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Effect of florfenicol and thiamphenicol exposure on the photosynthesis and antioxidant system of *Microcystis flos-aquae*

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ABSTRACT

Florfenicol (FF) and thiamphenicol (TAP) are two typical pharmaceuticals used widely as therapeutica antibiotic agents in aquaculture. However, little is known about the potential adverse effects of these two antibiotics on non-target organisms in the aquatic ecosystem. In this study we investigated the effects of FF and TAP on photosynthesis and the antioxidant system of the cyanobacteria *Microcystis flos-aquae*. Over a concentration range of 0.001–1 μg/L, the results showed that both FF and TAP significantly increased the chlorophyll a content of *M. flos-aquae*, while the superoxide dismutase (SOD) activity, catalase (CAT) activity and the levels of malondialdehyde (MDA) changed slightly. In contrast, the chlorophyll a content of *M. flos-aqua* was significantly inhibited ($p < 0.01$) at high concentrations (>1 μg/L) of FF and TAP, reaching a 46% inhibition level at 50 μg/L FF and 56% inhibition at 100 μg/L TAP. At the same time, the activities of SOD and CAT along with MDA content also increased significantly ($p < 0.01$), indicating that the high concentrations of both FF and TAP led to oxidative stress in the algae. In addition, the *M. flos-aquae* fluorescence parameters (Fv/Fm, Fv/Fo, alpha, ETRmax and Ik) increased with increasing concentration of both FF and TAP, which may be the result of the increasing photoprotection capacity.

1. Introduction

Since humans invented the penicillin in 1929, antibiotics have become the boon of human and animal health. Thus far, antibiotics have been extensively used in medicine, animal husbandry and aquaculture, and play a huge role in the prevention and control of human (Acker et al., 2016; Salimi et al., 2016), animal and fish diseases (Kołodziejska et al., 2013; Liu et al., 2012). They are also used in animal feed additives to promote animal growth (Wegener, 2003), among other things (Gothwal and Shashidhar, 2015). In 2013, approximately 162 000 tons of antibiotics were used in agriculture and medicine in China, of which 48% was for human use and 52% was for veterinary use (Zhang et al., 2015). However, a significant fraction is excreted in the parent and active metabolic forms in the urine and faeces (Kümmerer and Henninger, 2003). In intensive fish farms, approximately 70–80% of all the antibiotics used end up in the environment (Ye and Zhang, 2015). Therefore, the aquaculture industry has become a major source of antibiotic contamination in the water environment.

Among the many antibiotics, phenicol antibiotics are commonly used in animal husbandry and aquaculture (Zhang et al., 2015). These include florfenicol (FF), thiamphenicol (TAP) and chloramphenicol (CAP). CAP was the first synthetic antibacterial drug and can inhibit a variety of aerobic and anaerobic microorganism. However, the CAP toxicity in human bone marrow has been linked to blood disorders such as aplastic anaemia (Anadón et al., 1994). Many countries, including China, have classified it as prohibited drugs banned from use in animal husbandry and aquaculture (Sarmah et al., 2006). In terms of toxicity, florfenicol and thiamphenicol are the ideal drugs for the replacement of chloramphenicol and are approved for aquaculture (Sapkota et al., 2008). Due to their wide use, these antibiotics have been detected in various aquatic environments as well, including sewage, surface, ground and drinking waters, with detected concentrations ranging from nanograms per litre to micrograms per litre (Jia et al., 2011; Chen, 2015; Ou et al., 2013; Zhang et al., 2014). Hence, the ecological risk of antibiotics in the aquatic environment has attracted concern. There is limited information on the effects of florfenicol and thiamphenicol may have on the microalgae. In the study by Li (Li et al., 2012), florfenicol and thiamphenicol were found to inhibit the growth of *Chlorella pyenoidosa* in a time-and dose-dependent

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manner, with an EC_{50} (48 h) of 10 and 84 mg/L, respectively. Liu (Liu et al., 2012) indicated that florfenicol stimulates *S. costatum* growth at concentrations of 0.5, 1.0 and 2.0 mg/L, and significantly inhibits *S. costatum* growth at >2.0 mg/L, with the highest inhibition rate at up to 86% at 16.0 mg/L and an IC_{50} for 96 h growth was 5 mg/L.

In previous studies about florfenicol and thiamphenicol, the concentrations were nearly beyond the detection limits in the aquatic environment (Ferreira et al., 2007; Kołodziejska et al., 2013; Lai et al., 2009; Liu et al., 2012; Li et al., 2012). Recently, the frequent outbreak of cyanobacterial blooms have become a ubiquitous phenomenon in freshwater ecosystems (Kutser et al., 2006; Paerl and Otten, 2013), leading to serious harm to fisheries, the ecological environment, and human health (Merel et al., 2013). Microalgae are primary producers and they play a very important role in the balance and stability of the aquatic ecosystem. Many studies only investigated the EC_{50} or IC_{50} of florfenicol and thiamphenicol on algae, but did not focus on their deeper influence on algae, such as the influence on photosynthetic parameters, oxidative stress damage and response. Moreover, because cyanobacteria are unicellular, they are easy to culture and respond rapidly to environmental changes. Additionally, some studies have reported that blue-green algae are more sensitive to antibiotics than green algae. *Microcystis flos-aquae*) is common blue-green algae, which is widely distributed in many kinds of freshwater bodies and it is the main algae found in cyanobacterial blooms. Therefore, we need to study the toxic effects of florfenicol and thiamphenicol on microalgae in detail.

In this paper, we investigated the effects of florfenicol and thiamphenicol on growth in a cyanobacterium (*Microcystis flos-aquae*). The concentration range of florfenicol and thiamphenicol covered the concentrations detected in aquatic environments. The objective of the present work is to gain insight into the effects and mechanisms of florfenicol and thiamphenicol on cyanobacteria.

2. Material and methods

2.1. 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)in Wuhan City, China. All the experimental apparatuses used to culture *M. flos-aquae* were sterilized before use by autoclaving at 121 °C for 20 min. The algae were grown in 250 mL Erlenmeyer flasks containing 100 mL of BG-11 medium, cultivated at 25 ± 1 °C and illuminated with 50 μmol photons m^{-2} s^{-1} (cool white fluorescent tube) with a 12 h/12 h light/dark interval. They were incubated in an oscillation incubator with the speed of 100 r/min (Ma et al., 2001). To reduce any effect caused by minor differences in photon irradiance, the flasks were arranged randomly.

2.2. Antibiotic treatment

FF and TAP with >99% purity were purchased from Shanghai Yiji Industrial Co., Ltd. in Shanghai, China, The stock solution was prepared with sterilized water and then diluted to various test concentrations before use. Tests were performed according to guideline no. 201 of the organization for economic cooperation and development (OECD) with modifications (OECD, 1984). The experiment was carried out in a 250 mL Erlenmeyer flasks with 100 mL of algal and antibiotics mixture solution. We added the stock solution into the algal medium to make the final concentrations of 0, 0.001, 0.01, 0.1, 1, 10, 25, and 50 μg/L (FF), and 0, 0.001, 0.01, 0.1, 1, 10, 40, 70, and 100 μg/L (TAP). Since the molar masses of the two compoundsare very similar (FF, 358.22; TAP, 356.23), we used the mass concentration to report their concentrations. We used 0 μg/L algal medium as the control. Each test concentration was replicated three times, and all operations were carried out under sterile conditions to avoid contamination from bacteria.

2.3. Determination of chlorophyll a content and photosynthetic activity parameters

In this study, the chlorophyll a content and photosynthetic activity parameters of *M. flos-aquae* were determined by a pulse amplitude-modulated fluorometer (Phyto-PAM Walz, Effeltrich, Germany). The chlorophyll a content was determined by analysing samples (1 mL) of each treatment every day by using an irradiance of 32 μmol photons m^{-2} s^{-1} PAR (PAR: illumination intensity). The other photosynthetic activity parameters (Fv/Fm, Fv/Fo, RLCs, alpha, ETRmax and Ik) were also tested every day. Reviews of the fluorescence measurements are given by Schreiber (Schreiber et al., 1995). The minimal fluorescence yield, Fo, and the maximal fluorescence yield, Fm, were measured after the samples were dark-adapted for at least 15 min. Based on these measurements, the photochemical efficiency (Fv/Fm) and potential photosynthetic activity of PSII (Fv/Fo) can be determined by the equation (Rohacek and Bartak, 1999):

$$\frac{Fv}{Fm} = \frac{Fm - Fo}{Fm}$$

$$\frac{Fv}{Fo} = \frac{Fm - Fo}{Fo}$$

The fluorometer control software computed the ETR from the yield by multiplying the effective quantum yield of PSII by half of the applied PAR and a factor of 0.84 for absorbed light (Körner and Nicklisch, 2002; Schreiber et al., 1995). An RLC was constructed by exposing the samples to each of 11 increasing actinic light levels for 20 s. The nonlinear curve was fit using the model of by Platt et al. (Krause, 1988) and applied to estimate the initial slope (alpha), the maximum photo synthetic rate (ETRmax) and the semi-light saturation point (Ik). Alpha indicate the solar energy utilization efficiency of algae, the ETRmax shows the maximum electron transport rate and Ik represents the tolerance of algae to strong light.

2.4. Analysis of antioxidant responses

After *M. flos-aquae* was exposed to FF and TAP for 7 days, 45 mL of culture medium containing each antibiotic was centrifuged at 3,000*g* and 4 °C for 15 min to harvest the cyanobacteria cells. Collected cells were resuspended in 0.05 M phosphate buffer (pH 7.8), placed for 30 min in an ice-bath with ultra-sonication (5 W of output power) and then centrifuged at 13,050*g* at 4 °C for 10 min (Nie et al., 2013; Wan et al., 2015). The supernatant was used for the lipid peroxidation assay and antioxidative enzyme activity. The SOD activity was measured according to a SOD kit (Total Superoxide Dismutase Assay Kit with WST-8, Beyotime Company, China). The CAT activity was determined by the catalase (CAT) assay kit (Visible light, Nanjing Jiancheng Bioengineering Institute, China). Malondialdehyde (MDA), which is a product of lipid peroxidation, was measured using a malondialdehyde (MDA) assay kit (TBA method, Nanjing Jiancheng Bioengineering Institute, China). The activity of the antioxidant enzymes and the non-enzymatic antioxidant content in *M. flos-aquae* were expressed in units per mg or μg of protein (U/mgprot or U/μgprot) and nmol/mg of protein, respectively, to eliminate the measurement deviation caused by the varied extraction efficiency of protein. Total protein was determined according to the Bradford method (1976) using bovine serum albumin (BSA) as the protein standard.