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Cellular responses and biodegradation of amoxicillin in *Microcystis aeruginosa* at different nitrogen levels

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ABSTRACT

The influence of nitrogen on the interactions between amoxicillin and *Microcystis aeruginosa* was investigated using a 7-day exposure test. Growth of *M. aeruginosa* was not significantly ($p > 0.05$) affected by amoxicillin at the lowest nitrogen level of 0.05 mg L^{-1}, stimulated by 500 ng L^{-1} of amoxicillin at a moderate nitrogen level of 0.5 mg L^{-1} and enhanced by 200–500 ng L^{-1} of amoxicillin at the highest nitrogen level of 5 mg L^{-1}. The generation of reactive oxygen species (ROS) and the synthesis of glutathione S-transferases (GST) and glutathione (GSH) were more sensitive to amoxicillin and were stimulated at all nitrogen levels. At the lowest nitrogen level of 0.05 mg L^{-1}, superoxide dismutase and peroxidase were not effective at eliminating amoxicillin-induced ROS, resulting in the highest malondialdehyde content in *M. aeruginosa*. The biodegradation of 18.5–30.5% of amoxicillin by *M. aeruginosa* was coupled to increasing GST activity and GSH content. Elevated nitrogen concentrations significantly enhanced ($p < 0.05$) the stimulation effect of amoxicillin on the growth of *M. aeruginosa*, the antioxidant responses to amoxicillin and the biodegradation of amoxicillin in *M. aeruginosa*. The nitrogen-dependent hormesis effect of the coexisting amoxicillin contaminant on the *M. aeruginosa* bloom should be fully considered during the control of *M. aeruginosa* bloom.

1. Introduction

Cyanobacterial blooms are frequently observed in freshwater environments (Paerl and Huisman, 2009). Excessive growth of cyanobacteria significantly reduces water quality and aquatic ecosystem function, and cyanotoxins pose serious threats to animals and humans (Veldhuis and Wassmann, 2005). Therefore, cyanobacterial blooms have become a worldwide public health and ecological concern. Most studies on cyanobacterial blooms use *Microcystis aeruginosa* as a model species. *M. aeruginosa* is one of the most widely distributed cyanobacterial species and produces microcystins (MCs), which is a major group of cyanotoxins (Babica et al., 2006). *M. aeruginosa* was normally considered to be regulated by conventional factors, including nitrogen (N), phosphorous (P), N:P ratio, light intensity, trace metals, temperature, and pH (Davis et al., 2009; Jiang et al., 2008). In the last two decades, numerous domestic and industrial contaminants were released into aquatic environments from human activities. Some of these contaminants were found to interact with *M. aeruginosa*, such as heavy metals, pesticides (Qian et al., 2012), benzene compounds (Perrona and Juneau, 2011) and pharmaceuticals (Hu et al., 2014). Reported interactions were mainly growth inhibition effects of these contaminants at exposure concentrations much higher than the actual contamination levels.

Very recently, several studies observed the stimulation effects of anthropogenic contaminants on *M. aeruginosa* blooms at environmentally relevant concentrations. For instance, an herbicide pentachlorophenol (de Morais et al., 2014) was reported to promote the growth of *M. aeruginosa* at a concentration of 1 μg L^{-1}. A study performed by our group found that the antibiotic amoxicillin enhanced the growth of *M. aeruginosa* and the production and release of MCs at its currently detected concentrations in aquatic environments (Liu et al., 2012a). The above studies suggested that the regulation of cyanobacterial blooms by these contaminants became a reality in aquatic environments and should be fully considered during the control of cyanobacterial blooms. However, interaction mechanisms between anthropogenic contaminants and *M. aeruginosa* remained unclear.

Exposure to exogenous chemicals was found to generate reactive oxygen species (ROS), cause oxidative stress and induce antioxidant responses in microalgae (Wang et al., 2011). Antioxidant responses have proven to be correlated with

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morphological, physiological and biochemical changes in microalgae (Mallick and Mohn, 2000). Wang and Xie (2007) observed that increased antioxidant activities could protect *M. aeruginosa* from oxidative damage caused by low concentrations of nonylphenols (NPs) and consequently led to stimulated growth as a hormesis response. Additionally, antioxidants, including glutathione S-transferases (GST) and glutathione (GSH), not only participate in the elimination of ROS but also play an important role in the metabolism of exogenous chemicals (Belchik and Xun, 2011). Elevated activities of GST and increased content of GSH, coupled with the degradation of amoxicillin, was found to alleviate the toxicity of amoxicillin in *M. aeruginosa* (Liu et al., 2012b). The above studies suggested that antioxidant responses may be a promising route for investigating the interaction mechanisms between anthropogenic contaminants and *M. aeruginosa*.

M. aeruginosa in aquatic environments was simultaneously exposed to anthropogenic contaminants and various environmental factors, and the environmental factors have the potential to affect the interactions between *M. aeruginosa* and anthropogenic contaminants. For instance, low temperature was demonstrated to enhance the inhibition effect of an herbicide, atrazine, on the growth and photosynthesis of *M. aeruginosa* (Chalifour and Juneau, 2011). Studies on the combined effects of other anthropogenic contaminants and other environmental factors on cyanobacteria were still limited. Nitrogen is usually considered to be the dominant environmental factor regulating cyanobacteria as a major nutrient for growth, and it is also an indispensable element in the molecules of various antioxidants (Downing et al., 2005). Therefore, altered nitrogen concentrations may affect the antioxidant responses in *M. aeruginosa* under exposure to anthropogenic contaminants.

In the present study, amoxicillin was selected as the target chemical, which is a widely used antibiotic with high biological activity, and has been verified to interact with *M. aeruginosa* at environmentally relevant concentrations (Liu et al., 2012a). The variation of the growth rate, the generation of ROS and the responses of various antioxidants in *M. aeruginosa* following exposure to amoxicillin, as well as the biodegradation of amoxicillin by *M. aeruginosa*, were investigated at different nitrogen levels. Combined effects of nitrogen and antibiotic contaminants on *M. aeruginosa* have not been reported by previous studies. The observed results would contribute to a better understanding of the formation of cyanobacterial bloom with the coexistence of antibiotic contaminants.

2. Materials and methods

2.1. Cellular response test

An axenic culture of the cyanobacterium *M. aeruginosa* PCC7806 was supplied by the Pasteur Culture Collection of Cyanobacteria (Paris, France). Amoxicillin was purchased from Sigma-Aldrich, Inc. (Shanghai, China), and the stock solution was prepared in methanol and stored at −20 °C prior to use. *M. aeruginosa* was pre-cultivated under aseptic conditions for two weeks in sterile BG11 medium at 25 ± 1 °C under a 16:8 light:dark cycle provided by cool white fluorescent lights at an intensity of 40 μmol photons m^{-2} s^{-1}. The BG11 medium contained nitrate as the source of nitrogen, with a nitrogen concentration of 247 mg L^{-1}. After pre-cultivation, the *M. aeruginosa* cells reaching the exponential growth phase were collected by centrifugation (4000*g*, 4 °C, 5 min) and used as the inoculums for the cellular response test and the biodegradation test. Modified BG11 media were used in the cellular response test and the biodegradation test, which were divided into three test groups according to the nitrogen concentration in the modified BG11 medium, which were 0.05, 0.5 and 5 mg L^{-1}, respectively. In each test group, 250-mL Erlenmeyer flasks, each containing 150 mL of the modified BG11 medium, were spiked with different concentrations of amoxicillin, each in triplicate. The test concentrations of amoxicillin in each test group were 200 ng L^{-1} and 500 ng L^{-1}. Amoxicillin was replenished regularly to maintain a stable exposure dose, and the determined test concentrations deviated within an acceptable range (from −9% to +12%) compared with their nominal values. The final concentrations of methanol in the test media were below 0.01% (v/v). At each nitrogen level, three flasks containing modified BG11 media with the same nitrogen concentration and 0.01% (v/v) methanol, but without amoxicillin, were prepared as the non-antibiotic-treated control. The initial cell density of *M. aeruginosa* in each flask was 4×10^5 cells mL^{-1}. After inoculation, the *M. aeruginosa* cells were cultured under the same aseptic condition as the pre-cultivation for seven days. The entire experimental apparatus used for the culture of *M. aeruginosa* and addition of amoxicillin were sterilized by autoclaving at 121 °C for 20 min prior to use. The flasks were shaken well before each sampling. Sampling was conducted in a laminar flow cabinet using sterile apparatus. One milliliter of algal culture was aseptically sampled from each flask every day and used for cell density counting. The specific growth rate was calculated according to Eq. (1):

$$\mu(/\text{day}) = (LnX_1 - LnX_0)/(t_1 - t_0) \quad (1)$$

where X_0 and X_1 were the cell density at the beginning (t_0) and the end (t_1) of the selected time interval during the exponential phase of growth, respectively.

2.2. Analysis of ROS and antioxidant responses

In the cellular response test, the culture medium was aseptically sampled from each flask after 4 and 7 days of exposure. For the analysis of ROS, the *M. aeruginosa* cells were collected by centrifugation at 2000*g* at 4 °C for 10 min, loaded with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probes and then measured with a fluorescence plate reader (Bio-TEK, USA), according to the method of Knauert and Knauer (2008), with an excitation filter of 485 nm and an emission filter of 530 nm. DCFH-DA is a cell-permeable indicator, which was hydrolyzed by the cellular esterase to form the non-fluorescent DCFH after penetrating into the cells. DCFH is immediately transformed to highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS, and the DCF fluorescence indicates the ROS level. For the analysis of the antioxidants, the *M. aeruginosa* cells harvested by centrifugation were re-suspended in 1.5 mL of phosphate buffer (100 mM, pH=7.4) containing 1% (w/v) of polyvinylpyrrolidone and homogenized on ice by an ultrasonic cell pulverizer at 200 W with total time of 5 min (ultrasonic time: 2 s; rest time: 4 s). The homogenate was then centrifuged at 12,000*g* at 4 °C for 10 min. The supernatant was used for the determination of antioxidants. The activities of superoxide dismutase (SOD), peroxidase (POD) and GST and the contents of malondialdehyde (MDA) and GSH were determined according to previous studies (Gao and Tam, 2011; Wang et al., 2013a). One unit of SOD activity was defined as the amount of enzyme that caused a 50% decrease in the SOD-inhibited nitro-blue tetrazolium reduction. One unit of POD activity was defined as the amount of enzyme required for the formation of purpurogallin from pyrogallol in 20 s at pH 6.0. One unit of GST activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of S-(2.4-dinitrophenyl)-glutathione per minute at 37 °C. The activities of the antioxidant enzymes and the contents of non-enzymatic antioxidants in *M. aeruginosa* were