L berberine for 24 hours. Chlorophyll *a* (Chl *a*) was determined spectrophotometrically at 647 and 664.5 nm according to the method of Inskip & Bloom (1985). After being harvested by centrifugation and resuspended in BG11 medium with or without 0.47 mg/L berberine addition (control), algal photosynthetic O_2 evolution and electron transport activities were assayed using a Clark type oxygen electrode (Hansatech, UK) at 950 $\mu\text{mol photons/m}^2/\text{s}$ and 25 °C according to the method of Chen et al. (2007). Dark respiration was estimated from the O_2 consumed in darkness. The true photosynthesis rate was equivalent to the sum of O_2 evolution and consumption in dark respiration.

PSII electron transport activities were determined based on O_2 evolution in the assay mixture comprising 25 mM bis-tris propane (BTP, pH 7.8) and 1 mM *p*-benzoquinone (*p*-BQ). PSI electron transport activities were determined by O_2 consumption in the assay mixture containing 25 mM BTP (pH 7.8), 0.1 mM 2,6-dichlorophenol indophenol (DCPIP), 5 mM ascorbate (ASC) to reduce DCPIP to DCPIPH₂, 0.1 mM methyl viologen (MV), 1 mM NaN_3 and 10 μM 3-(3,4 dichlorophenyl)-1, 1-dimethyl urea (DCMU). Whole electron chain transport activities were estimated in terms of O_2 consumption in the assay mixture containing 25 mM BTP (pH 7.8), 1 mM NaN_3 and 0.1 mM MV. Inhibitors, electron donors and acceptors used in the measurement of electron transport activities are shown in Table 1.

Statistical analysis

Data are expressed as means \pm SD and the t-test was used to evaluate the statistical significance of differences between

Table 1 | Inhibitors, electron donors and acceptors used in the measurement of electron transport activities

	Electron donor	Electron acceptor	Respiration inhibitor	PSII activity inhibitor
Whole chain	H_2O	MV	NaN_3	
PSI	DCPIP	MV	NaN_3	DCMU
PSII	H_2O	<i>p</i> -BQ		

Abbreviations: MV, methyl viologen; DCPIP, 2,6-dichlorophenol indophenol; *p*-BQ, *p*-benzoquinone; DCMU, 3-(3,4 dichlorophenyl)-1,1-dimethyl urea.

controls and treatments. Values of $P < 0.05$ were considered to indicate significance.

RESULTS

Effects of berberine on the growth of *M. aeruginosa* and *C. pyrenoidosa*

As shown in Figure 1(a), under light-dark conditions, 4 mg/L berberine could eliminate *M. aeruginosa* effectively and the cell density dropped to zero on day 6. By contrast, berberine exhibited no sterilizing effects on *M. aeruginosa* under constant darkness. As shown in Figure 1(b), whether under normal light-dark conditions or constant dark conditions, there was no significant difference between cell densities of *C. pyrenoidosa* with and without berberine exposure ($P > 0.05$), suggesting that berberine had no algicidal activity on *C. pyrenoidosa*.

Effects of berberine on chlorophyll fluorescence parameters of *M. aeruginosa* and *C. pyrenoidosa*

Changes in the chlorophyll fluorescence parameters of *M. aeruginosa* exposed to berberine are shown in Figure 2(a). Compared to *M. aeruginosa* without berberine exposure, 4 mg/L berberine significantly decreased F_v/F_m and ETR under light-dark conditions ($P < 0.05$). After being exposed to berberine for 1 day, F_v/F_m and ETR of *M. aeruginosa* dropped to nearly zero or zero. Under constant darkness, there were no significant differences in F_v/F_m and ETR between *M. aeruginosa* with and without berberine exposure ($P > 0.05$). The measurement of the chlorophyll fluorescence parameters was performed in the light, which might be responsible for berberine-induced insignificant decreases in F_v/F_m and ETR of *M. aeruginosa*. Consistent with the growth performance of *C. pyrenoidosa*, no

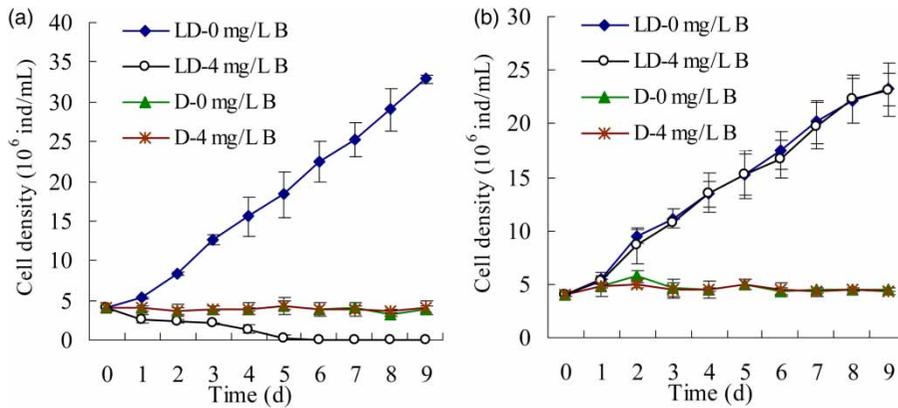


Figure 1 | Effects of berberine on the growth of *M. aeruginosa* (a) and *C. pyrenoidosa* (b) with or without light exposure. LD stands for light-dark conditions ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 12:12 LD cycle); D stands for constant dark conditions; B stands for berberine. Data are the mean \pm SD ($n=3$).

berberine-induced changes in both F_v/F_m and ETR of *C. pyrenoidosa* were observed under light-dark and constant dark conditions (Figure 2(b)). Under constant darkness, the chlorophyll fluorescence parameters of both *M. aeruginosa* and *C. pyrenoidosa* decreased with the prolongation of culture time.

As shown in Figure 3, compared to the control group, berberine decreased F_v/F_m and ETR significantly for *M. aeruginosa* in the first 10 hours under light conditions ($P < 0.05$). After 10 hours of exposure to berberine, F_v/F_m and ETR dropped to 0.04 and $0 \mu\text{mol electrons/m}^2/\text{s}$, respectively.

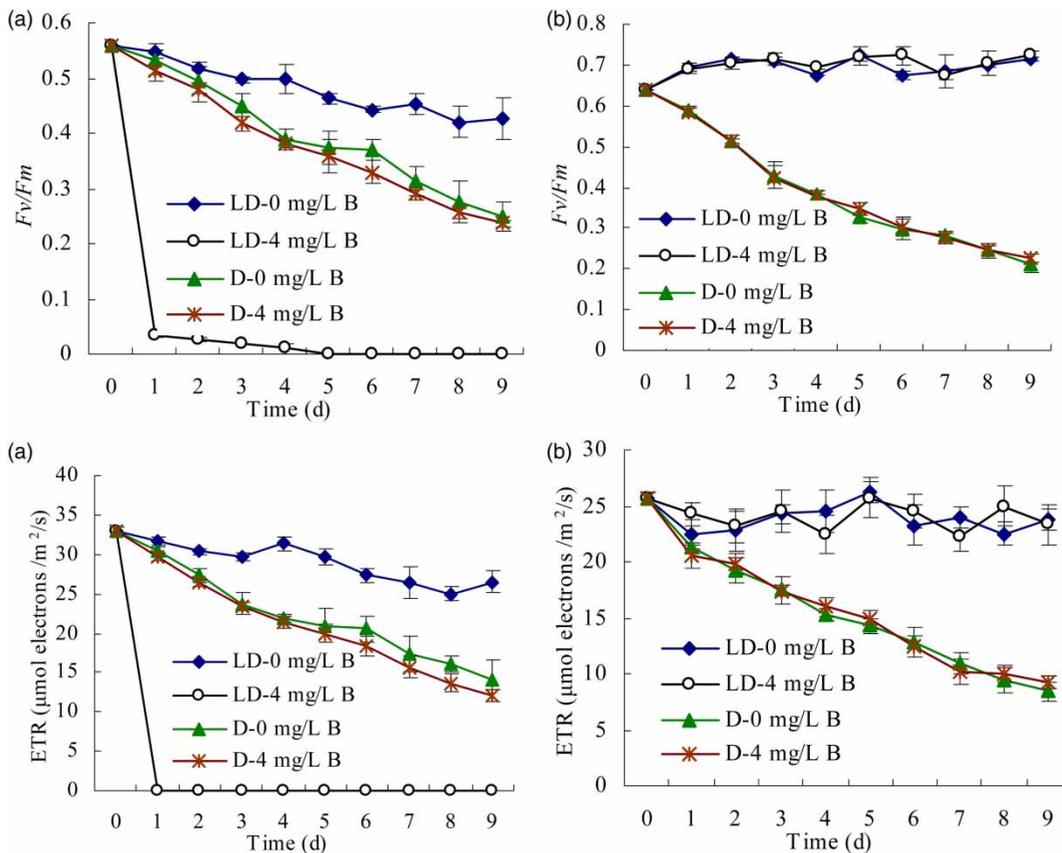


Figure 2 | Effects of berberine on the chlorophyll fluorescence parameters of *M. aeruginosa* (a) and *C. pyrenoidosa* (b) with or without light exposure. LD stands for light-dark conditions ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 12:12 LD cycle); D stands for constant dark conditions; B stands for berberine. Data are the mean \pm SD ($n=3$).

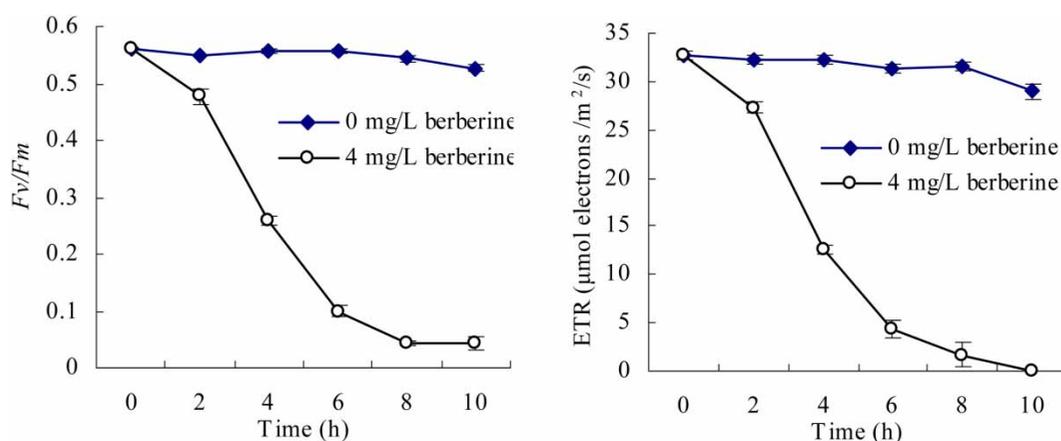


Figure 3 | Changes in the chlorophyll fluorescence parameters of *M. aeruginosa* during the first 10 hours of berberine exposure under light conditions ($40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). Data are the mean \pm SD ($n = 3$).

Effects of berberine on the dark respiration, photosynthetic oxygen evolution and electron transport activities of *M. aeruginosa* and *C. pyrenoidosa*

As shown in Table 2, 0.47 mg/L berberine inhibited the true photosynthesis rate of *M. aeruginosa* significantly ($P < 0.05$). However, the dark respiration rate was not significantly affected by 0.47 mg/L berberine treatment ($P > 0.05$). Compared to the control, no significant changes were observed in the true photosynthesis rate and dark respiration rate of *C. pyrenoidosa* exposed to 0.47 mg/L berberine ($P > 0.05$).

After being treated with 0.47 mg/L berberine, the whole chain electron transport activity and PSII electron transport activity of *M. aeruginosa* were significantly decreased by 28% and 57%, respectively ($P < 0.05$; $P < 0.01$). However, 0.47 mg/L berberine did not affect the PSI electron transport activity of *M. aeruginosa* significantly ($P > 0.05$). No significant difference in the photosynthetic electron transport activities of *C. pyrenoidosa* were observed in the presence or absence of 0.47 mg/L berberine ($P > 0.05$).

DISCUSSION

M. aeruginosa, as a photoautotroph species, relies heavily on its photosynthetic systems for energy conversion, which is also the main target of allelopathic substances (Dziga et al. 2007; Shao et al. 2011). The light-dependent algicidal effects of berberine on *M. aeruginosa* indicated that berberine might be a photosynthesis-inhibiting algicidal allelochemical. Chlorophyll fluorescence has been proven to be useful for indicating the changes in photosynthesis under stress conditions (Vonshak et al. 2001; Deng et al. 2015). Berberine could noticeably decrease the chlorophyll fluorescence parameters in *M. aeruginosa* after short-term light exposure, which decreased sooner and more rapidly than the cell density. This is consistent with the previous research results of Rzymiski et al. (2013), that the fluorescence changes of *M. aeruginosa* can be preceded by changes in cell density when cells are exposed to toxic metals. Changes in the chlorophyll fluorescence further support our previous speculation

Table 2 | Effects of 0.47 mol/L berberine on the dark respiration, true photosynthesis and electron transport activities of *M. aeruginosa* and *C. pyrenoidosa*

Rate ($\mu\text{mol O}_2/\text{mg Chl a/h}$)	<i>M. aeruginosa</i>		<i>C. pyrenoidosa</i>	
	Control	0.47 mg/L berberine	Control	0.47 mg/L berberine
Dark respiration	49.52 \pm 4.36	41.93 \pm 4.87	21.40 \pm 0.34	22.83 \pm 1.55
True photosynthesis	131.20 \pm 8.86	107.82 \pm 7.80*	357.83 \pm 68.06	336.20 \pm 43.83
Whole chain activity	107.93 \pm 6.54	78.02 \pm 14.55*	185.36 \pm 9.50	190.85 \pm 26.30
PSII activity	186.52 \pm 7.91	80.94 \pm 9.39**	185.36 \pm 23.24	189.44 \pm 30.17
PSI activity	43.89 \pm 9.81	47.89 \pm 10.03	343.24 \pm 80.31	312.49 \pm 31.95

Data are the mean \pm SD ($n = 3$) and are marked with asterisk(s) when significantly different (* $P < 0.05$; ** $P < 0.01$) compared to the rate in the control.

that berberine could eliminate *M. aeruginosa* by inhibiting photosynthesis.

The many reactions that occur during photosynthesis in plants can be grouped into two broad categories: the photosynthetic electron-transfer reactions (also called the 'light reactions') in the thylakoid and the carbon-fixation reactions (also called the 'dark reactions') in the stroma (Alberts *et al.* 2002). The formation of ATP, NADPH, and O₂ in the photosynthetic electron-transfer reactions requires light energy directly (Trebst 2003), and two photosystems (PSI and PSII) are responsible for converting light energy into redox processes (Lunde *et al.* 2000; Rast *et al.* 2015). The interruption of electron flow in PSII or diversion of electrons in PSI are the main targets for some photosynthesis-inhibiting herbicides and phytotoxins (Leu *et al.* 2002; Muller *et al.* 2008; Dayan & Zaccaro 2012; Deng *et al.* 2015). Some commercial photosynthesis-inhibiting herbicides, such as diuron and atrazine, are PSII inhibitors: they compete with the binding of plastoquinone at its BQ binding site and inhibit energy transfer. Other herbicides, such as paraquat, act on PSI (Dayan & Zaccaro 2012).

PSII reaction centers, as the main photosynthetic apparatus, play a key role in photosynthesis in cyanobacteria (Ou *et al.* 2012). It is more susceptible to various environmental stresses than PSI (Singh *et al.* 2012). For algicidal allelochemicals, Zhu *et al.* (2010) found pyrogallol acid and gallic acid could reduce photosynthetic activity in *M. aeruginosa* and PSII in cyanobacteria was considered to be one of the target sites attacked by allelopathic polyphenols. In the present study, berberine could significantly inhibit PSII electron transport activity in *M. aeruginosa* while no significant changes in PSI electron transport activity were observed, suggesting the targeted action sites of berberine are located on the electron transport chain PSII of *M. aeruginosa*.

Berberine could eliminate *M. aeruginosa* effectively by interrupting the electron flow on PSII but had no algicidal effects on *C. pyrenoidosa*. No berberine-induced inhibition in photosynthesis and electron transport activities were observed in *C. pyrenoidosa*. A possible explanation for the different sensitivities might be related to the different cell structures of these two algae. Zhu *et al.* (2010) found that the opposite inhibitory effects of allelopathic polyphenols on *M. aeruginosa* and *Selenastrum capricornutum* might be due to the different photosynthetic apparatuses in cyanobacteria and chlorophytes (Zhu *et al.* 2010). The cyanobacteria *Microcystis* are phototrophic prokaryotes, and their photosynthetic thylakoids are directly exposed to the cytoplasm (Stanier & Cohen-Bazire 1977). In contrast

to cyanobacteria, photosynthetic thylakoids in phototrophic eukaryotic algae are enclosed by chloroplast envelope consisting of a double membrane, a highly permeable outer membrane and a much less permeable inner membrane (Amils 2011). The inner membrane of the chloroplast has a selective permeability, reflecting the presence of specific carrier proteins (Alberts *et al.* 2002). It's worth mentioning that *M. aeruginosa*, as a kind of bacteria, is more sensitive to berberine as an antibacterial agent, while *C. pyrenoidosa*, as a eukaryote, is not sensitive to it (Cerňáková & Košťálová 2002). The permeability status of the chloroplast membrane to berberine could play an important role in mediating its algicidal activity in eukaryotic algae. Because there were changes in the photosynthetic activity of *C. pyrenoidosa*, we speculated that berberine might have no opportunity to permeate through the inner membrane of its chloroplast, leading to no disturbance in photosynthesis. This might be the reason why berberine had no algicidal effects on *C. pyrenoidosa*. To maximize the benefits of using berberine to control cyanobacterial blooms, the toxicity of berberine to more target cyanobacteria species and more non-target algal species needs to be studied. Studies have reported that berberine can have some beneficial effects on other aquatic animals, such as farmed fish, including inhibiting fish oxidative stress, enhancing fish immunity, and reducing fatty liver and diseases (Chen *et al.* 2016; Zhou *et al.* 2018).

Finally, it is worth noting that berberine-induced lysis of toxic *Microcystis* will release microcystins into the surrounding water and result in the rapid increase of microcystin concentrations in the water column. To avoid the potential threats from microcystins, it is important to eliminate them using effective measures, such as biodegradation, photodegradation, ozonation and activated carbon adsorption.

CONCLUSION

As a photosynthesis-inhibiting allelochemical, berberine could eliminate *M. aeruginosa* effectively and its inhibitory site in *M. aeruginosa* is located in PSII. The target *M. aeruginosa* and non-target *C. pyrenoidosa* showed totally different tolerance to berberine. The difference in the photosystem structures of these two algae might be responsible for their different sensitivities to berberine. It is reasonable to predict that the selective algicidal activity of berberine would lead to the replacement of susceptible target *M. aeruginosa* by tolerant non-target *C. pyrenoidosa*.

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