The Response of *Microcystis aeruginosa* to the First, Second and Third Exposure of Ofloxacin

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Abstract. In recent years, the problem of antibiotics abuse has been serious and aroused the attention of the scholars around the world. The effects of ofloxacin’s first, second and third exposure to the algae *Microcystis aeruginosa* were studied in this paper. The results indicated that the growth of *Microcystis aeruginosa* was inhibited by the first and repeated exposure of ofloxacin (OFC) in different degrees. The algae showed obvious resistance to OFC in second and third exposure, and the treatment with higher concentration (500μg/L) of OFC to algae could encourage higher resistance. The chlorophyll fluorescence and photosynthetic pigment contents varied with a relation of dose effect response. The OFC displayed inhibition for the synthesis of soluble protein. The content of malondialdehyde and the activity of SOD increased with the increase of OFC concentration. Since the SOD was more active in second and third exposure, the increase of SOD may be one of mechanisms for the resistance of algae to OFC.

1. Introduction

Antibiotics as one of the common pharmaceutical and personal care products are a kind of compounds generated by organisms like fungi, actinomycetes and bacteria in the metabolic process to inhibit growth of other microorganisms. The antibiotics can inhibit the synthesis of cell wall, cell membrane and protein, and disturb the transcription of RNA and the replication of RNA and DNA. They can also promote the formation of the anti-metabolites lysozyme to achieve the goal of bacteriostatic. Ofloxacin (OFC) is one of the most commonly used antibiotic of quinolones, and has been widely used on human, animals and plants disease treatment. However, because of the abuse, antibiotic residues have been detected in water environment, which can impact on aquatic organisms and water ecosystem, finally threat the water ecological security.

The researches on the effects of antibiotics to algae are mostly focused on the acute toxicity for just once exposure. Compared to cyanobacteria, green algae had been paid more attention. Despite the short half-life of some antibiotics, a large amount of antibiotics inputting to the environment can cause the phenomenon of pulse pollution. The study on response of micro algae to antibiotics repeatedly exposure is very necessary. As cyanobacteria are prokaryotes, the antibiotic can act on algae cells with antibacterial mechanism, so the cyanobacteria are more sensitive to antibiotics than green algae.
Microcystis aeruginosa as a kind of bloom-forming cyanobacteria abound in eutrophic water is widely used in the study of aquatic ecotoxicology.

In order to evaluate ecological risks of ofloxacin to algae, primary and repeated exposure of OFC with different concentrations to Microcystis aeruginosa were studied.

2. Materials and methods
Microcystis aeruginos (FACHB-524) was purchased from Freshwater Algae Culture Collection at the Institute of Hydrobiology. The algae were cultivated with BG11 medium in illumination incubator under the conditions as: the temperature of 25 °C, the light intensity of 2000 lux and the photoperiod of light to dark at 12h: 12 h. The algae were inoculated in fresh medium each week and algae in logarithmic phase were carried out for toxicity experiments.

The apparatus and BG11 medium were used in experiments after sterilization at 121 °C, under 0.105 MPa. The algae were added to a series of 250mL Erlenmeyer flasks under sterile conditions with the same concentration, subsequently different concentrations of OFC solutions were mixed after filtered by 0.22 μm filters. The final concentrations of OFC in solutions were 0, 0.1, 0.2, 0.5, 0.75, 1 and 2 mg/L respectively. The flasks were shaken three times a day and randomly changed the position in the incubator. The algae dealed with OFC were taken out of previous solution in the sterile environment by centrifugation and then inoculated in clean BG11 medium without antibiotics after washed with sterile phosphate buffer solution three times. The second exposure experiment were conducted as the first one after the algae cultivation five days. The procedure of the third exposure of OFC were similar to the second exposure.

The microalgae biomass and Fv/Fm values were measured every 24h using spectrophotometry and Handy-PEA respectively. The contents of photosynthetic pigments, malondialdehyde (MDA) and soluble protein, as well as superoxide dismutase (SOD) activity were determined at 96h. The determination for content of photosynthetic pigments was based on alcohol extraction method. The MDA content and SOD activity were analyzed by reference [1], and the soluble protein content was tested followed referenced [2].

3. Results and Discussion

3.1. Effects of OFC on growth of Microcystis aeruginosa
The effects of algal growth of the initial exposure of OFC have been shown in figure 1 (a). Along with the increase of the concentration of OFC, the inhibition rates on algae increased significantly, and displayed obvious dose and time effects. The reason was speculated that when algae was exposed to OFC of low concentration, the number of cells could still stably increase as the extension of exposure time, and each cell experienced low stress from OFC, so that the growth speed was steady even greater than the control groups. However, the growth of algae cells in high concentration OFC groups were significantly inhibited, as the forces caused by OFC exceeded the tolerance limits of cells. When the algae grew on the force of 0.1 and 0.5 mg/L OFC resuspended in the same OFC solutions as the first exposure, the inhibition rates also increased with the increase of OFC concentration, but were distinctly lower than the ones in initial (as shown in figure 1 (b) and (c)). The inhibition rates of OFC to the algae treated by 0.1mg/L OFC twice were all below 20% except for the concentration of 2mg/L. The 96h-EC$_{50}$ were 1.07mg/L for initial exposure, 1.93mg/L for second exposure after treatment by 0.1mg/L OFC, 5.26mg/L for 0.5mg/L OFC and 12.68mg/L for third one, indicating that the resistance of algae to OFC enhanced through three times’ treatment.
Figure 1. The effects of OFC in the first exposure(a), the second exposure after 0.1mg/L OFC treatment (b), the second exposure after 0.5mg/L OFC treatment (c) and the third exposure after 0.1mg/L OFC’s second exposure (d) on the growth inhibition rates of Microcystis aeruginosa.

3.2. Effects of OFC on photosynthesis of Microcystis aeruginosa

The effects of OFC on the photosynthetic pigment contents of algae presented dose-effect as growth inhibition. The treatment of higher concentration of OFC to algae may bring out irreversible damages, as contents of chlorophyll a and carotenoid in the groups of second exposure by 0.1mg/L OFC were both higher than in the initial exposure, whereas the contents of two pigments for second exposure by 0.5mg/L OFC were lower. Moreover, the activities of two photosynthetic pigments in third exposure were both higher than that of primary and second exposure, especially in solutions with high concentration of OFC (> 0.5mg/L). In three times’ exposure, the effects of chlorophyll a content by OFC were more significant than carotenoids. The reason may be carotenoids could not only play an important role in photosynthesis but also absorb the remaining light and quenching reactive oxygen, thus preventing membrane lipid peroxidation and protecting chlorophyll and photosynthetic function.
Figure 2. The effects of OFC on the contents of chlorophyll a (a) and carotenoid (b) on *Microcystis aeruginosa*. E1 presents the results of the initial exposure, E2-1 presents the second exposure after 0.1mg/L OFC treatment, E2-2 presents the second exposure after 0.5mg/L OFC treatment and E3 presents the third exposure after 0.1mg/L OFC’s second exposure.

Chlorophyll fluorescence kinetics is a kind of novel determination and diagnosis technology using chlorophyll in plants as the probe to explore plant photosynthetic physiology and the subtle influence of various kinds of external factors. It has the advantages of rapid, sensitive and no damage to the cell. The Fv/Fm is the most commonly used parameters in fluorescence analysis. When the microalgae suffers from high temperature or heavy metal pollution, chlorophyll fluorescence parameters reflecting the algae photosynthesis intensity will be affected and the values of Fv/Fm will decrease with the treatment time increasing. The effects of OFC on Fv/Fm of algae were shown in figure 3. The values of Fv/Fm decreased with the increase of antibiotic concentration in the initial exposure and displayed a significant concentration and time-effect relationship. In the second stress of 0.1mg/L OFC, the values of Fv/Fm at 96h in the groups with 0.2mg/L OFC had a certain degree of recovery. The second stress of 0.5mg/L OFC had the similar patterns with initial one. In the third time of exposure of 0.1mg/L
OFC, Fv/Fm expressed no significant differences with control, except for 2mg/L OFC. The changes of Fv/Fm brought by OFC treatment could be discovered at 24h, illustrating Fv/Fm was more sensitive than other indicators like growth rated and chlorophyll pigments.
3.3. Effects of OFC on MDA of Microcystis aeruginosa

MDA is an important indicator for the degree of lipid oxidation in algae cells. The results of effects of OFC with different concentrations on MDA contents to Microcystis aeruginosa were shown in Figure 4 (a). In initial exposure, the MDA contents of microalgae showed increasing trend with the increasing of OFC concentration. The reason was conjectured that after exposure to OFC, a large amount of reactive oxygen species (ROS) were produced in the solution leading to serious membrane lipid peroxidation of micro algae, and then damaged the membrane structure and function, finally affected the normal cells’ growth. The MDA contents in the second and third exposure were both lower than the initial, indicating that the levels of lipid peroxidation caused by OFC decreased. It can be seen that a certain mechanism
of algae resistance to the presence of OFC has been produced in the exposure, and the processes of membrane lipid peroxidation can be inhibited by the algae’s own regulation.

3.4. Effects of OFC on soluble protein of Microcystis aeruginosa
Protein is an important structural substance of organisms and can act as a catalyst in cell metabolism. Therefore, the change of protein contents can indirectly reflect whether the metabolic activities are normal. The effects of different concentrations of OFC on the soluble protein contents of the Microcystis aeruginosa were performed in Figure 4(b). The synthesis of soluble protein in algae was inhibited in the treatment of OFC, and its content decreased with the increase of the concentration of OFC. Although compared with the initial exposure, the number of algal cells in second exposure by 0.5mg/L OFC and third exposure by 0.1mg/L OFC was higher in the OFC solution with same concentration, the soluble protein content significantly reduced in the second and third treatment, explaining that the OFC may cause irreversible damage to some protein of Microcystis aeruginosa.

3.5. Effects of OFC on SOD of Microcystis aeruginosa
The effects of different concentrations of OFC on superoxide dismutase (SOD) activities were demonstrated in Figure 4 (c). In the initial exposure, there was no significant difference between the treatment group and the blank with low concentration of OFC, while the SOD in the high concentration groups increased with the increase of concentration. This indicated that large amounts ROS were produced under the exposure of OFC and the algae cells could improve the activity of SOD to eliminate excessive ROS. The changes of SOD activity in second and third exposure followed a similar pattern as the initial one, and both showed the concentration- effect. Moreover, the SOD activities were higher in second exposure than the initial, and this demonstrated that the algae may gain a certain mechanism to increase the activity of SOD in OFC solution with the same concentration for inhibition of membrane lipid peroxide process through the early exposure of OFC, thereby reduce cell damage by ROS. However, in third exposure, when the concentration of OFC was greater than 0.2mg/L, the SOD activities were lower than the second treatment, resulting from the tough stress of OFC to algae through three times’ treatment. The increase of SOD activity may be one of the reasons for the resistance of Microcystis aeruginosa to OFC.
Figure 4. The effects of OFC on MDA (a), soluble protein (b) and SOD (c) of *Microcystis aeruginosa*.

4. Conclusion
The effects of OFC with different concentration on the growth of *Microcystis aeruginosa* in the first, second and third exposure were studied in this paper. The impacts of OFC on growth inhibition showed obvious concentration - effect and time - effect relationship. The algae displayed resistance to OFC in second and third treatment. This kind of resistance was reflected in the experiments of the effects on growth, photosynthetic pigments, Fv/Fm, MDA and soluble protein. The activity of SOD was significantly increased in the second and third exposure, which may be one of the mechanisms of the resistance.

References
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