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Effects of multiple antibiotics exposure on denitrification process in the Yangtze Estuary sediments

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HIGHLIGHTS

- Multiple antibiotics exposure has synergistic inhibition on denitrification rates.
- Different species of antibiotics have different effect on N_2O release.
- Combined effects of antibiotics lead to stimulation on N_2O release.
- Multiple antibiotics exposure inhibits the abundances of *nirS* and *nosZ* genes.
- Different inhibition on *nirS* and *nosZ* may explain the variations of N_2O release.

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ABSTRACT

Denitrification is a dominant reactive nitrogen removal pathway in most estuarine and coastal ecosystems, and plays a significant role in regulating N_2O release. Although multiple antibiotics residues are widely detected in aquatic environment, combined effects of antibiotics on denitrification remain indistinct. In this work, 5 classes of antibiotics (sulfonamides, chloramphenicols, tetracyclines, macrolides, and fluoroquinolones) were selected to conduct orthogonal experiments in order to explore their combined effects on denitrification. ^{15}N-based denitrification and N_2O release rates were determined in the orthogonal experiments, while denitrifying functional genes were examined to illustrate the microbial mechanism of the combined antibiotics effect. Denitrification rates were inhibited by antibiotics treatments, and synergistic inhibition effect was observed for multiple antibiotics exposure. Different classes of antibiotics had different influence on N_2O release rates, but multiple antibiotics exposure mostly led to stimulatory effect. Abundances of denitrifying functional genes were inhibited by multiple antibiotics exposure due to the antimicrobial properties, and different inhibition on denitrifiers may be the major mechanism for the variations of N_2O release rates. Combined effects of antibiotics on denitrification may lead to nitrate retention and N_2O release in estuarine and coastal ecosystems, and consequently cause cascading environmental problems, such as greenhouse effects and hyper-eutrophication.

1. Introduction

The loading of reactive nitrogen (mainly nitrate) has increased significantly in the past few decades, as a result of human activities (Cui et al., 2013; Galloway et al., 2008; Liu et al., 2013; Yin et al., 2014a). A huge quantity of this reactive nitrogen, however, has been transported into estuarine and coastal areas, consequently leading to various environmental problems in these aquatic ecosystems (Burgin and Hamilton, 2007; Chen et al., 2016; Hou et al., 2015; Seitzinger, 2008; Zheng et al., 2016). As the dominant microbial nitrogen removal pathway in most estuarine and coastal ecosystems, denitrification has received much attention (Fernandes et al., 2012; Gomez-Velez et al., 2015; Hou et al., 2014; Yin et al., 2014a). Denitrification can mitigate eutrophication by eliminating reactive nitrogen from aquatic ecosystems permanently in the form of nitrogen gas (Deng et al., 2015; Devol, 2015; Hou et al., 2012; Yin

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et al., 2014a). Denitrification is also intimately related to N_2O generation and consumption, as N_2O is an intermediate product of denitrification process (Dong et al., 2011; Hou et al., 2014; Yin et al., 2016). The radiative forcing potential of N_2O is approximately 4 times of CH_4 and 200 times of CO_2, thus it is considered to be a major greenhouse gas affecting ozone depletion in atmospheric environment (Ravishankara et al., 2009; Reay et al., 2012). Since denitrification plays an important role in regulating eutrophication and the release of greenhouse gas, an understanding of anthropogenic influence on denitrification process is of great importance (Conley et al., 2009; Hou et al., 2014; Yin et al., 2014a).

Nowadays, up to 200,000 tons of antibiotics are used annually around the world (Shi et al., 2014), due to the huge demand of medical care, aquaculture, farming, and veterinary drug usage (Looft et al., 2012; Yang et al., 2011; Zhang et al., 2015; Zhou et al., 2013). Consistent antibiotics input leads to wide distribution and occurrence of antibiotics residues in aquatic ecosystems (Kümmerer, 2009a; Moreno-González et al., 2015; Yan et al., 2013a), and antibiotics residues may disturb natural microbial processes because of the antibacterial properties (Kümmerer, 2009b; Novo et al., 2013; Yan et al., 2013a). The effects of antibiotics on nitrate reduction processes have become an emerging topic (Brandt et al., 2015; DeVries and Zhang, 2016; Roose-Amsaleg and Laverman, 2015), and several recent studies have been operated to investigate the antibiotics influence on denitrification process (DeVries et al., 2015; Hou et al., 2014; Yan et al., 2013b; Yin et al., 2016). However, these studies are mostly carried out with single antibiotic exposure. Actually, multiple antibiotics occurrence is widely detected in aquatic ecosystems (Chen and Zhou, 2014; Looft et al., 2012; Yan et al., 2013a; Zhao et al., 2015). Combined effects of multiple antibiotics exposure may disturb N_2O release and nitrate reduction in denitrification process, thus leading to various environmental problems, such as greenhouse effect and eutrophication (Canfield et al., 2010; Deegan et al., 2012; Ravishankara et al., 2009; Reay et al., 2012). Therefore, study of combined effects of antibiotics on denitrification is required to give a further understanding of reactive nitrogen and antibiotics pollution control (DeVries and Zhang, 2016; Roose-Amsaleg and Laverman, 2015).

The Yangtze Estuary, one of the most heavily populated and industrialized estuarine and coastal regions in China (Deng et al., 2015), was selected as the study area. Consistent inputs of industrial and domestic sewage lead to sustained antibiotics residues and hyper-eutrophication in this area (Chen et al., 2016; Yan et al., 2013a). The most widely detected antibiotics residues in the Yangtze Estuary are sulfonamides, chloramphenicols, tetracyclines, macrolides, and fluoroquinolones (Chen and Zhou, 2014; Yan et al., 2013a; Zhang et al., 2015; Zhao et al., 2015), thus sulfamethazine (SMT), thiamphenicol (TAP), oxytetracycline (OTC), erythromycin (ERY), and norfloxacin (NOR) were selected to represent the five classes of these antibiotics, respectively. In this work, orthogonal experiments were employed to illustrate the combined effects of antibiotics on denitrification and N_2O release rates, and abundances of denitrifying functional genes were also detected to investigate the microbial mechanisms of multiple antibiotics exposure at genic level. This work provides more realistic data for the understanding of denitrification process responding to multiple antibiotics exposure in aquatics ecosystems, which may help to control the pollution of both reactive nitrogen and antibiotics.

2. Materials and methods

2.1. Sampling and pretreatment

Box corers were used to collect surface sediments (0–5 cm) from the intertidal zone of the Yangtze Estuary in December 2015 (Fig. S1, Supporting Information). The sediment samples were transported to the laboratory within 2 h. A pre-incubation was conducted to eliminate ambient antibiotics in the sediment samples in order to resemble a pristine status. Briefly, the sediment corers were pre-incubated at room temperature (25 °C) in a recirculating glass container which was filled with artificial seawater having near *in situ* nutrient levels and salinity (Yin et al., 2016). The pre-incubation experiment lasted for about 2 months. After the pre-incubation, the sediments were extracted and the antibiotics concentrations were examined to confirm that no antibiotics residuals were left (Shi et al., 2014; Yan et al., 2013a). Subsequently, the sediment samples were used for the slurry experiments and functional gene abundance measurements.

2.2. Orthogonal experimental design

The L_{16} (4^5) orthogonal experiment was applied to study the effects of multiple antibiotics exposure on denitrification rates. Five antibiotics (SMT, TAP, OTC, ERY, and NOR) were designated as five influence factors to represent the most widespread antibiotics residues in the study area (Chen and Zhou, 2014; Shi et al., 2014; Yan et al., 2013a). Four concentration levels were applied for each antibiotic, which were specifically assigned as 0, minimum concentration detected, average concentration detected, and maximum concentration detected in both estuarine water and sediments of the study area (Chen and Zhou, 2014; Yan et al., 2013a; Zhao et al., 2015). Detailed information on antibiotics concentrations and assignments in the orthogonal experimental design is exhibited in Table S1 (Supporting Information). Range analysis was conducted to make a brief description of different antibiotics influence. K value and Rj value were defined in the range analysis as two parameters. K value is referred to as the average value of each result at the same antibiotic concentration. Rj value is referred to as subtracting the minimum K value from the maximum K value in each antibiotic group. A large Rj value means a great influence of the antibiotic (Shen et al., 2016). Analysis of variance was also done to examine the significance of antibiotics influence (Yang et al., 2014).

2.3. Slurry experiments

The surface sediment samples were homogenized with artificial seawater having near *in situ* salinity at a ratio of 1:7 (Hou et al., 2014). The slurries were purged with helium and stirred vigorously for 15 min (Hou et al., 2013). The mixed slurries were filled into respective 12 mL gas-tight vials (Exetainer, Labco, High Wycombe, UK), and then the vials were sealed with butyl-rubber septa and screw caps. A 24-h pre-incubation was operated to eliminate the background nitrate/nitrite (Hou et al., 2014). After the pre-incubation, the vials were spiked with $^{15}NO_3^-$ (final concentration ca. 100 μmol L^{-1}, final % ^{15}N ca. 90–99%) (Hou et al., 2012). Meanwhile, solutions of selected antibiotics were injected into the respective vials following the orthogonal experimental design. An initial group was designated for each trial and preserved with 250 μl 50% $ZnCl_2$ solution (Yin et al., 2016), and 8 replicates were prepared for each group. The slurries were incubated for 8 h at room temperature on a shaker table (200 rpm) (Hou et al., 2012). After the incubation, replicates of all the groups were preserved with $ZnCl_2$ as described for the initial groups. Half of the replicates in each group were used to analyze the dissolved nitrogen gases ($^{29}N_2$ and $^{30}N_2$) generated during the incubations by membrane inlet mass spectrometry (HPR-40, Hiden Analytical, UK), with a detection limit of 0.1 μmol L^{-1} for N_2 (Yin et al., 2014b). The denitrification rates were calculated according to the following equation (Hou et al., 2014):