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RESEARCH ARTICLE

Effects of chitosan, gallic acid, and algicide on the physiological and biochemical properties of *Microcystis flos-aquae*

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Abstract The effects of chitosan, gallic acid, and algicide chitosan-gallate on the activities of antioxidant enzymes, malonaldehyde (MDA) content, and photosynthetic activity of *Microcystis flos-aquae* were investigated to explore the physiological and biochemical mechanisms of algicides. Results demonstrated that chitosan did not significantly affect catalase (CAT) and superoxide dismutase (SOD) activities, MDA content, and photosynthetic activity in this alga. At 30 mg L^{-1}, gallic acid, CAT, and SOD activities and MDA of *M. flos-aquae* cells showed maximums of 2.872×10^{-10} mg·cell^{-1} min^{-1}, 0.787×10^{-8} U·cell^{-1}, and 0.626×10^{-8} nmol·cell^{-1}, respectively. Photosynthetic organs in algal cells were severely damaged under the stress of high gallic acid concentrations, inducing blockage of photosynthetic electron transport and resulting in the inability to restore normal photosynthetic activity. CAT and SOD activities and MDA content with lower algicide concentration were significantly higher than the control group ($p<0.05$) and, in higher algicide groups, significantly lower than the control ($p<0.05$). Algicide releasing gallic acid in groups treated with 60, 90, and 130 mg/L algicide was strong enough to cause severe damage to photosynthetic organs in these algal cells. The algicide suppression time was longer than that of directly added gallic acid.

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Introduction

Inhibition of algae and algae removal are important in the treatment of water supplies, such as drinking water and industrial circulating cooling water (Gao and Guo 2012). Many researchers have studied the inhibitory effect of various algicides (Greenfield et al. 2014; Spencer et al. 2013; Tilney et al. 2014; Ebenezer et al. 2014). Some investigations of industrial water treatment with algicide have focused on allelopathic inhibition of algae growth because of the algicide's biodegradability and ecological safety (Haddadchi and Gerivani 2009; You et al. 2011; Huang et al. 2014). The allelopathic substance phenolic acid, which is abundant in higher plants (Haddadchi and Gerivani 2009; You et al. 2011), has strong allelopathic effects at low concentrations. Kamaya et al. (2006), Kovacik et al. (2010), and Raman and Ravi (2011) have shown that salicylic acid exerts clear inhibitory effects on several types of green alga. Laue et al. (2014) have shown that tannic and gallic acids reduce the growth rate, maximum photosystem II (PS-II) quantum yield, and chlorophyll a during the first 2 days after administration. *Microcystis aeruginosa* is more sensitive to polyphenols than *Desmodesmus armatus*. In such research, however, most allelochemical phenolic acids are delivered directly to the simulated water environments, thus causing overly high local concentrations and having a serious impact on other organisms. Furthermore, the effective duration of such algal inhibition is short. Thus, using algicides with a slow-release function to inhibit algal growth might be a more effective method for controlling algal blooms. Therefore, exploring and developing algicides with slow-release functions has broad prospect and appeal (Chen et al. 2011). Chitosan

and its derivatives are a class of biodegradable natural polymers with characteristics of biocompatibility, high charge density, nontoxicity, and mucoadhesion, and are environmentally friendly adsorption materials with high potential in algal control. Furthermore, chitosan molecules have pore structures and contain hydroxyl and amino functional groups as well as strong coordination ability with phenolic acids and heavy metals (Bailey et al. 1999; Kumar 2000). Preliminary research has been carried out here on the desorption capacities of copper ions from a chitosan carrier and its efficiency in removing red tide organisms. Copper ions are gradually eluted from chitosan carriers that have adsorbed copper when placed into water. These ions could kill and control red tide organisms and have a prolonged control time (Liang et al. 2001). Chen et al. (2011) have shown that copper-containing algaecide clearly suppresses *Chlorella pyrenoidosa* growth, and algae removal increased with increasing algaecide dosage. Gallic acid-algicide carried by chitosan has been previously produced by the present authors, and its release ability and long-term inhibition on *Microcystis flos-aquae* growth investigated (Liu et al. 2014). However, the algicidal mechanism of this inhibition has not yet been studied. Research has shown that production of hydrogen peroxide and quinine can be induced by phenolic acid autoxidation, and hydrogen peroxide also causes lipid peroxidation (Furukawa et al. 2003). As a potential peroxide, quinone produces reactive oxygen species through oxidation reduction cycles, thereby affecting algal cell growth (Wolf et al. 2000). Therefore, the effects of chitosan, gallic acid, and algicide chitosan-gallate on the activities of antioxidant enzymes, malonaldehyde (MDA) content, and photosynthetic activity of *M. flos-aquae* were analyzed to reveal the algicidal mechanism and provide a scientific basis for investigating and developing ecologically safe algicides with a slow-release feature.

Materials and methods

Experimental materials

The *M. flos-aquae* (FACHB-1028) algal bloom species was used in this study because it can release toxins. The alga was purchased from the Freshwater Algae Species Pool of Wuhan Aquatic Research Institute (Chinese Academy of Sciences, Wuhan, China). BG-11 culture medium was used in the present study. Chitosan was purchased from the Sinopharm Chemical Reagent Co., Ltd. (Beijing, China) with a deacetylation degree of >80 %. Analytically pure gallic acid was purchased from Meiko Chemical Co., Ltd. (Tokyo, Japan).

The algicide used in the present experiment had an adsorption ratio of 623 mg g^{-1} for gallic acid/chitosan. The novel algicide has a significant inhibitory effect on *M. flos-aquae* growth (Liu et al. 2014). When the algicide concentration was 30 and 45 mg L^{-1}, the inhibition time reached 15 days. At 60, 90, and 130 mg L^{-1}, the inhibition time reached 24 days with an inhibition rate of >99 %. A 43 mg L^{-1} dose of gallic acid (equivalent to 130 mg L^{-1} of algicide containing gallic acid) produced an inhibition rate up to 95 %. However, algal growth showed recovery after 15 days, indicating that gallic acid could not maintain long-term inhibition.

Experimental methods

Algal cell culture and density measurement

Algae in the logarithmic phase were seeded into a 1-L conical flask containing 400 mL of newly prepared culture medium. When the algal cell density reached the logarithmic growth phase, the cells were loaded into 100-mL empty, sterilized, conical flasks. After further culturing for 1 day, the cells were treated with the quantities of additives at final concentrations of 4–87 mg L^{-1} in groups treated with chitosan; 4–43 mg L^{-1}, in groups treated with gallic acid; and 10–130 mg L^{-1}, in groups treated with algicide. The "ck" group was the blank control group and three parallel samples were set in every group. The culture temperature was 25±1 °C and light/dark ratio was 12 h/12 h, with light intensity at 50 μmol photons m^{-2} s^{-1}. The flasks were shaken three times a day and, to reduce accidental error from orientation, their positions changed randomly to ensure the luminous flux in each bottle was consistent.

Algal cell density was measured using blood cell counting plate microtechnology. The spectroscopic absorbance was measured at 680 nm to determine the relationship between cell density of *M. flos-aquae* (Y, 5×10^4 cells·mL^{-1}) and absorbance of the alga liquid $D_{680}(X)$:

$$Y = 847.99X - 13.962 \left(R^2 = 0.9997\right)$$

As the correlation between these two factors was good, the optical density of the algal liquid (D) was measured at regular times to assess algal growth.

Determination of antioxidant enzyme activity and MDA content

After treatment of algal liquid by addition of chitosan, gallic acid, or algicide for 16 days, algal cells were harvested by centrifugation and quickly placed in an ice bath. Harvested cells were then suspended in 50 mmol L^{-1} phosphate buffer solution (pH 7.8) and crushed by an ultrasonic cell crusher (power at 500 W). After 20 min, samples were centrifuged at 12,000 g at 4 °C for 15 min. The resulting supernatants were crude enzyme extracts, which were preserved under cryopreservation conditions for future use. Catalase (CAT) activity was

determined by potassium permanganate titration (Li et al. 2000). Superoxide dismutase (SOD) activity was determined by nitroblue tetrazolium staining (Li et al. 2000). MDA content in algal cells was measured by the thiobarbituric acid (TBA) method (Li et al. 2000).

Determination of photosynthetic activity

The content of chlorophyll and its fluorescence parameters were measured using a Phyto-PAM Phytoplankton Analyzer (Heinz Walz GmbH, Effeltrich, Germany) once every 48 h, as described previously (Heinz Walz GmbH 2003). Thirteen actinic lights were used in the rapid light response curves, with irradiation time of each actinic light at 20 s. An Origin 8.0 fitting light response curve (OriginLab Corp., Northampton, MA, USA) was used. The least square method was employed for curve fitting and the fitting model, as described previously (Platt et al. 1980).

$$P = \mathrm{Ps}\bullet(1\text{-}e^{-\alpha}\bullet \mathrm{PAR}/\mathrm{Ps})\bullet e^{-\beta}\bullet \mathrm{PAR}/\mathrm{Ps} \quad (1)$$

In Eq. 1, P is the photosynthetic rate (rETR), namely the relative electron transport rate, Ps the maximum potential and relative electron transport rate when light is inhibited, PAR the effective light intensity, α the initial slope of the P-I curve, reflecting the efficiency of light energy use, and β the light inhibition parameters (Ralph and Gademann 2005).

$$\mathrm{rETR}_{\max} = \mathrm{Ps}[\alpha/(\alpha+\beta)]\ [\beta/(\alpha+\beta)]^{\beta/\alpha} \quad (2)$$

It can be concluded that half saturation light intensity I_k

$$I_k = \mathrm{rETR}_{\max}/\alpha \quad (3)$$

Data processing

Data were analyzed by SPSS18.0 software and Origin 8.0 used to produce figures. The control and treatment groups were analyzed by single-factor analysis of variance, with $p<0.05$ representing significant differences.

Experimental results and analysis

Effects of chitosan, gallic acid, and algicide on CAT activity in *M. flos-aquae*

The effects of different chitosan dosages on CAT activity in individual *M. flos-aquae* cells showed no significant differences in groups treated with different chitosan concentrations and the control group ($p>0.05$, Fig. 1a). The effects of different gallic acid dosages on CAT activity in individual *M. flos-aquae* cells showed significant differences among gallic acid groups with 10, 15, 20, and 30 mg L^{-1} gallic acid ($p<0.05$), and CAT activity was higher than the control ($p<0.05$). A maximum value of 2.872×10^{-10} mg·cell^{-1} min^{-1} was obtained with 30 mg L^{-1} gallic acid. The effects of different algicide

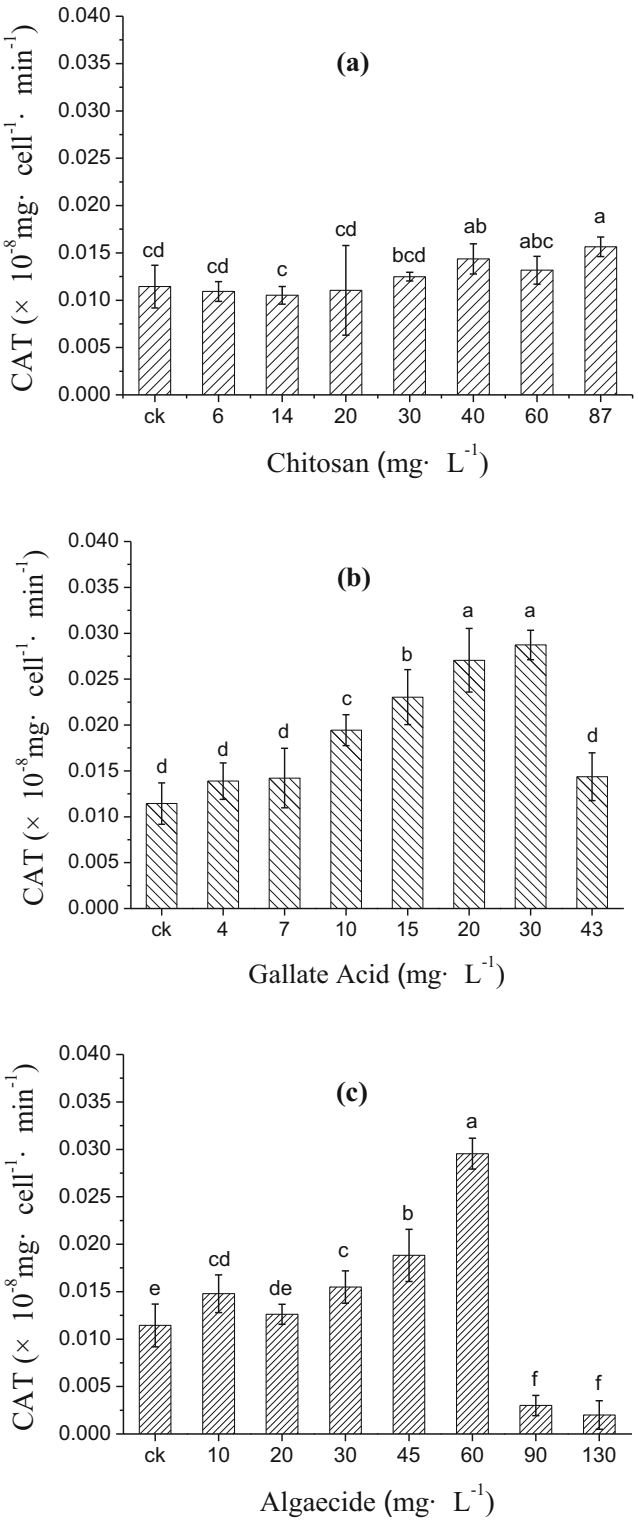

Fig. 1 Effects of chitosan (**a**), gallic acid (**b**), and algicide (**c**) on CAT activity of *Microcystis flos-aqua*

dosages on CAT enzyme activity in individual *M. flos-aquae* cells showed that CAT activity in the 60 mg L^{-1} algicide group was significantly higher than the control ($p<0.05$, Fig. 1c). The CAT enzyme activities in groups with 90 and 130 mg L^{-1} algicide were significantly lower than the control ($p<0.05$).

Effects of chitosan, gallic acid, and algicide on SOD activity in *M. flos-aquae*

The effects of different chitosan dosages on SOD enzyme activity in individual *M. flos-aquae* cells showed no significant differences in SOD activity between chitosan-treated groups and the control group ($p>0.05$, Fig. 2a). The effects of different gallic acid dosages on SOD enzyme activity in individual cells showed that SOD activities in gallic acid groups at 20 and 30 mg L^{-1} were significantly higher than the control ($p<0.05$, Fig. 2b). At 30 mg L^{-1} gallic acid, the maximum SOD activity was 0.787×10^{-8} U·$cell^{-1}$. The effects of different algicide dosages on SOD enzyme activity in individual cells showed that SOD activity in the 60 mg L^{-1} algicide group was significantly higher than the control ($p<0.05$, Fig. 2c). SOD enzyme activities at 90 and 130 mg L^{-1} algicide were significantly lower than the control ($p<0.05$).

Effects of chitosan, gallic acid, and algicide on MDA activity in *M. flos-aquae*

The effects of different chitosan dosages on MDA content in individual *M. flos-aquae* cells showed no significant differences in MDA among the different groups ($p>0.05$, Fig. 3a). The effects of gallic acid different dosages on MDA in individual cells showed that the 7, 20, and 30 mg L^{-1} gallic acid groups were significantly higher in MDA than the control ($p<0.05$, Fig. 3b). At 30 mg L^{-1} gallic acid, the maximum MDA content was 0.626×10^{-8} nmol·$cell^{-1}$. The effects of different algicide dosages on MDA in individual cells showed that the 10, 20, 30, 45, and 60 mg L^{-1} algicide groups were significantly higher in MDA than the control ($p<0.05$, Fig. 3c). MDA in the 90 and 130 mg L^{-1} algicide groups were significantly lower than the control ($p<0.05$).

Effects of chitosan, gallic acid, and algicide on photosynthetic activity in *M. flos-aquae*

The rETR reflects the apparent electron transfer efficiency of PS-II under actual light intensity (Bilger and Björkman 1990). The effects of chitosan, gallic acid, and algicide on the rapid light response curves of *M. flos-aquae* on 1, 7, and 15 days produced rapid light response curves of algal cells, fitted by actinic flux density and rETR (Fig. 4). There were no significant differences in rETR between the different algicide concentration groups and the control group ($p>0.05$, Fig. 4a–c). Therefore, chitosan exhibited no effects on the photosynthetic electron transport system in these algal cells. The rETR in algal cells increased with enhanced light

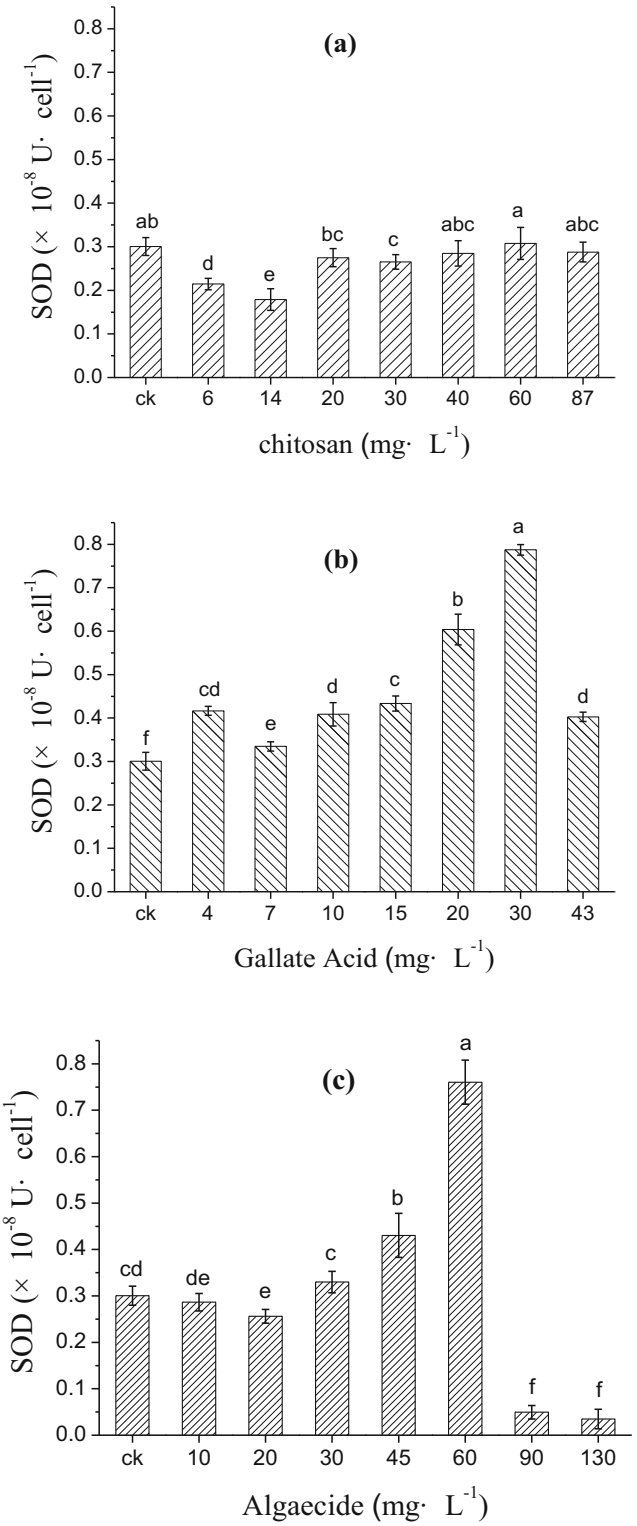

Fig. 2 Effects of chitosan (**a**), gallic acid (**b**), and algicide (**c**) on SOD activity of *Microcystis flos-aquae*

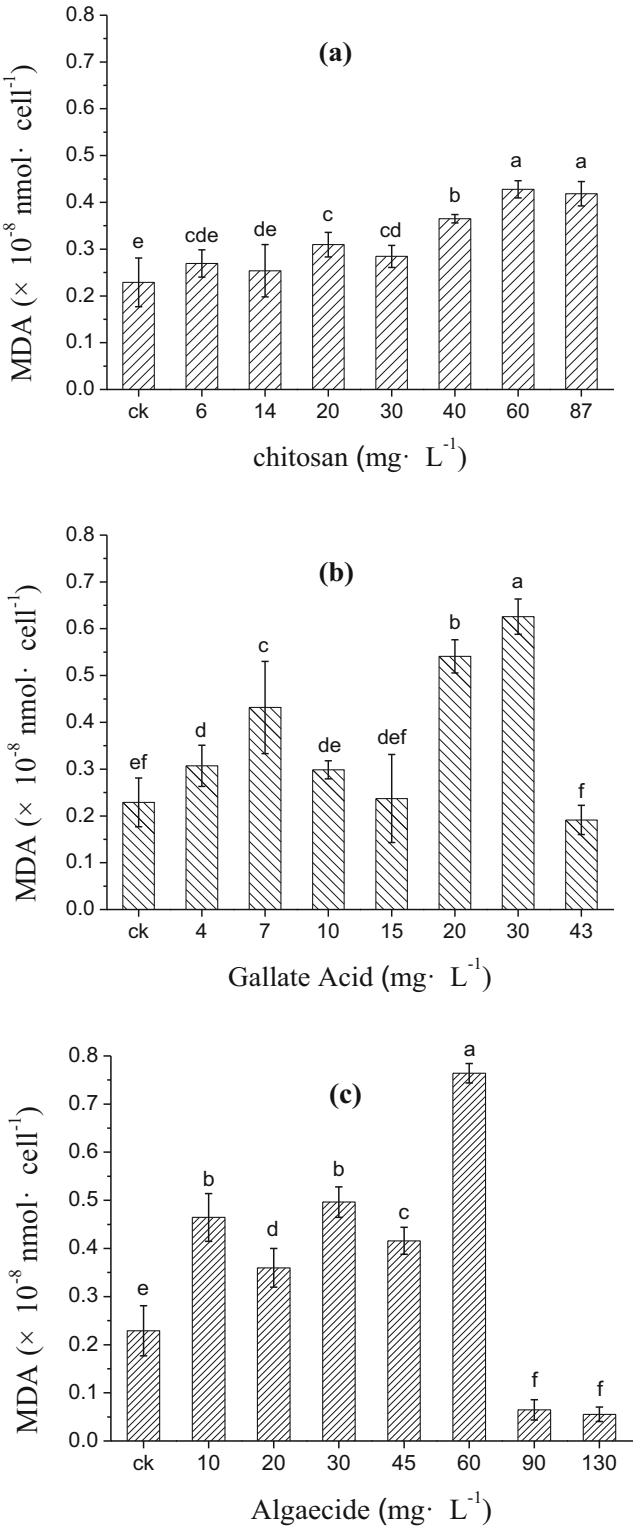

Fig. 3 Effects of chitosan (**a**), gallic acid (**b**), and algicide (**c**) on MDA content of *Microcystis flos-aquae*

intensity in the different gallic acid concentration groups treated at 1 day. In addition, rETR decreased with increased gallic acid concentration, with rETR values lower than 20 mol m^{-2} s^{-1} in the 20, 30, and 43 mg/L gallic acid groups. These results indicated that the photosynthetic electron transport system in these algal cells was inhibited strongly. However, compared with 1 day, rETR in algal cells from the low gallic acid groups (4, 7, 10, and 15 mg/L) at 7 days showed different degrees of recovery, such that were no significant differences compared with the control group ($p>0.05$). Compared with 1 day, rETR was clearly enhanced in the 20 mg/L gallic acid group. However, rETR rates were still low in algal cells from low gallic acid groups (30 and 43 mg/L). The rETR in algal cells from all treatment groups exhibited further recovery 15 days after treatment, but rETR remained low in the 43 mg/L gallic acid group. It was speculated, therefore, that the stress of high gallic acid concentrations severely damaged photosynthetic organs in these algal cells, inducing blockage of photosynthetic electron transport and resulting in the inability to recover normal photosynthetic activity.

There were no apparent differences in rETR between the 10 and 20 mg/L algicide groups and the control after 1 day of treatment ($p>0.05$, Fig. 4f–h). The rETR in the 30 and 45 mg/L algicide groups were lower than the 10 and 15 mg/L gallic acid groups. This suggested that, compared with groups treated with equal gallic acid concentrations, the algal inhibition ability of gallic acid released in the 30 and 45 mg/L algicide groups treated was clearly lower. Compared with the 20, 30, and 43 mg/L gallic acid groups (Fig. 4d), rETR in the 60, 90, and 130 mg/L algicide groups showed no significant differences, indicating that gallic acid released by the 60, 90, and 130 mg/L algicide groups was strong enough to cause severe damage to photosynthetic organs in these algal cells. The rETR clearly recovered in the 30, 45, and 60 mg/L algicide groups 7 days after treatment, but the decreasing trends of algicide algal inhibition ability from these three concentrations were slower than in the corresponding gallic acid treatments (Fig. 4e), indicating that the chitosan in algicide showed some degree of protection against gallic acid. Fifteen days after treatment, only the 90 and 130 mL/L algicide groups still showed low rETR rates.

Discussion

Effects of chitosan, gallic acid, and algicide on SOD, CAT, and MDA in *M. flos-aquae*

Chitosan is a biodegradable natural polymer and the only alkaline polysaccharide in the natural world (Pillai et al. 2009). Because there are a large number of active hydroxyl and amino groups in chitosan molecular chains, it can produce adsorption by interacting with aqueous phenolic acids (Yang et al. 2008; Imran et al. 2012). Antioxidant system enzymes, such as CAT and SOD in *M. flos-aquae* cells, can remove free radicals and comprised an important defense system against

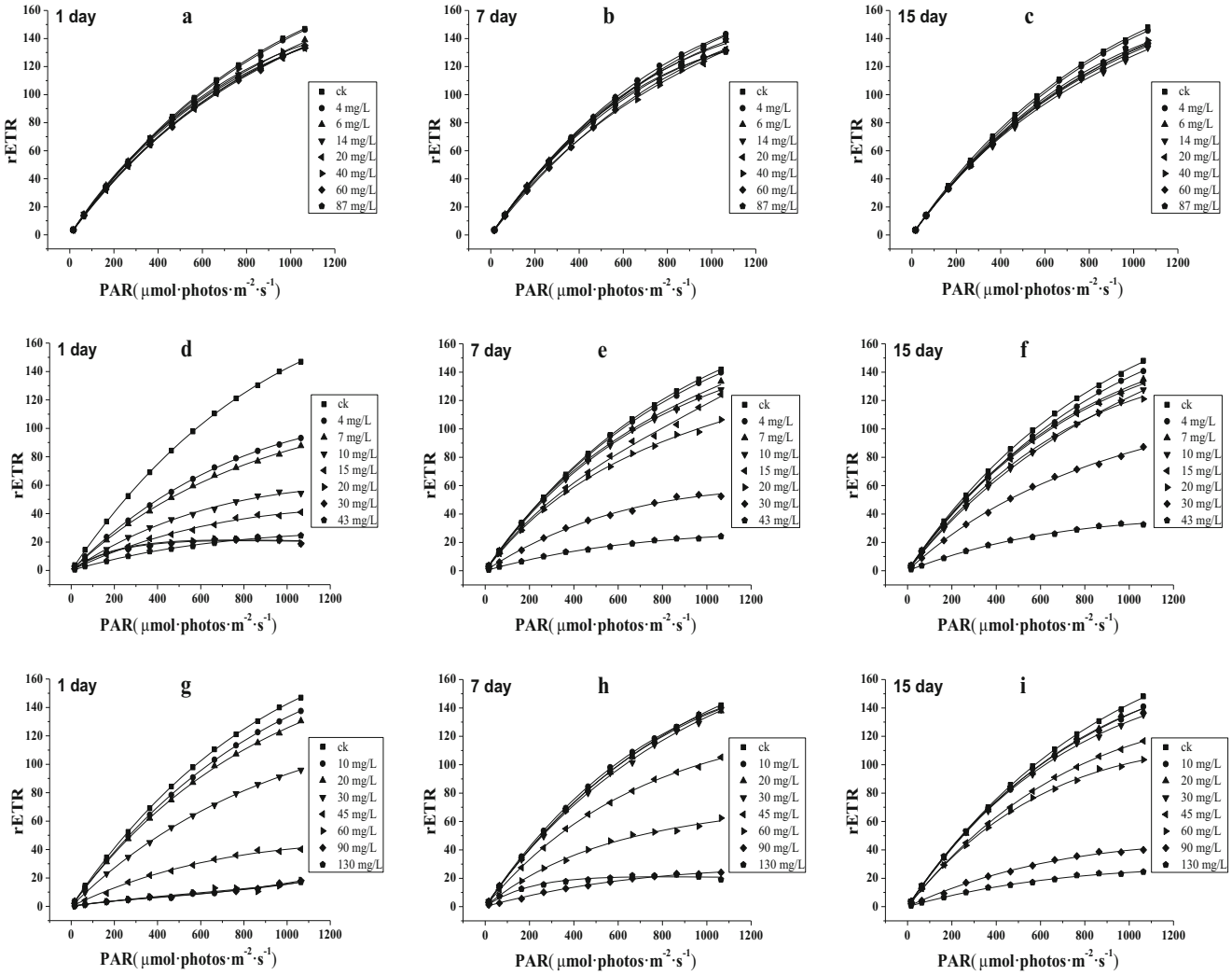

Fig. 4 Effects of chitosan (**a**, **b**, **c**), gallic acid (**d**, **e**, **f**), and algicide (**g**, **h**, **i**) on the rapid light curves of *Microcystis flos-aquae*

oxidative damage in the present experiment. This antioxidant system can improve enzyme activity and thus maintain metabolic balance in cells under stress conditions, protecting cells from oxidative injury. CAT, which exists in almost all organisms, functions to catalyze intracellular H_2O_2 to oxygen and water, such that cells can avoid its toxicity and resulting damage. There are direct correlations between CAT activity and accumulation of H_2O_2, and CAT is a key enzyme of the biological defense system (Bocova et al. 2012). SOD is a type of oxidase widely found in algal cells. SOD can effectively remove active oxygen and superoxide anion free radicals in cells grown in stressful environments, thereby preventing cellular damage. SOD is, therefore, considered a protective enzyme (Albert and Mfarkhart 1986). Concentrations of intracellular reactive oxygen free radicals increase in algal cells under external stress. Active oxygen free radicals cause membrane lipid peroxidation and MDA generation, which seriously damages the cell membrane system. The present experimental results showed that chitosan did not affect CAT and SOD in *M. flos-aquae*, and these cells also did not exhibit MDA accumulation. The results obtained here suggested that the functional groups in chitosan did not produce free radicals or affect cell growth. Thus, the effects on cell growth might have been from gallic acid.

Experimental results also illustrated that gallic acid in the high concentration group induced increased activities in antioxidant enzymes in *M. flos-aquae* cells and that MDA content increased significantly. Gallic acid is an important class of phenolic acid, in which three adjacent phenolic hydroxyls are the main active groups (Inoue et al. 1995). These phenolic hydroxyl groups exhibit autoxidation reactions by interacting with transition metal ions, such as Cu^{2+} and Fe^{3+}, in culture medium and generating H_2O_2. Algal cells remove excess H_2O_2 by increasing the activities of their antioxidant enzyme system, thus protecting from cellular oxidative damage. Under normal conditions, cells can maintain a balance between generation and elimination of active oxygen through their protective mechanisms. If free radicals are excessively accumulated,

however, lipid peroxidation will be induced, the membrane system and function of algal cells will be damaged, and membrane permeability will increase. MDA is a product of membrane lipid peroxidation, and its content in cells can reflect the degree of damage to cell membrane structure (Panda et al. 2003). Under high concentration gallic acid stress, if production and elimination of free radicals in algal cells is not balanced, it will cause excessive accumulation of free radicals. When the H_2O_2 concentration is beyond a certain threshold, cells cannot degrade it rapidly enough, which leads to cell damage and slow cell repair as well as causing decreased enzyme activity.

Effects of chitosan, gallic acid, and algicide on photosynthetic activity of *M. flos-aquae*

Rapid light curves are a relatively new tool that uses the modulated fluorescence principle to measure the rETR with short-term photosynthetic effective radiation, or PAR, changes to clarify the photosynthetic characteristics of cell samples (Schreiber et al. 1997; White and Critchley 1999). Light adaptation time is very short at different PAR gradients, and the measurement process has little interference from natural photosynthetic states of samples (White and Critchley 1999). In the present study, little difference was observed under weak light intensity conditions (<464 μmol m^{-2} s^{-1}). When the light intensity was >1164 μmol m^{-2} s^{-1}, the *M. flos-aquae* electron transfer chain limited ability for photosynthesis, and the curve attained a steady state when the time response capacity was maximum. The obtained data indicated that the PS-II reaction center was partly closed or inactivated, and excess energy could only be dissipated through a nonphotochemical quenching mode (Wang et al. 2010). Studies have confirmed that a good linear relationship exists between rETR, the photosynthetic oxygen release rate, and the CO_2-fixation rate before light saturation (Geel et al. 1997; Genty et al. 1989; Gilbert et al. 2000; Figueroa et al. 2003). Gallic acid and gallic acid in algicide in the present study showed marked effects on the relative electron transport rates in *M. flos-aquae*. Compared with the effects of gallic acid and algicide on chlorophyll a in *M. flos-aquae*, algae initially exhibited recovery of photosynthetic capacity during the growth recovery process. Furthermore, only after photosynthesis ability recovery could these algal cells resume rapid growth and reproduction.

Conclusion

The effects of chitosan on CAT and SOD activities and MDA content in *M. flos-aquae* were not significant, suggesting that the inhibitory effect of algicide chitosan-gallic acid on this alga was caused by gallic acid. The mechanism of gallic acid algal inhibition was because of excess H_2O_2 produced by phenolic hydroxyl produced by autoxidation, leading to increased CAT and SOD activities and thus further resulting in irreversible repair and cell death. Gallic acid and algicide affected the photosynthetic activity of this alga to some degree, but photosynthetic activity was restored in later stages. Gallic acid and algicide showed reversible inhibition effects on algal photosynthetic activity. The lower the gallic acid and algicide concentrations, the better the alga's photosynthetic activity recovery. Gallic acid adsorption by chitosan did not cause changes in the gallic acid reactive groups, which showed the same inhibitory mechanism as that of only gallic acid, although the time suppression was longer than that of only gallic acid.

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