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Aquatic Toxicity of Antibiotic Contaminant Doxycycline Hydrochloride on Cyanobacterium *Microcystis aeruginosa*

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Abstract: Veterinary antibiotics have been used extensively in many countries to treat diseases and protect the health of animals. As they are poorly adsorbed in the gut of the animals, the majority of antibiotics are excreted unchanged in faeces and urine. Therefore, antibiotic contaminants in aquatic ecosystems may pose physiological effects on aquatic lives. In the present study, growth inhibition and oxidative damage in cyanobacteria *Microcystis aeruginosa* exposed to doxycycline hydrochloride were investigated. The results showed that doxycycline hydrochloride could inhibit the growth of *M. aeruginosa* under laboratory conditions. The inhibition percentages after 24 h, 48 h, 72 h and 96 h exposure at the concentration of 1 mg/L were 8.13%, 16.49%, 39.56% and 55.31%, respectively. The activities of superoxide dismutase (SOD), catalase (CAT), and the concentrations of malondialdehyde (MDA) in *M. aeruginosa* were stimulated by doxycycline hydrochloride after 24 h exposure under a series of concentrations. The results are useful for environmental assessment of antibiotics. Besides, it is also helpful for guiding the application of doxycycline hydrochloride in agricultural settings.

Key words: Cyanobacteria, doxycycline hydrochloride, oxidative stress, lipid peroxidation.

Introduction

The use of veterinary antibiotics has become integral of the growing animal food industry. For example, in the European Union, 4700 tons of antibiotics were administered to farm animals and 8500 tons to humans in 1999 (FEDESA, 2003). In China, 15,770 tons of antibiotics were used as non-prescription therapeutics in 2004 (Richardson et al., 2005). Due to incomplete absorption and metabolism, up to 85% of administered antibiotics including the parent compound and their metabolites may be excreted into the environment via animal manures and human wastes (Hartmann et al., 1998). They can be discharged into soil and water system through different exposure routes such as raining, surface runoff and infiltration. Eventually, they may pose adverse effects on aquatic lives and threaten human health. As one of the destinations of antibiotics, aquatic system requires a particular attention.

Water blooms caused by cyanobacteria in aquatic system are major global environmental issues in recent years. They can bring about serious environmental problems such as water anoxia, off-flavour in water bodies, and toxin release from toxic cyanobacterial strains (Graham et al., 2010; Pan et al., 2006; Veldhuis and Wassmann, 2005). Many studies have investigated the factors causing the blooms (Pliński and Jozwiak, 1999; Davis et al., 2009) and various approaches

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to deal with the environmental problems caused by cyanobacteria blooms (Harada et al., 1988; Hoeger et al., 2002). However, the physiological effects on cyanobacteria caused by environmental pollutants including antibiotics are insufficient. In China, tetracycline and other antibiotics have been detected widely in the Yangtze River, Yellow River and other water systems (Zou et al., 2011). The antibiotic contaminants in aquatic ecosystems may pose toxic effects on cyanobacteria in water reservoirs. Several studies have found that antibiotic contaminants could affect the growth and photosynthetic efficiency of *M. aeruginosa* at environmentally relevant concentrations (Halling-Sørensen, 2000; Van der Grinten et al., 2010).

In the present study, cyanobacterium *M. aeruginosa* was selected as a test organism to explore the ecotoxicity of doxycycline hydrochloride. *M. aeruginosa*, both on an absolute advantage in number and frequency, is not only the dominant species which leads to water bloom, but also a major source of microcystin. The growth inhibition and the response of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and lipid peroxidation indicator malondialdehyde (MDA) concentration after exposure to doxycycline hydrochloride were investigated. Doxycycline hydrochloride, also called deoxytetracycline or vibramycin, is the second generation of tetracycline antibiotics, and its antibacterial activity is two-fold of tetracycline (Chopra and Roberts, 2001). Doxycycline hydrochloride has a high biological activity and persistence that may pose potential ecological risks on nontarget organisms in the environment. Therefore, studying the toxic effects of doxycycline hydrochloride on this blue-green algae is important both for rational application of this kind of antibiotics and for ecological safety evaluation.

Materials and Methods

Chemicals and Cell Cultures

Doxycycline hydrochloride [(4S,4aR,5S,5aR,6R,12aS)-4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide hydrochloride] with chemical purities ≥ 98% were obtained from YuanYe Biotechnology Co. Ltd (Shanghai City, China).

Cyanobacterium *M. aeruginosa* was obtained from Institute of Hydrobiology, Chinese Academy of Sciences. The unicellular inoculant was cultured in sterile BG11 medium under an irradiance of 40 μmol/m^2·s and a photoperiod of 12 h light/12 h dark at 28 ± 1ºC. The strain *M. aeruginosa* is single-cell under laboratory conditions. The growth tests were conducted under different concentrations (0, 1, 2, 5, 10 mg/L) of doxycycline hydrochloride. Three replicates of each concentration were prepared in Erlenmeyer flasks (50 mL) containing 5 mL of algal inoculant and 45 mL of culture medium. The Erlenmeyer flasks were maintained at 28 ± 1ºC and a humidity of 60% in a culture chamber with alternation periods of light and dark (12 h/12 h). The irradiance was kept at 40 μmol/m^2·s. The initial algal density in every flask was (5.5–6.7) × 10^6 cells/mL. The linear equation (R^2 = 0.9992) between cell number and optical density of algal culture at 685 nm was established, and algal densities were tested every 24 h using UV/VIS spectrometer (TU-1810, China) to obtain the growth curves under different conditions.

Analysis of Exposure Concentrations

After the chemicals added into culture medium, triplicate culture samples were filtered through a 0.45-μm filter and analyzed by HPLC. The analyses were performed on a LC600 HPLC system (HW-001, Beijing, China) with a LabTech P600 high-pressure constant flow pump, and a UV600 plus UV/VIS detector. The operation conditions were a ODS (C18) column (4.6 mm × 250 mm, LabTech, Beijing, China), a flow rate of 1.0 mL/min, a mobile phase of methanol/water (50:50, v/v), a detection wavelength of 350 nm, an injection volume of 10 μL at room temperature.

Samples Preparation

Algal cells were concentrated by centrifugation at 1500 × g for 10 min at 4 °C and the cell pellets were transferred into 10 mL centrifuge tubes. The pellets were washed twice with sterilized media and recentrifuged. The recentrifuged cell pellets were resuspended in 2 mL of PBS solution (50 mM, pH 7.0) and homogenized by an ultrasonic cell pulverizer (Microson XL 2000, New York, USA) at 200 W for a total time of 2 min (ultrasonic time: 2 s; rest time: 8 s) while being cooled in an ice-bath. Then the homogenate was centrifuged at 1200 × g for 10 min at 4 °C. The cell-free enzyme extract supernatant was used for enzymatic and antioxidant measurements.

Analysis of Antioxidant Responses

The SOD activity assay was conducted at 550 nm using the method of Beauchamp and Fridovich (1971). The determination of MDA was performed at 532 nm using the thiobarbituric acid (TBA) reactive substances method of Hagege et al. (1990). The CAT activity assay

was conducted at 405 nm using the method of Góth (1991). After 24 h of treatments, activities of SOD and CAT and MDA concentrations were determined. We purchased the SOD assay kit, MDA assay kit and CAT assay kit (Jiancheng Bioengineering Institute, Nanjing, China) and conducted the measurements according to the handling instruction.

Data Analysis

Statistical analysis was performed using Origin 8.0 (Microcal Software, Northampt, MA, USA) and SPSS 16.0 (SPSS, USA) to determine the significance among the treatments. One-way analysis of variance (ANOVA) was used to determine the differences in the concentrations. $p < 0.05$ was considered statistically significant.

Results and Discussion

Exposure Concentrations of the Chemicals

For nominal exposure concentrations of 1, 2, 5 and 10 mg/L, the actual exposure concentrations of doxycycline hydrochloride were 0.93 ± 0.096, 1.69 ± 0.119, 4.16 ± 0.355 and 8.33 ± 0.724 mg/L, respectively.

Growth Inhibition in *M. aeruginosa*

Growth curves of *M. aeruginosa* exposed to doxycycline hydrochloride are illustrated in Figure 1. Apparently, the inhibition percentages were related to the compound concentration. After 24 h exposure, doxycycline hydrochloride slightly inhibited the growth of *M. aeruginosa* at all concentrations. After 48 h exposure, the inhibition percentages were 8.24%, 16.49%, 16.50% and 12.37% for 1, 2, 5 and 10 mg/L, respectively. After 72 h exposure, the inhibition percentages were 25.17%, 35.25%, 39.56% and 40.28% for 1, 2, 5 and 10 mg/L, respectively. After 96 h exposure, doxycycline hydrochloride significantly inhibited the growth of *M. aeruginosa*. The inhibition percentages were 36.17%, 51.06%, 55.31% and 56.38% for 1, 2, 5 and 10 mg/L, respectively. After 144 h exposure, the inhibition percentages were 45.68%, 63.84%, 67.67% and 70.22% for 1, 2, 5 and 10 mg/L, respectively.

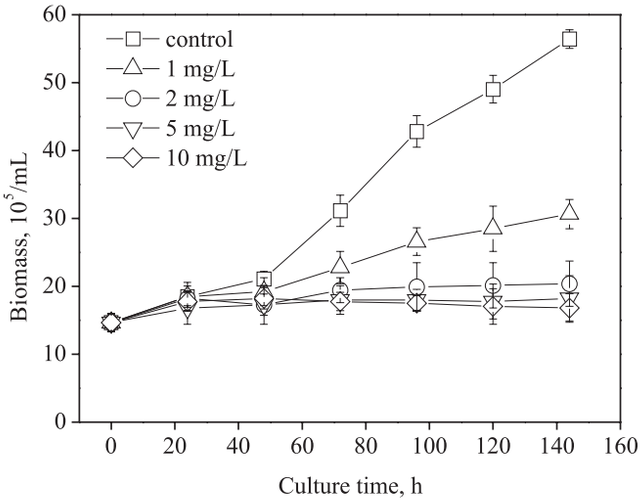

Figure 1. Growth curves of *M. aeruginosa* exposed to doxycycline hydrochloride.

Antioxidant Responses in *M. aeruginosa*

SOD activities in *M. aeruginosa* showed slight increases in response to doxycycline hydrochloride after 24 h exposure (Figure 2). The highest SOD activity was observed at the concentration of 5 mg/L, which showed an increase of 2.77% compared to the control. However, the activities of CAT in *M. aeruginosa* were significantly stimulated by doxycycline hydrochloride after 24 h exposure (Figure 3). At 1, 2, 5 and 10 mg/L, the increases were 2.25, 2.17, 2.11 and 2.94-fold compared to the control, respectively. Stimulation of MDA concentrations in response to doxycycline hydrochloride was also observed at 24 h exposure (Figure 4). At 1, 2, 5 and 10 mg/L, the stimulation percentages were 20%, 40%, 80% and 60%, respectively.

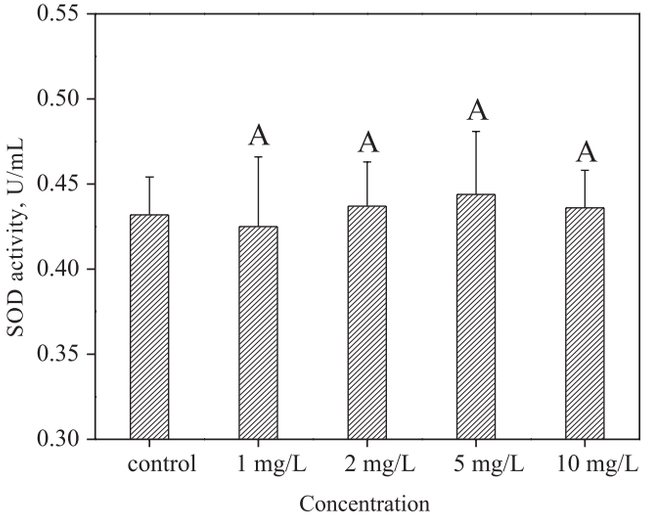

Figure 2. SOD activities in *M. aeruginosa* cells exposed to 0, 1, 2, 5 and 10 mg/L doxycycline hydrochloride after 24 h. Results are presented as mean ± SD of three independent assays. The capitalized letter 'A' indicates no significant differences ($p < 0.05$) among different exposure concentrations.

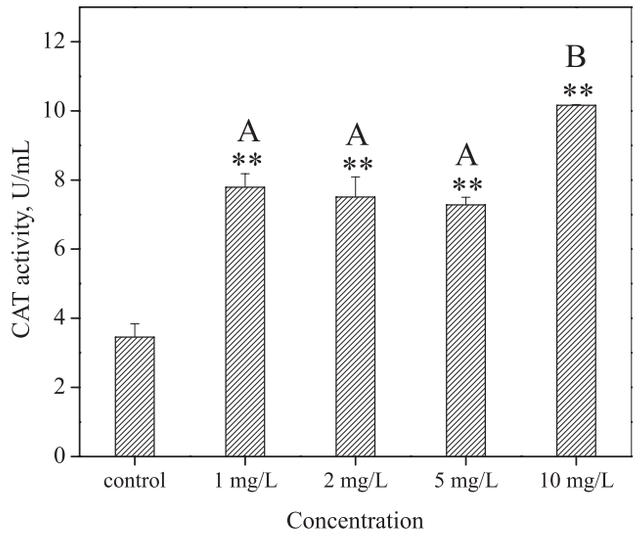

Figure 3. CAT activities in *M. aeruginosa* cells exposed to 0, 1, 2, 5 and 10 mg/L doxycycline hydrochloride after 24 h. Results are presented as mean ± SD of three independent assays (indicates $p < 0.01$ relative to the control by ANOVA). Different capitalized letters indicate significant differences ($p < 0.05$) among different exposure concentrations at 24 h, while the same letter indicates no significant differences.**

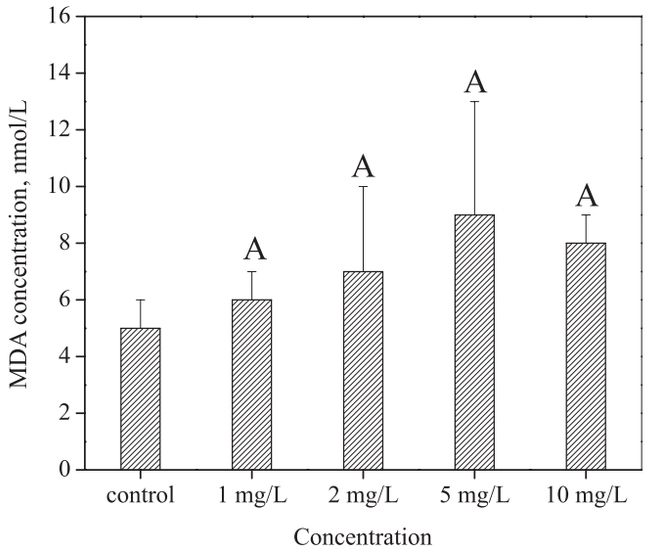

Figure 4. MDA concentrations in *M. aeruginosa* cells exposed to 0, 1, 2, 5 and 10 mg/L doxycycline hydrochloride after 24 h. Results are presented as mean ± SD of three independent assays. The capitalized letter 'A' indicates no significant differences ($p < 0.05$) among different exposure concentrations.

Toxicology of Doxycycline Hydrochloride

Antioxidant responses could alleviate the oxidative damage caused by environmental stress through scavenging reactive oxygen species (ROS) (Torres et al., 2008). SOD is normally regarded as the first defense against ROS among the antioxidant system (Alscher et al., 2002) and it converts superoxide radicals to hydrogen peroxide, which could be further eliminated by CAT (Cho and Seo, 2005). CAT is an antioxidant enzyme, which can remove H_2O_2 in cells by transforming it into non-toxic H_2O and O_2. Morelli and Scarano (2004) found that the activities of SOD and CAT in marine diatom were increased in response to the copper treatment. Liu et al. (2012) reported that the activities of SOD and CAT in amoxicillin-treated *M. aeruginosa* cells were intensified. Similar to the previous studies, the activities of SOD and CAT in the present study were also stimulated, indicating that the algal cells were under oxidative stress. At 1 mg/L, the SOD activity decreased slightly. In contrast, the CAT activity increased significantly.

It is possible that the CAT is more sensitive compared to the SOD. However, stimulation of SOD and inhibition of CAT were observed in *M. aeruginosa* exposed to doxycycline hydrochloride at test concentrations ranging from 2 to 5 mg/L. At 5 mg/L, the highest SOD activity and the lowest CAT activity were observed, which indicated that the hydrogen peroxide generated through the SOD catalytic reaction may be accumulated in the algal cells due to the inhibition of CAT. Compared to 5 mg/L, SOD activity decreased slightly while the CAT activity increased significantly at 10 mg/L. This may be resulted from the damage of antioxidant system and the stimulation of high concentration of hydrogen peroxide in algal cells. MDA concentration is an important indicator of lipid peroxidation that reflects cellular oxidative damage under environmental stress (Bailly et al., 1996). Several studies have reported that increased MDA concentration was commonly observed in cyanobacteria in response to various environmental stress, including herbicides (Ye et al., 2013), allelochemicals (Zhang et al., 2011), and antibiotics (Stoichev et al., 2011).

In the present study, MDA concentration was increased at the concentrations ranging from 1 to 5 mg/L, and the highest MDA concentration was observed at 5 mg/L. The results indicated that the doxycycline hydrochloride initiated lipid peroxidation in *M. aeruginosa*. Besides, the most inhibited CAT activity was observed at this concentration, which may also be responsible for the highest lipid peroxidation in doxycycline hydrochloride treated algal cells. At 10 mg/L, the MDA concentration decreased while the CAT activity increased significantly, it is possible

that doxycycline hydrochloride treated algal cells may have a lower biological activity compared to its initial biological activity.

Conclusions

According to the activities of SOD, CAT and MDA concentration, we can infer that doxycycline hydrochloride caused oxidative damage to *M. aeruginosa*. It also posed certain inhibition effects on the growth of *M. aeruginosa*. The results are helpful in understanding the aquatic toxicity of doxycycline hydrochloride on cyanobacterium. In addition, the application of tetracycline antibiotics requires more direct supervision and training.

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