

# Physiological Responses of *Aphanizomenon flos-aquae* Under the Stress of *Sagittaria sagittifolia* Extract

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**Abstract** The algal growth and physiological characters of *Aphanizomenon flos-aquae* were studied under the stress of *Sagittaria sagittifolia* extract. The results showed that the growth of *A. flos-aquae* was significantly inhibited by *S. sagittifolia* extract. The exopolysaccharide (EPS), total soluble protein, intracellular phosphorus (o-PO<sub>4</sub>-P) contents and malondialdehyde (MDA) contents in *A. flos-aquae* cells increased significantly. These results suggested that *A. flos-aquae* can adapt to stress by increasing its normal metabolic activity. The algal cellular antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), were triggered to different degrees when exposed to *S. sagittifolia* extract. The MDA contents and activities of SOD, CAT and POD in algal cells suggested that oxidative damage induced by *S. sagittifolia* extract via the oxidation of ROS (O<sub>2</sub><sup>-</sup>) might be an important factor responsible for the inhibition of the growth of *A. flos-aquae*. In addition, SOD may be an important site for the inhibition of *S. sagittifolia* extract on *A. flos-aquae* cells. These results indicate that *S. sagittifolia* may be a good candidate for controlling *A. flos-aquae* blooms.

**Keywords** *Sagittaria sagittifolia* · *Aphanizomenon flos-aquae* · Reactive oxygen species · Antioxidant enzyme · Exopolysaccharide

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Cyanobacterial blooms are ubiquitous phenomenon in eutrophic aquatic ecosystems, having deleterious effects on the biodiversity and equilibrium of aquatic ecosystems. Therefore, the control and elimination of the growth of cyanobacterial blooms is a crucial step in the recovery and protection of aquatic ecosystems, especially lakes. Natural algicides from natural biomaterials have received attention as alternatives to chemical agents, as algicides are likely to be specific and biodegradable and may, therefore, offer an environmentally friendly method for the control of algal blooms (Yi et al. 2012). The polyphenols produced by aquatic plants influenced the activity of different extracellular or membrane-bound enzymes produced by *M. aeruginosa* (Dziga et al. 2009). Linoleic acid (LA) could also influence the antioxidant enzymes of *M. aeruginosa* (Ni et al. 2015).

*Aphanizomenon flos-aquae* is a bloom-forming cyanobacteria species and can release toxins into the surrounding water (Liu et al. 2006; Zhang et al. 2015b). In Dianchi Lake, occurrences of *Microcystis* blooms alternate with *Aphanizomenon* blooms, and *A. flos-aquae* is the dominant species from March to May (Liu et al. 2006). Although there have been several studies involving the isolation of natural algicides for the control of *Microcystis* blooms (Nakai et al. 2000; Hong et al. 2009; Zhang et al. 2012, 2016), less attention has been paid to the search for algicides against *A. flos-aquae*. *Sagittaria sagittifolia* is an emergent macrophyte used in the ecological restoration of eutrophic bodies of water. In our previous study, the extract of *S. sagittifolia* showed inhibitory activity on the growth of *A. flos-aquae* (Zhang et al. 2015b). The aim of the present study is to further explore the mechanisms of *S. sagittifolia* extract activity on *A. flos-aquae*.

## Materials and Methods

*Sagittaria sagittifolia* was collected from a small lake in a suburb of Kunming City, Yunnan Province, China, dried at normal ambient temperature. Powdered *S. sagittifolia* (20 g) was immersed in hexane (600 mL) for 3 days at room temperature and was filtered with filter paper to remove the insoluble residue. The filtrate was concentrated under vacuum to remove hexane and to yield the extract. The extract was then stored in a freezer ( $-20^{\circ}\text{C}$ ). The cyanobacteria species *A. flos-aquae* (FACHB 1170) was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology, the Chinese Academy of Sciences, and was pre-cultivated in BG11 medium at  $25 \pm 2^{\circ}\text{C}$  with a 14 h:10 h light/dark cycle, with illumination at  $43.2 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  provided by cool-white fluorescent lamps. The algae were shaken three times each day. In the study, organisms in the exponential growth phase were used. The bacterial biomass in the cultures was negligible.

The growth inhibition of *A. flos-aquae* was performed using the ISO 8692 method (1989) with some modifications. Conical flasks (250 mL) were prepared and autoclaved, with each containing 100 mL culture media. After its dissolution into dimethyl sulfoxide (DMSO), the initial concentration gradients of macrophyte extract were as follows: 0, 5, 10, 20, 50 and 100  $\text{mg L}^{-1}$ . Media with only DMSO were used as the controls. The DMSO level in the test flasks did not exceed 0.1% (v/v). Prior to these experiments, we found that 0.1% (v/v) DMSO had no obvious effect on the growth and other tested physiological characteristics of *A. flos-aquae*. The initial algal density (IADs) was  $1 \times 10^6$  cells  $\text{mL}^{-1}$ . Each experiment included triplicate treatments, and the experiments were repeated twice.

The chlorophyll a concentrations of *A. flos-aquae* were measured using the colorimetric method (SEPA 2002), and the algal cell number was examined with a light microscope (Olympus CX23) and hemocytometers (STD, 20 mm  $\times$  20 mm, 0.1 mL), with 10  $\mu\text{m}$  defined as one unit

(Larson and Passy 2012). These measurements were performed every day.

The algal cell-free enzyme extract was obtained according to the procedure described by Zhang et al. (2015a) and it was used to measure the enzyme activities and malondialdehyde (MDA). The activity of superoxide dismutase (SOD) was determined using the nitroblue tetrazolium (NBT) photoreduction (Beauchamp and Frodovich 1971) test. The antioxidant enzyme activities of catalase (CAT) and peroxidase (POD) were determined according to the method of Rao et al. (1996) and Dias and Costa (1983) respectively. The MDA content was assayed by the method described by Shiu and Lee (2005).

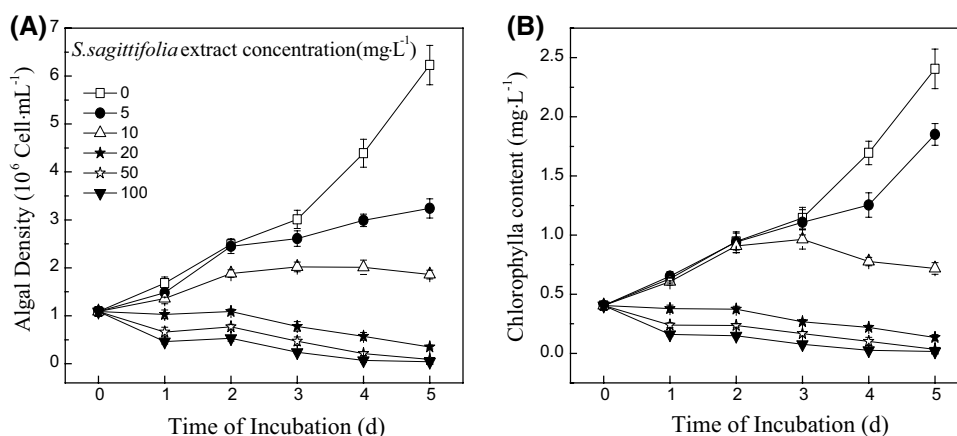
The total soluble protein content was measured according to the Coomassie brilliant blue G-250 dye-binding method (Bradford 1976) using bovine serum albumin as a standard. The total exopolysaccharide (EPS) concentration was obtained by measurement of the bound and the soluble exopolysaccharide according to the methods described by Hellebust and Craigie (1978) and Staats et al. (1999). The orthophosphate concentration in the cells of *A. flos-aquae* was obtained and measured according to the method described by Shi et al. (2003).

The mass units of the enzymatic activities and non-enzymatic substance contents in this study were defined using the cell-counting method. All experiments were performed in triplicate, and the data were expressed as the means  $\pm$  standard deviation (SD). Analysis for significance was performed with SPSS software (13.0) (SPSS Inc., Chicago, IL, USA) using the independent-samples t-test, and the differences were considered to be significant at  $p < 0.05$ .

## Results and Discussion

The growth inhibition of *A. flos-aquae* caused by the hexane extract from *S. sagittifolia* is illustrated in Fig. 1. After 3 days, the cell densities of *A. flos-aquae* exposed to 5, 10, 20,

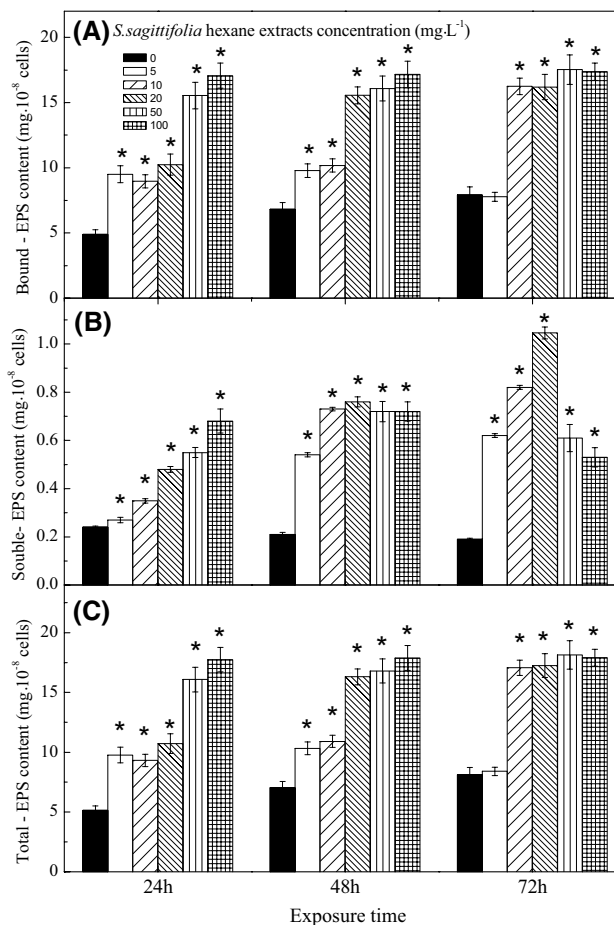
**Fig. 1** Effects of *Sagittaria sagittifolia* hexane extract on **a** the algal density and **b** the Chl-a content of *Aphanizomenon flos-aquae*. (n=3, the data are the mean, and the error bars indicate the SD of three replicates)



50, and 100 mg L<sup>-1</sup> macrophyte extract were 87%, 67%, 26%, 16% and 8%, relative to the controls, respectively. The changes in the algal Chl-a concentration showed the same tendency as that of the algal densities. Zhang et al. (2012) found that methanol extract of *Hydrilla verticillata* (Linn.f.) Royle had the highest inhibition on the growth of *Anabaena flos-aquae* and *Chlorella pyrenoidosa*. *Thalia dealbata* root aqueous extract showed growth inhibition against *Anabaena flos-aquae*, *M. aeruginosa* and natural phytoplankton assemblages (Zhang et al. 2011). In this study, both the algal cells and Chl-a concentration decreased obviously in the *S. sagittifolia* extract treatment (Fig. 1,  $p < 0.05$ ).

Carbohydrate and cellular proteins are two basic indicators of the physiological state of cells (Henderson et al. 2008). Phytoplankton can adapt to stress by increasing their normal metabolic activity (Prado et al. 2009). One of the functions of EPS is to form a protective layer to resist toxins or other environmental stresses. The EPS concentration of *Phormidium autumnale* increased significantly to defend against three ciliate grazer species (Pajdak-Stos et al. 2001). In our study, EPS showed a concentration-dependent increase in response to *S. sagittifolia* hexane extract exposure (Fig. 2). The bound, soluble and total EPS contents of *A. flos-aquae* increased with increasing *S. sagittifolia* extract concentration after 24 h exposure. The increased EPS concentrations suggested that new polysaccharides were synthesized to resist environmental stress. Soluble proteins in *Scenedesmus obliquus* cells are accumulated under heavy metal stress (Mohamed et al. 2004). In the present experiment, the total soluble protein content of *A. flos-aquae* cells increased significantly with the increasing exposure concentration of *S. sagittifolia* hexane extract (Fig. 3a). Accumulation of protein may be one of the ways through which *A. flos-aquae* abolishes the toxic effects of the *S. sagittifolia* hexane extract.

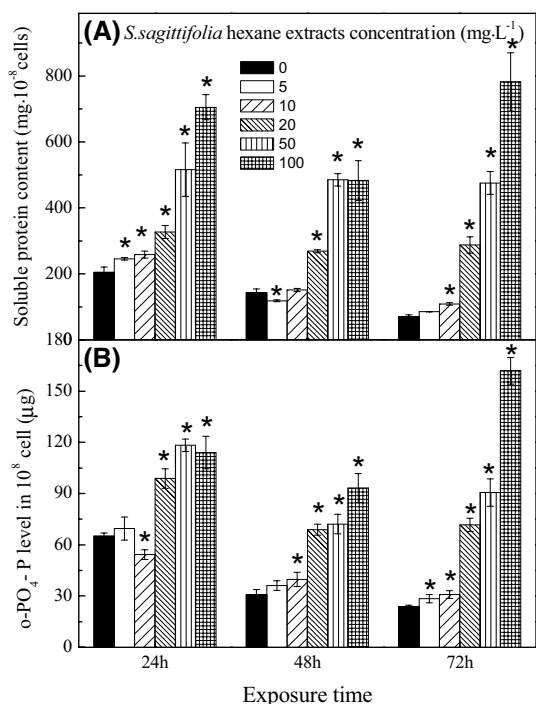
The change in phosphorus (o-PO<sub>4</sub>-P) (B) content (Fig. 3b) showed a similar pattern to that of the soluble protein content in that the o-PO<sub>4</sub>-P content increased after exposure to all treatment concentrations between 24 and 72 h. Orthophosphate (PO<sub>4</sub><sup>3-</sup>) is a substance in high-energy phosphate-bond synthesis. Kasemets et al. (2006) showed that the addition of low concentrations of octanoic and decanoic acids increased Q<sub>ATP</sub> (specific ATP production rate) and m<sub>e</sub> (maintenance energy requirement for growth) in *Saccharomyces cerevisiae*. Nonanoic acid stress stimulated the phosphorus uptake of *M. aeruginosa* (Shao et al. 2009); thus, the *S. cerevisiae* and *M. aeruginosa* required more energy to cope with the stress of allelochemicals. The contents of o-PO<sub>4</sub>-P in *A. flos-aquae* cells showed a concentration-dependent increase in response to *S. sagittifolia* extract exposure. We speculate that *A. flos-aquae*, similar to *M. aeruginosa* and *S. cerevisiae*, requires more energy to cope with *S. sagittifolia* extract stress, and more high-energy phosphate bonds were



**Fig. 2** Effects of the *Sagittaria sagittifolia* hexane extract on the contents of bound (a), soluble (b) and total exopolysaccharides (EPS) (c) of *Aphanizomenon flos-aquae*. (Data are given as the means  $\pm$  SD of three replicates, \* $p < 0.05$  indicates significant differences compared with the corresponding controls without *S. sagittifolia* extract)

required under different doses of *S. sagittifolia* extract, suggesting that *S. sagittifolia* extract stress stimulated phosphorus uptake by *A. flos-aquae*.

Enhanced MDA content is a vital sign of cellular oxidative damage (Shao et al. 2009). In the present study, MDA showed a concentration-dependent increase in response to *S. sagittifolia* hexane extract exposure (Fig. 4d). The MDA contents of the control group remained relatively steady, but those of the treatment groups exhibited significant changes. Increases in the MDA concentrations of *A. flos-aquae* were observed after 24 h incubation with increased exposure concentration. In addition, the MDA concentration in *A. flos-aquae* cells exposed to low concentrations of *S. sagittifolia* extract increased sharply at first and then decreased to slightly higher than that of the control. These results indicated that the *S. sagittifolia* extract caused algal cell membrane damage, and the oxidative damage might be reduced by the cellular defense system under exposure to low concentrations.

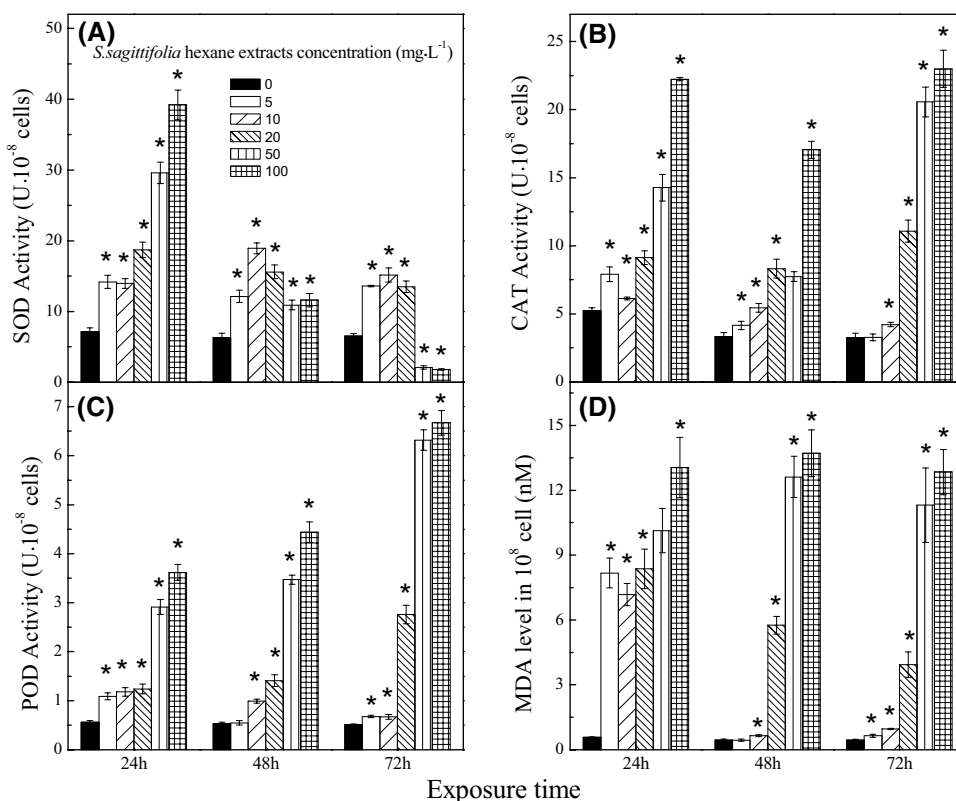


**Fig. 3** Soluble protein (a) and phosphorus (o-PO<sub>4</sub>-P) (b) contents in *Aphanizomenon flos-aquae* cells exposed to varying concentrations of *Sagittaria sagittifolia* hexane extract. (Data are given as the means ±SD of three replicates, \**p*<0.05 indicates significant differences compared with the corresponding controls without *S. sagittifolia* extract)

Scavenging of ROS occurs mainly through antioxidant pathways that consist of enzymatic and non-enzymatic scavenging systems. Antioxidant enzymes in algal cells were activated when exposed to allelochemicals (Latifi et al. 2009; Zhang et al. 2015a). Our results indicated that algal cellular antioxidant enzymes were triggered to different degrees when exposed to *S. sagittifolia* extract (Fig. 4a–c). The change in SOD activity was different depending on the *S. sagittifolia* hexane extract concentration (Fig. 4a). With exposure to lower concentrations (5, 10 and 20 mg L<sup>-1</sup>) of *S. sagittifolia* extract, the SOD activity increased throughout the 72 h period. In contrast, the SOD activities increased at first and then decreased when exposed to the 50 and 100 mg L<sup>-1</sup> concentrations of *S. sagittifolia* extract. This result is in accordance with the effects of the algicidal bacterium *Pseudomonas mendocina* on the antioxidant system of *A. flos-aquae* (Shi et al. 2009). However, the effects of *S. sagittifolia* hexane extract on CAT and POD activities were different (Fig. 4b, c). The CAT and POD activities both increased initially after exposure to *S. sagittifolia* hexane extract, and increased as the *S. sagittifolia* extract concentration increased and exposure time extended.

The CAT and POD activities in the treated group remained at high levels throughout the 72 h cultivation period. The SOD activity in the treated *A. flos-aquae* showed significant increases during the first 24 h, thereafter, the SOD activity showed a declining tendency (Shi et al.

**Fig. 4** Activities of superoxide dismutase (a), catalase (b), peroxidase (c), and malondialdehyde content (d) in *Aphanizomenon flos-aquae* exposed to varying concentrations of *Sagittaria sagittifolia* hexane extract. The y-axis represents the activities of the enzymes (or the content of MDA) expressed as the mean ±SD of three replicate cultures. The asterisks indicate statistically significant differences compared with the corresponding control, with *p*<0.05



2009). The resistance through SOD disappeared later due to the durative or acute increase of  $O_2\cdot^-$  (Hong et al. 2009). Therefore, the decrease in SOD activities in *A. flos-aquae* cells under higher exposure concentrations is a result of the acute increase in ROS in cells. The major function of CAT and POD in cells is the direct removal of  $H_2O_2$  to protect from oxidative damage (Latifi et al. 2009). The CAT and POD activities in treated cells were significantly higher than those in the control under all the treatment concentrations, indicating that CAT and POD were also active in eliminating ROS directly or indirectly. The major ROS include the superoxide radical ( $O_2\cdot^-$ ), hydrogen peroxide ( $H_2O_2$ ), and their derivatives. Primarily,  $O_2\cdot^-$  is converted into  $H_2O_2$  by superoxide dismutase (SOD). Subsequently,  $H_2O_2$  is scavenged by hydroperoxidases. Therefore, SOD is the first line of resistance against ROS. The *S. sagittifolia* hexane extract increased SOD activity in *A. flos-aquae* cells after 24 h incubation with increased exposure concentration, and SOD activity transitioned from an increase to a decrease with the prolongation of exposure time when exposed to 50 and 100 mg L<sup>-1</sup> concentrations of *S. sagittifolia* extract. These results are consistent with previous reports in which the  $O_2\cdot^-$  in cells increased and SOD activities decreased when treated with allelochemicals (Park et al. 2007; Hong et al. 2009). Therefore, our results suggest that oxidative damage induced by *S. sagittifolia* hexane extract via the oxidation of ROS ( $O_2\cdot^-$ ) might be an important factor responsible for the inhibition of the growth of *A. flos-aquae*. The results also suggest that SOD is an important site for the activities of *S. sagittifolia* hexane extract in *A. flos-aquae* cells.

*Aphanizomenon flos-aquae* is rarely used as the target algae in the search for the algicides. In our previous study, the extract of *S. sagittifolia* showed inhibitory activity on the growth of *A. flos-aquae* (Zhang et al. 2015b), but the mechanisms for the inhibition were not explored. As far as we know, this is the first report elucidating the effects of *S. sagittifolia* extract on the physiological characteristics of *A. flos-aquae*. In conclusion, the growth of *A. flos-aquae* was inhibited by *S. sagittifolia* hexane extract. On the basis of the results for EPS, protein, o-PO<sub>4</sub>-P, our present study suggests that *A. flos-aquae* could adapt to stress by increasing the normal metabolic activity. The MDA contents and SOD, CAT and POD activities in algal cells suggested that oxidative damage induced by *S. sagittifolia* extract via the oxidation of  $O_2\cdot^-$  is an important factor responsible for the inhibition of the growth of *A. flos-aquae* and that SOD is an important site of action of *S. sagittifolia* extract in *A. flos-aquae* cells. These results indicate that *S. sagittifolia* would be a potential species for *A. flos-aquae* blooms control.

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