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RESEARCH ARTICLE

Response of the cyanobacterium *Microcystis flos-aquae* to levofloxacin

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Abstract The effects of levofloxacin (LEV) on *Microcystis flos-aquae* and its mechanism were investigated by determining the responses of some parameters of *M. flos-aquae* to LEV stress, including growth inhibition ratio, chlorophyll *a* content, superoxide dismutase (SOD) and catalase (CAT) activities, malondialdehyde (MDA) content, F_v/F_0 and F_v/F_m, etc. The results indicated that LEV at 0.001–0.1 μg L^{-1} could stimulate the growth of *M. flos-aquae* and increase the chlorophyll *a* content but did not induce a significant increase in the activity of antioxidant enzymes (SOD and CAT) and the content of MDA. When the LEV concentration exceeds 10 μg L^{-1}, the growth of *M. flos-aquae* could be significantly inhibited (the highest inhibition ratio can be up to 88.38 % at 100 μg L^{-1}) and chlorophyll *a* content, SOD and CAT activities, and MDA content also significantly decreased in a concentration-dependent manner, indicating that high concentrations of LEV caused a severe oxidative stress on algal cells, resulting in a large number of reactive oxygen species produced in algal cells and thereby inhibiting the growth of algae. At the same time, the F_v/F_m and F_v/F_0 values of *M. flos-aquae* decreased significantly with both exposure time and increasing test concentration of LEV, showing that the process of photosynthesis was inhibited.

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Introduction

In recent years, antibiotics have been widely used in medicine and health, aquaculture industry, food processing, poultry farming, etc. In China, 15,770 tons of antibiotics were used as non-prescription therapeutics in 2004 (Richardson et al. 2005). Wise (2002) calculated that antibiotic consumption has been estimated worldwide to lie between 100,000 and 200,000 tons per annum, among which about 40,000 tons were used in China. Because antibiotics have short half-lives in living organisms and low metabolic rate, about 70–80 % of antibiotics are excreted by organisms in their parent form and then enter the aquatic ecosystem through many ways. More than 50 kinds of antibiotics have been detected in various aquatic environments, including sewage, surface, ground, and drinking waters, and the detected concentration was in the range of nanograms per liter to micrograms per liter (Tan et al. 2007; Ye et al. 2007). Previous studies have shown that macrolide and sulfonamide antibiotics had the highest residue levels, followed by quinolone antibiotics (Lalumera et al. 2004; Yang and Carlson 2004; Santos et al. 2010). Therefore, the potential hazard of antibiotics upon aquatic ecosystems has attracted increasing attention among people.

Quinolone antibiotics have been widely used in human and animal disease therapeutics as well as agricultural industry because of their very broad spectrum antimicrobial activity and high efficacy. Their environmental behavior and ecological effects are of increasing concern. Levofloxacin (LEV) is the levo isomer of ofloxacin, one of the representatives of quinolone antibacterial drugs. It kills bacteria by inhibiting key bacterial enzymes (DNA gyrase and topoisomerase IV)

involved in unwinding the DNA helix for replication and transcription (Robinson et al. 2005). Robinson et al. (2005) investigated the toxicity of levofloxacin to five aquatic organisms but only determined the EC_{50} value. Pan et al. (2009) reported the effects of levofloxacin hydrochloride (LH) on the photosystem II activity and heterogeneity of *Synechocystis* sp.; the results suggested that O_2 evolution and the photosystem II (PSII) activity were clearly inhibited by LH. These studies showed that LEV has adverse effects on aquatic organisms.

Recently, the frequent outbreak of cyanobacterial blooms has become a ubiquitous phenomenon in freshwater ecosystems, leading to serious harm to fishery, ecological environment, and human health. Cyanobacterial blooms have become one of the important environmental problems. Formation of cyanobacterial blooms is normally regulated by various factors, including eutrophication, light intensity, trace metals, temperature, and salinity (Jiang et al. 2008; Davis et al. 2009). However, the correlation between the residue of antibiotics in water and the outbreak of algal blooms has not been reported. Thus, in this paper, we investigated the effects of LEV on growth in a cyanobacterium (*Microcystis flos-aquae*). *M. flos-aquae* is a common freshwater alga that causes water bloom. Also, physiological and biochemical parameters including chlorophyll *a* content, superoxide dismutase (SOD) and catalase (CAT) activities, malondialdehyde (MDA) content, F_v/F_0, and F_v/F_m were analyzed. The objective of the present work is to study the effects and the physiological and biochemical mechanisms of antibiotics to a cyanobacterium. Results from this study will help us evaluate the effects of antibiotic residues to the frequent outbreak and die out of cyanobacterial blooms.

Material and methods

The algae material and culture condition

M. flos-aquae (FACHB-1028) was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology (FACHB-Collection), Wuhan City, China. The algae were grown in 250-mL Erlenmeyer flasks containing 100 mL of BG11 medium, cultivated at 25±1 °C and 50 μmol photons m^{-2} s^{-1} illumination (cool white fluorescent tube) with a 12-h light/dark cycle. In order to reduce any effect caused by minor difference in photon irradiance, the flasks were shaken manually three times each day and rearranged randomly.

Antibiotic treatment

LEV was purchased from Beijing Jorferin Bio-Technology Co., Ltd, Beijing, China, with purity >98 %. The stock solution was prepared with sterilized water and then diluted to various test concentrations before use. When the algae were at the logarithmic growth phase, 1 mL of sterilized water or LEV in various concentrations was added into the algal medium to make final concentrations of 0, 0.001, 0.01, 0.1, 1, 10, 40, 70, and 100 μg L^{-1}. The algal medium with 0 μg L^{-1} was used as the control. Each test concentration was replicated three times, and all operations were carried out under sterile conditions to avoid contamination from bacteria. All cultures were cultivated according to the culture condition described above.

Algal growth rate

Cells were counted everyday using a hemocytometer under a microscope (Olympus CX41, Japan). Meanwhile, the optical density OD_{680} was also measured everyday using a spectrophotometer (UV1800, Shimadzu, Japan). The linear relationship was built up between cell density and optical density, and the result showed that the relevant index $R^2>0.99$, which is well in agreement with the previous report (Ge et al. 2010). The growth inhibition ratio was calculated as follows:

$$\mathrm{IR} = (1-N/N_0) \times 100\%$$

where N is the cell count of the treated group, N_0 is the cell count of the control group, and IR is the inhibition ratio.

Chlorophyll a content and photosynthetic activity

Chlorophyll *a* content and fluorescence parameters were determined using a pulse amplitude-modulated fluorometer (Phyto-PAM Walz, Effeltrich, Germany). In this study, chlorophyll *a* was recorded everyday, and fluorescence parameters were measured every other day. Reviews of fluorescence measurements are given by Schreiber et al. (1994). The minimal fluorescence yield F_0 and the maximal fluorescence yield F_m were measured after the samples were dark-adapted for at least 15 min. As a measure of the chlorophyll *a* concentration, the fluorescence was at an irradiance of 32 μmol photons m^{-2} s^{-1} PAR. Based on such measurements, the photosynthetic activity parameters can be formulated mathematically as (Rohacek and Bartak 1999)

$$F_v/F_m = (F_m-F_0)/F_m$$

$$F_v/F_0 = (F_m-F_0)/F_0$$

Analysis of antioxidant responses

The algal cells from 80 mL of culture medium were harvested after 7 days of exposure to LEV and centrifuged at 5,000*g* at 4 °C for 15 min, after which the supernatant was discarded. Collected algae were washed twice with 5 mL of buffer

(50 mM potassium phosphate and 150 mM potassium chloride, pH 7.5). The algae were then transferred into 5 mL of buffer solution, ground at 4 °C with quartz powder, and then centrifuged at 12,000*g* at 4 °C for 10 min (Geoffroy et al. 2003). The supernatant was used for enzyme activity assays.

The SOD activity was determined by the nitro blue tetrazolium (NBT) photochemical reduction method (Giannoplitis and Ries 1977). One unit of SOD activity was defined as the amount of enzyme that caused a 50 % decrease of the SOD-inhibited NBT reduction. The activity of SOD was expressed as units per cell. The activity of CAT was measured by the method of Goth (1991). One unit of CAT activity was defined as the amount of enzyme which degraded 1 mmol H_2O_2 per minute at 37 °C. The activity of CAT was expressed as units per cell. The content of MDA was determined by the thiobarbituric acid method as described by Hegedüs et al. (2001) and expressed as nanomoles per cell.

Statistical analysis

All data presented were expressed as mean ± standard deviation. Statistical analysis was performed using SPSS statistical package version 17.0. One-way analysis of variance (ANOVA) followed by the least significant difference test and Tukey's test was used to establish differences between the control group and treatments. The difference from the control group was considered significant at $P<0.05$ or very significant at $P<0.01$. All figures were produced using Origin 7.5.

Results

Algal growth rate

The effects of LEV on the growth curve and inhibition ratio of *M. flos-aquae* are depicted in Fig. 1a, b. As shown in Fig. 1, at 0.001–0.1 μg L^{-1} of LEV, the inhibition ratio of *M. flos-aquae* was negative, the growth was significantly stimulated ($P<0.05$), and the promotion began to decrease with further increases in the concentration of LEV but still higher than the control. After 7 days of exposure, the average increments over the control values were 4.99, 3.13, and 1.52 %, respectively. The growth of *M. flos-aquae* slightly increased at the concentration of 1 μg L^{-1}, but not obvious ($P>0.05$). At 10 μg L^{-1} of LEV, the growth of *M. flos-aquae* increased firstly and then decreased. A statistically significant inhibition of growth occurred when the concentrations of LEV were equal to and higher than 40 μg L^{-1}; the inhibition ratio increased in a concentration-dependent manner. At the concentration range of 10–100 μg L^{-1}, the inhibition ratios were 11.92, 81.57, 83.22, and 88.38 % at 7 days of exposure, respectively. On the whole, the stress on *M. flos-aquae* by LEV increased with increasing concentration of LEV, and the inhibition presented a concentration-dependent trend.

Chlorophyll a content

The effect of different concentrations of LEV on the chlorophyll *a* content of *M. flos-aquae* is depicted in Fig. 2. The content of chlorophyll *a* showed a significant increase ($P<0.01$) compared with the control during 1–7 days of exposure to the LEV concentration of 0.001 μg L^{-1}. There was a slight increase of chlorophyll *a* content by LEV at 0.01–1 μg L^{-1}; the degree of increase gradually declined with time delays but remained higher than the control. The chlorophyll *a* content in response to LEV was inhibited at the test concentration of 10 μg L^{-1} after 6 days of exposure, showing a significant decrease ($P<0.01$) compared with the control. A statistically significant inhibition of chlorophyll *a* content occurred when the concentration of LEV was equal to and higher than 40 μg L^{-1}, and the inhibition ratio went up to 73.07, 76.28, and 85.6 % after 7 days of exposure, respectively.

Photosynthetic activity

The F_v/F_m and F_v/F_0 ratios of photosystem II were determined to assess the impact of LEV on the maximum

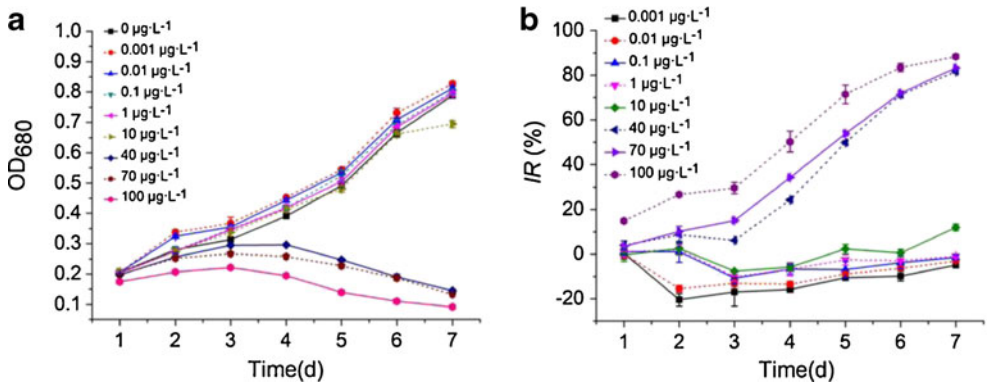

Fig. 1 Effects of levofloxacin on the growth curve (**a**) and inhibition ratio (**b**) of *M. flos-aquae*. Data shown are mean values±SE ($n=3$). *Error bars* indicate the standard deviation

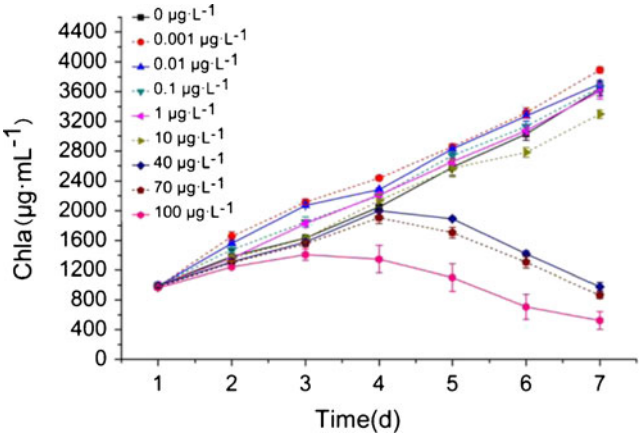

Fig. 2 Effects of levofloxacin on the chlorophyll *a* content of *M. flos-aquae*. Data shown are mean values±SE ($n=3$). *Error bars* indicate the standard deviation

photosynthetic capacity and the potential vitality of *M. flos-aquae* (Rohacek and Bartak 1999). As shown in Fig. 3a, b, the F_v/F_m and F_v/F_0 ratios increased slightly with low LEV concentrations (≤1 μg L^{-1}) during 1–7 days of exposure, only showing a significant increase at the third day ($P<0.01$). The F_v/F_m and F_v/F_0 ratios of *M. flos-aquae* showed an increase of 5.72 and 12.57 % relative to the control at the test concentration of 0.001 μg L^{-1} after 7 days of exposure. At a concentration range of 40–100 μg L^{-1}, the value of F_v/F_m and F_v/F_0 decreased sharply after 3 days of exposure. The F_v/F_m and F_v/F_0 ratios in 10 μg L^{-1} LEV exposure increased firstly and then decreased, showing a significant decrease below the control after 5 days of exposure ($P<0.01$).

The activities of CAT and SOD

To better understand the biochemical basis of resistance in *M. flos-aquae* caused by LEV exposure, SOD and CAT activities were measured, and the results are displayed in Fig. 4. The CAT activity was induced after *M. flos-aquae* was exposed to different concentrations of LEV (Fig. 4a) and showed a dose-dependent relationship. There were significant increases in the CAT activity by LEV at higher concentrations (10–100 μg L^{-1}), which were 5.47, 7.65, 25.09, and 29.44 times the control, respectively. As depicted in Fig. 4b, the SOD activity showed no significant difference with the control at low levels (0.001–10 μg L^{-1}) of LEV exposure, whereas LEV at high levels (40–100 μg L^{-1}) could significantly ($P<0.01$) stimulate the SOD activity, which increased to 1.32, 2.65, and 3.07 times the control, respectively.

MDA content

In the present study, the effect of LEV on the MDA content of *M. flos-aquae* was shown in Fig. 5. The MDA content of each treatment group was stimulated in almost all treatments to LEV with different concentrations. The MDA content increased with increasing LEV concentrations, that is, increased lipid peroxidation. Exposure of LEV at a lower concentration (<1 μg L^{-1}) augmented the MDA content slightly. However, significant increments in MDA level were observed at 10 μg L^{-1} and higher concentrations, showing a dose-dependent trend. When the concentration of LEV was 100 μg L^{-1}, the MDA content was 3.84 times the control.

Discussion

Effects of LEV on the growth of *M. flos-aquae*

The present study indicated that LEV had a dual effect (promotion and inhibition) on the growth of *M. flos-aquae*. The growth was promoted by LEV at lower concentrations whereas inhibited at higher concentrations. Currently, this "low-promoting and high-repressing" phenomenon has been reported. Florfenicol was found to stimulate marine diatom *Skeletonema costatum* growth at concentrations of 0.5, 1.0, and 2.0 mg L^{-1} and significantly inhibit algal growth higher than 2.0 mg L^{-1} (Liu et al. 2012). Tetracycline at 0.5–10 mg L^{-1} could stimulate seed germination, cell mitotic division, and growth of wheat seedlings. However, tetracycline at high concentrations (10–300 mg L^{-1}) could significantly inhibit these parameters in a concentration-dependent manner (Xie et al. 2011). LEV at

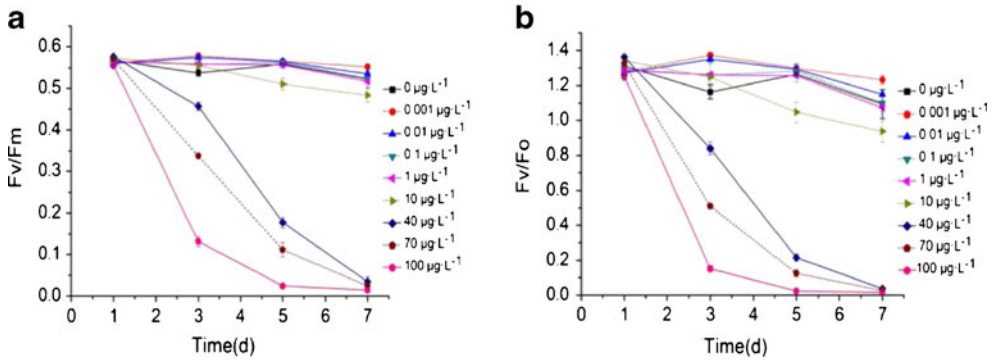

Fig. 3 Effects of levofloxacin on F_v/F_m (**a**) and F_v/F_0 (**b**) of *M. flos-aquae*. Data shown are mean values±SE ($n=3$). *Error bars* indicate the standard deviation

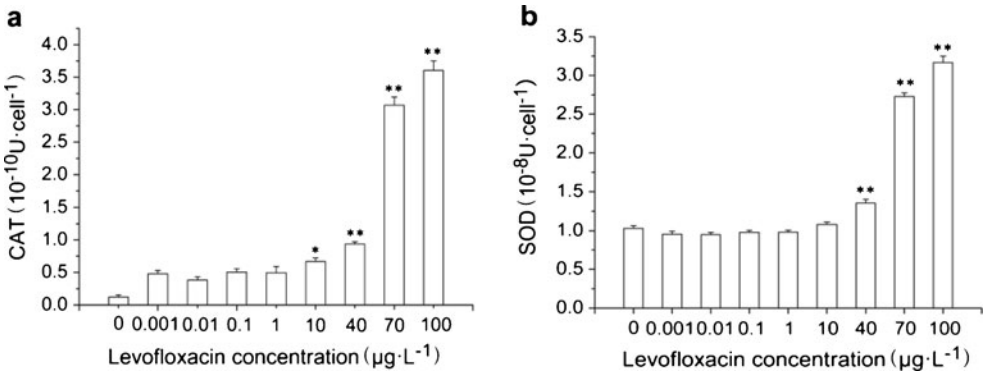

Fig. 4 Effects of levofloxacin on SOD (**a**) and CAT (**b**) activities of *M. flos-aquae*. Data shown are mean values±SE ($n=3$).*$P<0.05$ (statistically significant difference), **$P<0.01$ (statistical significance), when compared to the control (0 μg L^{-1}). *Error bars* indicate the standard deviation

lower concentrations (≤1 μg L^{-1}) could promote the growth of *M. flos-aquae*, meanwhile the chlorophyll *a* content was also increased; this may result from certain enzymes involved in some physiological and biochemical reactions that were induced by the stress of LEV at certain concentrations. Moreover, it is possible that the algae may partially degrade LEV and absorbed LEV as nutrients instead of toxic xenobiotics (Yue et al. 2006). However, the precise mechanism remains to be investigated. At higher concentrations, LEV had inhibitory effects on the growth of *M. flos-aquae* (a possible reason is that the concentration of LEV exceeds the tolerance limit of algal cells), the cell structure begins to crack and disintegrate, and algal cell growth is in the state of zero growth or negative growth, which resulted in growth that cannot be fully restored (Nie et al. 2007).

Effects of LEV on the chlorophyll a content and photosynthetic activity of *M. flos-aquae*

Plant photosynthesis is the process of converting solar energy into chemical energy. It is known that chlorophylls play a key role in all aspects of primary photosynthesis, including light harvesting, energy transfer, and light energy conversion. Chlorophyll *a* content can be used to estimate the primary productivity and also is an important symbol of vitality of phytoplankton. Under a certain environmental stress, the chlorophyll *a* content of microalgae will also be affected (Liu et al. 2011; Qian et al. 2012). At lower concentrations of LEV, the synthesis of chlorophyll *a* was stimulated, which may have promoted the photosynthesis of algae. However, when LEV exceeded a certain range, which is over the self-adjustment range of algae, the inhibitory effects gradually increased with the increase of LEV dose, algal cell structure was damaged, algae liquid turned white, and chlorophyll *a* content decreased, affecting the light harvesting and finally may lead to the photosynthesis pathway being blocked. The possible reasons for the decrease of chlorophyll *a* content may be as follows: Firstly, antibiotic stress caused thylakoid membrane disintegration, which resulted in the loss of chlorophylls from the tissue (Gao et al. 2007). Secondly, the accumulation of intracellular reactive oxygen species caused the cell structure to be damaged and the chlorophyll synthesis to be blocked (Geoffroy et al. 2003). Thirdly, antibiotics directly combined with some ingredients of algae, which inhibited the synthesis of the light-harvesting chlorophyll *a*/*b* protein complex and resulted in reduced the efficiency of energy transformation (Alberte et al. 1981).

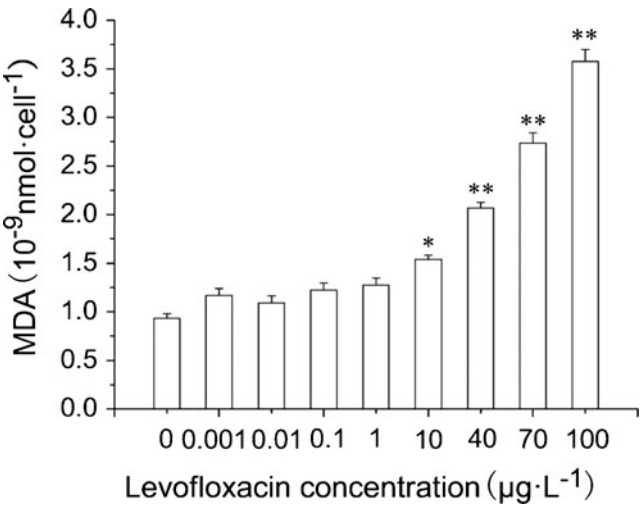

Fig. 5 Effects of levofloxacin on the MDA content of *M. flos-aquae*. Data shown are mean values±SE ($n=3$).*$P<0.05$ (statistically significant difference), **$P<0.01$ (statistical significance), when compared to the control (0 μg L^{-1}). *Error bars* indicate the standard deviation

Chlorophyll fluorescence is closely linked with photosynthesis processes; it can not only reflect light absorption, excited energy transfer, and photochemical reactions of photosynthesis primary reaction process but is also connected with electron transfer, the establishment of proton gradient, ATP synthesis, and CO_2 fixation. Thus, the chlorophyll fluorescence parameters can reflect changes in photosynthesis (Han et al. 2003). Adversity stress affects the photosynthesis of algae. The maximum quantum yield (F_v/F_m) indicates the maximum photochemical efficiency of PSII. The F_v/F_0 ratio is a useful parameter in assessing the potential photosynthetic activity of PSII (Krause 1988). Under normal conditions, F_v/F_m and F_v/F_0 remained relatively stable, freed from the influence of species and growing conditions, but changed significantly under stress. F_v/F_m and F_v/F_0 decreased which

meant that plants were photoinhibited (Razinger et al. 2007; Xing et al. 2010). In this study, the F_v/F_m and F_v/F_0 ratios of *M. flos-aquae* increased slightly with low LEV concentrations (≤1 μg L^{-1}) during 1–7 days of exposure, only showing a significant increase at the third day ($P<0.01$). The F_v/F_m and F_v/F_0 ratios were induced at the test concentration of 0.001 μg L^{-1} after 7 days of exposure. The results suggested that the low concentrations of LEV improved the maximum photochemical efficiency and the potential photosynthetic activity of PSII. The F_v/F_m and F_v/F_0 ratios increased and then decreased at the concentration of 10 μg L^{-1}, may be because *M. flos-aquae* rapidly started the synthesis of chlorophyll *a* to resist environmental stress during 1–3 days of exposure to LEV, increased the cell density, and accelerated the process of photosynthesis, but the inhibitory effect surpassed the defense effect with time delay; therefore, the F_v/F_m and F_v/F_0 ratios started to decrease significantly after 5 days of exposure. At higher concentrations (40–100 μg L^{-1}), F_v/F_m and F_v/F_0 were significantly inhibited, which implied that the primary light energy conversion efficiency of PSII was reduced, damaged the potential active center, blocked the primary reaction process of photosynthesis, and finally inhibited the growth of algae. This may be ascribed to higher concentrations of LEV that interrupted the photosynthetic electron transport between Q_A and Q_B, thus forming more Q_B-non-reducing PSII reaction centers, which resulted in the reduction of PSII reaction center oxygen evolution (Leu et al. 2002; Liang et al. 2006).

Effects of LEV on CAT and SOD activities and MDA content of *M. flos-aquae*

Under normal growth conditions, the generation and scavenging of reactive oxygen species (ROS) in plants are maintained in a dynamic equilibrium level, but under adverse conditions, this balance will be broken, which results in a large number of ROS generation and accumulation, triggers damaging effects to the membrane system of the organism, and finally inhibits growth (Tang and Li 2000). SOD and CAT are important enzymes associated with antioxidative stress in plants. SOD is an important antioxidative enzyme with free radicals as substrate, which can convert O^{2-} to H_2O_2 and O_2, thereby preventing the generation of superoxide anion radicals. CAT can catalyze H_2O_2 into H_2O and O_2 in order to alleviate the oxidative damage caused by H_2O_2. SOD and CAT make up the oxidation and antioxidant defense system which is indispensable for organisms for defense against the toxic effect of oxygen free radicals (Tripathi et al. 2006). At lower concentrations (≤10 μg L^{-1}) of LEV, the CAT activity showed a slight increase, whereas the activity of SOD slightly decreased, both showing no significant differences with the control. This illustrated that *M. flos-aquae* tolerated LEV by other mechanisms, the low concentrations of LEV did not stimulate the antioxidant system to scavenge ROS, or ROS produced by LEV did not obviously affect the metabolisms of algae (Xie et al. 2011). The SOD activity increased significantly ($P<0.01$) after exposure to 40 μg L^{-1} or higher concentrations of LEV. This may be attributed to the overproduction of superoxide, which is considered as the central component of the signal transduction, resulting in the activation of existing enzyme pools or increased expression of genes encoding SOD (Foyer et al. 1997; Mishra et al. 2006; Xie et al. 2011). In concurrence, the activity of CAT significantly increased, which was considered as an adaptive trait against the damage caused by oxidative stress (Rasheed and Mukerji 1991). In the present study, the activities of SOD and CAT were stimulated after exposure to higher concentrations of LEV and increased with increasing dose, indicating that the algal cells were under oxidative stress, resulting in the overproduction of ROS. However, the argument of SOD and CAT activities is still not enough to scavenge the ROS in algae, finally contributing to the inhibition of algal growth. A similar phenomenon was observed in the effect of tetracycline exposure on the growth of wheat (*Triticum aestivum* L.) (Xie et al. 2011) and the response of *Chlorella vulgaris* to trichloroisocyanuric acid (Nie et al. 2008).

MDA is an oxidized product of membrane lipids, and the content of MDA is commonly considered as an important index of lipid peroxidation to reflect cellular oxidative damage under environmental stress (Bailly et al. 1996; Chaoui et al. 1997). In the present investigation, the MDA content in algae exposed to 10 μg L^{-1} or higher concentrations of LEV was significantly higher than that in the control ($P<0.01$). The possible reason is that under the stress caused by higher concentrations of LEV, the balance between production and scavenging of ROS in algal cells was disturbed, resulting the overaccumulation of ROS, which triggered the peroxidation of membrane lipids, increased lipid peroxidative products and oxidative stress in algae, damaged the membrane systems and functions, and increased the membrane permeability, finally leading to the death of cells (Alaiz et al. 1999). However, there was a slight increase in the MDA content at LEV concentrations lower than 1 μg L^{-1}. The relatively low MDA content indicated less oxidative stress, which may account for no inhibition of algal growth (Xie et al. 2011).

Conclusion

The present study investigated the effects of LEV on *M. flos-aquae* and its mechanism. The results indicated that LEV could promote the growth of *M. flos-aquae* at lower concentrations and inhibit it at higher concentrations. After exposure to higher concentrations of LEV, the chlorophyll *a* content and photosynthetic activity decreased significantly, and the SOD and CAT activities and MDA content also increased

obviously. These results may confirm that higher concentrations of LEV can cause photosynthesis inhibition and serious oxidative stress on *M. flos-aquae.* However, at lower concentrations, the photosynthetic activity increased slightly and the SOD and CAT activities and MDA content were not obviously different from those of the control. Therefore, further studies should be conducted to understand the mechanism of promotion on algae by lower concentrations of antibiotics.

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