Ecotoxicology and Environmental Safety 77 (2012) 79–87

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Influences of two antibiotic contaminants on the production, release and toxicity of microcystins

Ying Liu [a], Baoyu Gao [a,*], Qinyan Yue [a], Yuntao Guan [b], Yan Wang [a], Lihui Huang [a]

[a] Shandong Provincial Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Shandong University, Jinan 250100, People's Republic of China

[b] Research Center of Environmental Engineering and Management, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, People's Republic of China

ARTICLE INFO

Article history:
Received 28 July 2011
Received in revised form
19 October 2011
Accepted 25 October 2011
Available online 9 November 2011

Keywords:
Pharmaceuticals and personal care products
Spiramycin
Amoxicillin
Liquid chromatography–tandem mass spectrometry
Combined toxicity
Photobacterium phosphoreum

ABSTRACT

The influences of spiramycin and amoxicillin on the algal growth, production and release of target microcystins (MCs), MC-LR, MC-RR and MC-YR, in *Microcystis aeruginosa* were investigated through the seven-day exposure test. Spiramycin were more toxic to *M. aeruginosa* than amoxicillin according to their 50 percent effective concentrations (EC_{50}) in algal growth, which were 1.15 and 8.03 μg/l, respectively. At environmentally relevant concentrations of 100 ng/l–1 μg/l, spiramycin reduced the total MC content per algal cell and inhibited the algal growth, while exposure to amoxicillin led to increases in the total MC content per algal cell and the percentage of extracellular MCs, without affecting the algal growth. Toxicity of MCs in combination with each antibiotic was assessed in the luminescent bacteria test using the toxic unit (TU) approach. The 50 percent effective concentrations for the mixtures (EC_{50mix}) were 0.56 TU and 0.48 TU for MCs in combination with spiramycin and amoxicillin, respectively, indicating a synergistic interaction between MCs and each antibiotic ($EC_{50mix} < 1$ TU). After seven-day exposure to 100 ng/l–1 μg/l of antibiotics, spiramycin-treated algal media and amoxicillin-treated algal media showed significantly lower ($p < 0.05$) and higher ($p < 0.05$) inhibition on the luminescence of *Photobacterium phosphoreum*, respectively, compared with the untreated algal medium. These results indicated that the toxicity of MCs were alleviated by spiramycin and enhanced by amoxicillin, and the latter effect would increase threats to the aquatic environment.

1. Introduction

Outbreaks of cyanobacterial blooms are frequently observed in eutrophic lakes and reservoirs all over the world, causing hypoxia and off-flavor in water bodies and producing various cyanotoxins (Chorus et al., 2000; Pan et al., 2006; Veldhuis and Wassmann, 2005). As one of the most widely distributed toxic cyanobacteria, *Microcystis aeruginosa* is the major producer of microcystins (MCs), which are lethal to zooplankton and fish (Penaloza et al., 1990) and highly poisonous to higher animals (Carbis et al., 1994) and humans (Jochimsen et al., 1998) at environmentally relevant concentrations. MCs are a group of cyclic heptapeptide composed of a seven-membered ring containing five amino acids as a common moiety and two variable L-amino acids (Xu et al., 2008). To date, more than 70 variants of MCs were identified according to the variations of the two L-amino acids (Dietrich and Hoeger, 2005). Microcystins can inhibit the protein phosphatases 1 and 2A in animal and plant cells (Fujiki and Suganuma, 1993; MacKintosh et al., 1990), leading to cell cycle disruption, cytoskeletal rearrangement, necrosis and apoptosis (Dawson, 1998; Kameyama et al., 2004). Microcystins are synthesized by peptide synthetases in *Microcystis* cells and released through the cell wall (Sakai et al., 2009). Various environmental factors, including nitrogen (N), phosphorous (P), N:P ratio, light intensity, trace metals, temperature and pH, were found to affect the production and of MCs, either directly (Graham et al., 2004; Jiang et al., 2008) or *via* the influences on algal growth (Long et al., 2001; Oh et al., 2000). The regulation of MC-production by environmental factors is still insufficiently understood even though extensive studies have been conducted.

As a result of broad applications in personal health care, veterinary medicine and agriculture, various pharmaceuticals and personal care products (PPCPs) are released into the aquatic environment, among which antibiotics have attracted increasing attention due to their significant biological activities even at low concentrations (Cinquina et al., 2003; Heberer, 2002; Okuda et al., 2009). The global production amount of antibiotics has exceeded 100,000 t annually (Wise, 2002). Antibiotics are normally excreted unchanged or as active metabolites after administration due to their incomplete metabolism in human and livestock

* Corresponding author. Fax: +86 531 88364513.
E-mail address: liuying2010@sdu.edu.cn (B. Gao).

0147-6513/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2011.10.027

(Kasprzyk-Hordern et al., 2008). Antibiotics are mainly discharged through domestic and hospital wastewater containing human excreta (Brown et al., 2006) and industrial wastewater from related production processes (Li et al., 2008), which could be collected by wastewater treatment plants (WWTPs) and released into surface waters due to the ineffective removal by current wastewater treatment processes (Zorita et al., 2009). Antibiotics given to livestock can enter groundwater *via* manure dispersed in fields (Christian et al., 2003). Therefore, antibiotics are widely detected in effluents of WWTPs, surface and ground waters (Kümmerer, 2009; Lin and Tsai, 2009; Zuccato et al., 2010). Most antibiotics are designed to inhibit the bacterial growth or attack various bacterial components, such as cell wall, cell membrane and essential enzymes. Without a membrane-enclosed nucleus, cyanobacteria are different from other algae and more appropriate to be classified as bacteria (Stanier and Bazine, 1977). Therefore, antibiotic contaminant in the aquatic environment is likely to be an emerging environmental factor regulating the growth of cyanobacteria and the production and release of MCs in cyanobacteria. At present, only limited studies focused on the effects of antibiotics on cyanobacteria. Crane et al. (2006) compared the responses of algal growth to antibiotics among various algal species, and found that cyanobacteria, including *M. aeruginosa*, are more sensitive to antibiotics than other reported species. Halling-Sørensen (2000) reported the growth inhibition effects of five antibiotics in *M. aeruginosa* at environmentally relevant concentrations. Van der Grinten et al. (2010) indicated that the photosynthetic efficiency of cyanobacteria was affected by three antibiotics. The influences of antibiotics on the production and release of MCs in cyanobacteria have not been reported.

Toxicants in mixtures may interact with each other and alter the toxicity of each individual toxicant, and the combined toxicity can be antagonistic, additive or synergistic. Therefore antibiotic contaminants have a high potential to alter the toxicity of MCs, which has not be investigated in previous studies. As a rapidly responding, relatively inexpensive, simple and highly reproducible toxicity test (Hernando et al., 2003), the luminescent bacteria test is sensitive to a wide range of toxicants (Ulitzur et al., 2002). Luminescent bacteria test has been used to assess the single toxicity of antibiotics (Backhaus and Grimme, 1999) and MCs (Lawton et al., 1990) and the combined toxicity of various toxicants (Altenburger et al., 2000; Heinlaan et al., 2008). But the combined toxicity of MCs and antibiotics has not been assessed *via* this toxicity test.

In the present study, the influences of typical antibiotics on the specific growth rate of *M. aeruginosa*, the production and release of MCs were investigated. The combined toxicity of antibiotics and MCs was also assessed using a luminescent bacteria test. Spiramycin and amoxicillin were selected as target antibiotics. Spiramycin can inhibit the biosynthesis of functional proteins (Mazzei et al., 1993) in bacteria, which may affect the enzymes involved in MC-production. Amoxicillin can affect the construction of the bacterial cell wall (Gustafsson et al., 2001), which relate closely with the release of MCs. Three most frequently detected MCs, MC-LR, MC-RR and MC-YR (Gupta et al., 2003), were selected as target MCs.

2. Materials and methods

2.1. Reagents and materials

Spiramycin, amoxicillin, enkephalin (internal standard for quantification) and a mixture of MC-LR, MC-RR, and MC-YR (in a ratio of 1:1:1), were purchased from Sigma-Aldrich, Inc. (Shanghai, China). All of the stock solutions were prepared in methanol and stored at −20 °C prior to use. The cyanobacteria *M. aeruginosa* PCC7806 was supplied by the Pasteur Culture Collection of Cyanobacteria (Paris, France). The freeze-dried cells of *Photobacterium phosphoreum* 502 (PP502) were provided by Hamamatsu Photonics (Beijing, China) and stored at −20 °C prior to use. Oasis HLB glass cartridges (200 mg, 5 ml) were obtained from Waters Corporation (Massachusetts, USA). Visiprep twelve-port SPE Vacuum Manifold was supplied by Supelco (Bellefonte, USA). All the glass wares used for the determination of MCs were previously washed with tap water, Milli-Q water, methanol and Milli-Q water again, and finally dried at 550 °C for 4 h before use. All the experimental apparatus used to culture *M. aeruginosa* were sterilized by autoclaving at 121 °C for 20 min before use. All organic solvents, suitable for liquid chromatography–mass spectrometry (LC-MS) analysis, were provided by Merk & Co., Inc. (Shanghai, China).

2.2. Exposure test

M. aeruginosa was pre-cultivated for two weeks in sterile BG11 medium (Rippka et al., 1979) 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. After pre-cultivation, algal cells reached the exponential phase of growth were collected by centrifugation (4000 g, 4 °C, 5 min) and used as the inoculums for the exposure test.

In the exposure test, 1000 ml Erlenmeyer flasks, each containing 500 ml of the sterile BG11 medium, were spiked with different concentrations of target antibiotics, each in triplicate. Target antibiotics were replenished every 12 h to maintain a stable exposure dose. Test concentrations were measured as described by Zuccato et al. (2010) after each addition of target antibiotics, and the determined values deviated within an acceptable range (from −10 percent to +8 percent) compared with their nominal values. The final concentrations of methanol in the test media were below 0.01 percent (v/v). Another set of flasks containing sterile BG11 media spiked with 0.01 percent (v/v) methanol and without antibiotics, also in triplicate, was prepared as the control. Algal cells were inoculated into each flask at an initial cell density of 4×10^5 cells/ml. After inoculation, the flasks were closed with sterile cotton stoppers and cultured in the same condition as the pre-cultivation for seven days. The flasks were shaken well every 8 h and before each sampling. One ml of algal culture was aseptically sampled from each flask at regular time intervals. The cell number was counted with a Neubauer counting chamber under a Zeiss Axioskop microscope (Zeiss, USA) at magnification of 400X.

The specific growth rate was calculated according to Eq. (1):

$$\mu(/day) = (Ln X_1 - Ln X_0)/(t_1 - t_0) \quad (1)$$

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 algal growth, respectively.

Specific growth rates were normalized to percentages of growth inhibition (PGI) according to Eq. (2).

$$PGI(\%) = 100 \times (\mu_c - \mu_t)/\mu_c \quad (2)$$

where μ_c and μ_t were the specific growth rate in control and each test medium, respectively. The EC_{50} and HC_5 values are defined as the concentration of target antibiotic causing 50 percent and five percent inhibition on the algal growth, respectively. They were calculated according to the dose-response of PGI values to the test concentrations of each target antibiotic.

2.3. Extraction of MCs

Target MCs were measured in the stationary phase of algal growth, because the cell density and growth rate reached a steady state at this stage (Ward and Codd, 1999). On the seventh day of exposure test, 50 ml of the culture medium was sampled from the control and antibiotic-treated media at test concentrations of 10 ng/l, 100 ng/l and 1 μg/l, respectively. The sampled culture medium was filtered through a Whatman GF/B filtration membrane (one-micrometer pore size) and extracted with an Oasis HLB glass cartridge to measure the extracellular concentrations of MCs. The algal cells harvested by the filtration membrane were freeze-dried, combined with 10 ml of 40 percent (v/v) aqueous methanol, sonicated on ice under 400 W for 5 min and then centrifuged at 4000 g at 4 °C for 20 min to remove the cell debris. The extract was then mixed with Milli-Q water to make a final volume of 100 ml and further extracted with an Oasis HLB glass cartridge to measure the intracellular concentrations of MCs.

An Oasis HLB glass cartridge was conditioned with 6 ml of methanol and 6 ml of distilled water. The sample (50 ml of filtered culture medium or 100 ml of Milli-Q water combined with an algal extract) was extracted with a cartridge at a flow rate of 2 ml/min. After extraction, the cartridge was washed sequentially with 5 ml of Milli-Q water and 5 ml of 10 percent (v/v) aqueous methanol. The cartridge was finally eluted with 6 ml of methanol after it had been dried under a vacuum for two hours. The elution solvent was blown down to dryness under a gentle flow of nitrogen, spiked with 100 ng of enkephalin as an internal standard, dissolved with 500 μl of methanol, and subjected to a liquid chromatography–tandem mass spectrometry (LC–MS/MS) system.