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Impacts of Rac- and S-metolachlor on cyanobacterial cell integrity and release of microcystins at different nitrogen levels

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HIGHLIGHTS

- The impact of a chiral pesticide, metolachlor on cyanobacterial cells was assessed.
- Metolachlor could affect cyanobacterial growth and membrane integrity significantly.
- Impacts of metolachlor on *M. aeruginosa* cells related to the dosages of nitrogen.
- Metolachlor could induce increases in dissolved microcystins.

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ABSTRACT

Pesticide residues and nitrogen overload (which caused cyanobacteria blooms) have been two serious environmental concerns. In particular, chiral pesticides with different structures may have various impacts on cyanobacteria. Nitrogen may affect the behavior between pesticides and cyanobacteria (e.g., increase the adverse effects of pesticides on cyanobacteria). This study evaluated the impacts of Rac- and S-metolachlor on the cell integrity and toxin release of *Microcystis aeruginosa* cells at different nitrogen levels. The results showed that (both of the configurations: Rac-, S-) metolachlor could inhibit *M. aeruginosa* cell growth under most conditions, and the inhibition rates were increased with the growing concentrations of nitrogen and metolachlor. However, cyanobacterial growth was promoted in 48 h under environmental relevant condition (1 mg/L metolachlor and 0.15 mg/L nitrogen). Therefore, the water authorities should adjust the treatment parameters to remove possible larger numbers of cyaonbacteria under that condition. On the other hand, the inhibition degree of *M. aeruginosa* cell growth by S-metolachlor treatments was obviously larger than Rac-metolachlor treatments. S-metolachlor also had a stronger ability in compromising *M. aeruginosa* cells than Rac-metolachlor treatments. Compared to control samples, more extracellular toxins (12%–86% increases) were detected after 5 mg/L S-metolachlor treatment for 72 h at different nitrogen levels, but the variations of extracellular toxins caused by 5 mg/L Rac-metolachlor addition could be neglected. Consequently, higher concentrations of metolachlor in source waters are harmful to humans, but it may prevent cyanobacterial blooms. However, the potential risks (e.g. build-up of extracellular toxins) should be considered.

1. Introduction

The use of nitrogen and pesticides is an essential way to improve crop yields in agricultural countries, such as China. However, studies have revealed that overuse of nitrogen and pesticides have negative impacts on humans and their living environment. For instance, overuse of nitrogen can cause eutrophication in source waters and then induce cyanobacterial blooms, which is a critical water issue globally (Orr and Jones, 1998; Liu et al., 2015). *Microcystis aeruginosa* (*M. aeruginosa*) is one of the most common cyanobacterial species. It could produce various secondary metabolites such as geosmin and 2-methylisoborneol, leading to poor quality of drinking water (Daly et al., 2007; Lin et al., 2009). Some *M. aeruginosa* strains can produce potent hepatotoxins, microcystins (MCs) (Codd et al., 1999; Fujii et al., 2011). Pouria et al.

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(1998) have reported that MC contamination in Brazil caused 100 patients having liver failure, and 52 of them were dead subsequently in 1996. In addition, Ueno et al. (1996) have reported that MCs is the main factor in drinking water inducing primary liver cancer in Haimen and Fusui, China.

Previous studies found that nitrogen could affect the interaction between cyanobacterial cells with other pollutants (Liu et al., 2015). It has been reported that low concentration (0.5 mg/L) of nitrogen could increase the growth of cyanobacteria and release of toxins in the presence of antibiotics; however, the cyanobacteria growth was inhibited with high concentration (100 mg/L) of nitrogen under the same condition. It should be noted that the percentage of herbicide consumption in chemical products in China has increased from 20% to 48% (1960–2005) (Ye et al., 2009; Wang et al., 2013). The residual pesticides could enter into freshwater bodies which may have negative influences to aquatic organisms and be harmful to humans through food chain (Zhang et al., 2016). Also, a wide range of herbicides have been detected in the areas where seagrass are growing in Australia, and these herbicides could have adverse effects to the photosystem-II of seagrass (Wilkinson et al., 2015). Among pesticides, chiral pesticides have attracted much attention due to their enantioselective ecotoxic effects (Zhang et al., 2012). Chiral pesticides which lack symmetry and have non-superimposable mirror images called enantiomers. Wen et al. (2011) have reported that a chiral herbicide (dichlorprop) could increase the reactive oxygen species of aquatic unicellular algae *Scenedesmus obliquus*. As for a common microorganism in fresh water sources, cyanobacteria may also be affected by herbicides (Ye et al., 2013). Meanwhile, overloads of nitrogen may affect the behavior of pesticides with cyanobacteria. These facts highlighted the need to explore the relationships between pesticides and nitrogen in source waters, but few literature have reported their relationships. Therefore, more attention should be paid to the effects of pesticides and nitrogen on the growth of cyanobacteria, in particular the potential production and release of toxins.

In this study, metolachlor (2 - methyl -6- ethyl -N- (1- methyl -2- ethyl) -N- acetyl aniline) was chosen as a typical herbicide (Fig. S1), because it is a broad spectrum herbicide which widely used for controlling annual weeds and some of the twin leaf weeds. It has two sets of enantiomers: αSS/αRR and αRS/αSR because of the carbon chiral center and hindered rotation about the Ar-N bond (Xie et al., 2016). As it has not yet been separated in this study, Rac-metolachlor (with approximately 50% R-metolachlor and 50% S-metolachlor) and S-metolachlor (Fig. S1) which widely used as commercial products at present were chosen. In order to explore the possible consequences of pesticides and cyanobacteria being present simultaneously at different nitrogen levels, the impacts of Rac- and S-metolachlor on cyanobacterial cell integrity and associated toxin release at various nitrogen concentrations were assessed in this study.

2. Materials and methods

2.1. Materials and reagents

A toxic strain of *M. aeruginosa* (FACHB-912, Institute of Aquatic Sciences, Chinese Academy of Sciences, China) which was a confirmed producer of MC-RR (MC-RR is one common isomer of microcystins) was chosen in this study. This strain maintained as unicellular cells in laboratory. BG-11 medium was prepared (Rippka et al., 1979) and adjusted to pH 7.2 ± 0.1 using either 0.1 M sterile filtered hydrochloric acid or sodium hydroxide. The *M. aeruginosa* strain was cultured in BG-11 medium and routinely sub-cultured to achieve exponential phase. All cultures were incubated in an incubation cabinet (in the absence of UV light) under constant cool-fluorescent light intensity of 2000 lux on a 12 h: 12 h light-dark cycle, at a constant temperature of 25 ± 1 °C. Samples for cell counts were treated with Lugol's iodine, and counted by using a microscopy (BX53, Olympus, Japan) (Fan et al., 2013a). Cultures having an initial cell density of 1.1×10^6 cells/mL were used in all experiments. PH 7.5 ± 0.1 adjusted using either 0.1 M sterile filtered hydrochloric acid or sodium hydroxide was used during all experiments.

All chemicals and reagents used were analytical grade except the methanol was chromatographic purity. The chemical solutions were made using ultra-pure water purified to a resistivity of 18.2 MU cm by a Milli-Q water purification system (Millipore Pty Ltd, USA). Rac-metolachlor (97.4%) was purchased from Shandong overseas Chemical Co., Ltd., (China), and S-metolachlor (96%) was from Novartis crop protection company (China). MC-RR was obtained from a commercial store (Beijing Express Company, China) and Sytox Green nucleic acid stain was from Molecular Probes, USA.

2.2. *M. aeruginosa* cells exposure to Rac-, S-metolachlor at different nitrogen levels

Surface waters were identified as types I, II, III, IV and V in China according to the standard of water quality. The concentrations of total nitrogen (TN) were less than 0.15, 0.5, 1, 1.5, 2 mg/L, respectively, for water types of I-V. However, the TN concentrations in real water bodies could be higher than the standard. Andreadakis et al. (2003) have reported that nitrogen load increased to 53% in Lake Plastira (Greece) during the touristic period. Paerl et al. (2011) have also documented the concentration of TN was approximately 6.0 mg/L in the inner bay of Lake Taihu, China. In this study, 0.15, 3, 9 and 15 mg/L of nitrogen concentrations were selected by changing the amounts of sodium nitrate in BG-11 medium to simulate the real water conditions. The procedures of preparing various concentrations of nitrogen in algal samples were: The *M. aeruginosa* in exponential growth phase were centrifuged at 5000 rpm for 5 min, the supernatant was discarded, and algae cells were washed and transferred with the sterilization BG-11 medium of desired concentrations (0.15, 3, 9 and 15 mg/L) of nitrogen. Following experiments were carried out after two days of re-cultivation. The detailed information could be referred to Mohapatra and Mohanty (1992a).

Triangular conical flasks (500 mL) were used as reactors in this study. The final concentrations of Rac- and S-metolachlor in the reactors were 1, 5, 7, 10 and 15 mg/L. *M. aeruginosa* cultures in the absence of herbicides were made as control. All of the experiments were conducted in triplicates, and 2 mL solution of algal sample was collected from each reactor for cell counts after treatments for 0, 24, 48 and 72 h. The inhibition rates of *M. aeruginosa* cell growth were calculated as follows:

$$\text{Inhibition rate (IR)} = (C_0 - C_T)/C_0 * 100\%$$

C_0: Cell densities of *M. aeruginosa* in the control samples
C_T: Cell densities of *M. aeruginosa* in the treatment samples

Positive number of IR represents inhibition; negative number of IR represents promotion.

2.3. Determination of *M. aeruginosa* cell integrity

Membrane integrity of *M. aeruginosa* cells was measured at 0, 24, 48, 72, 96 h after algal exposure to Rac- or S-metolachlor at different nitrogen levels. A FACSCalibur flow cytometer (Becton