



Tetracycline and sulfamethazine alter dissimilatory nitrate reduction processes and increase N₂O release in rice fields[☆]

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ABSTRACT

Effects of antibiotics on the transformation of nitrate and the associated N₂O release in paddy fields are obscure. Using soil slurry experiments combined with ¹⁵N tracer techniques, the influence of tetracycline and sulfamethazine (applied alone and in combination) on the denitrification, anaerobic ammonium oxidation (anammox), dissimilatory nitrate reduction to ammonium (DNRA) and N₂O release rates in the paddy soil were investigated, while genes related to nitrate reduction and antibiotic resistance were quantified to explore the microbial mechanisms behind the antibiotics' effects. The potential rates of denitrification, anammox, and DNRA were significantly ($p < 0.05$) reduced, which were mainly attributed to the inhibitory effects of the antibiotics on nitrate-reducing microbes. However, the N₂O release rates were significantly ($p < 0.05$) stimulated by the antibiotic treatments (0.6–6000 $\mu\text{g kg}^{-1}$ soil dry weight), which were caused by the different inhibition effects of antibiotics on N₂O production and N₂O reduction as suggest by the changes in abundance of *nirS* (nitrite reduction step) and *nosZ* (N₂O reduction to N₂ step) genes. Antibiotic resistance gene (*tetA*, *tetG*, *sulI*, and *sulIII*) abundances were significantly ($p < 0.05$) increased under high antibiotic exposure concentrations (>600 $\mu\text{g kg}^{-1}$ soil dry weight). Our results suggest that the widespread occurrence of antibiotics in paddy soils may pose significant eco-environmental risks (nitrate accumulation and greenhouse effects) by altering nitrate transformation processes.

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1. Introduction

As a plant of great agronomic importance, rice feeds more than half of the world's population (Ishii et al., 2011). To achieve high rice yield, synthetic nitrogen (N) fertilizers (commonly urea) have been intensively applied to paddy fields, but their excessive use causes the significant imbalance in the biogeochemical cycle of N (Galloway et al., 2004). Under aerobic conditions, microbial nitrification converts a large portion of urea-based fertilizers to nitrate in paddy fields, where the nitrate subsequently undergoes anaerobic transformation after the fields become waterlogged during the main stage of rice growth. The transformation and fate of nitrate are of great importance in paddy fields because they are responsible for N loss, production of nitrous oxide (N₂O) and N pollution in water bodies (Giles et al., 2012; Shan et al., 2016). Dissimilatory nitrate

reduction processes, including denitrification, anaerobic ammonium oxidation (anammox) and dissimilatory nitrate reduction to ammonium (DNRA), are the most important pathways of nitrate transformation (Burgin and Hamilton, 2007; Kraft et al., 2014) and exert various effects on nitrate dynamics in paddy soils (Shan et al., 2016). Denitrification and anammox remove nitrate permanently from paddy fields by converting it into dinitrogen (N₂), whereas DNRA reduces the nitrate into ammonium, resulting in N conservation in paddy soils. In a single habitat, denitrification, anammox, and DNRA will compete for nitrate as an electron acceptor (Kraft et al., 2014). The production of N₂O is also intimately related to dissimilatory nitrate reduction processes as it is the byproduct of denitrification and DNRA (Butterbach-Bahl et al., 2013; Hu et al., 2015). The size and composition of the nitrate reducing microorganisms are responsible for these transformation processes (Giles et al., 2012). And a number of abiotic factors such as moisture, nitrate concentration, soil organic carbon, and pH, are known to play important roles in terms of controlling the ratio of N₂O/(N₂O + N₂) in denitrification process (Ciario et al., 2008; Giles et al., 2012; Senbayram et al., 2012). Dissimilatory nitrate reduction

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processes are susceptible to environmental interference by anthropogenic activities such as fertilization, heavy metal and antibiotic exposure (Kraft et al., 2014; Vitousek et al., 1997; Roose-Amsaleg and Laverman, 2016), thus a better understanding of their responses to anthropogenic influence is essential for N regulation and management in paddy fields.

Antibiotics are widely used to treat microbial infections of humans and animals and constitute a new class of ubiquitous “persistent” contaminants (Hamscher et al., 2004). Most antibiotics are designed to be quickly excreted from the treated organism and are often overused, thus they occur in large quantities (up to several hundred mg kg⁻¹) in biosolids (treated sludge) and manure (Pan et al., 2011; Thiele-Bruhn, 2003; Thiele-Bruhn and Beck, 2005; Zhu et al., 2013). Increasing land application of biosolids and manure as fertilizers lead to the release of large amounts of antibiotics into the soil (Jechalke et al., 2014). Antibiotic residues in soils can reduce microbial activities and alter the microbial community structure (Ding and He, 2010; Kleineidam et al., 2010; Kotzerke et al., 2008; Liu et al., 2009; Thiele-Bruhn and Beck, 2005), which may consequently disturb related biogeochemical processes. A number of studies have investigated the effects of antibiotics on denitrification rates and the associated N₂O production in estuarine, coastal and riverine sediments (Hou et al., 2015; Roose-Amsaleg and Laverman, 2016; Yan et al., 2013; Yin et al., 2017; Yin et al., 2016), demonstrating that antibiotic residues significantly decreased the denitrification rates, resulting in more reactive N retention in these aquatic environments. Previous studies about influence of antibiotics on N transformation processes in soil mainly focused on nitrification (Roose-Amsaleg and Laverman, 2016), few studies have investigated the effects of antibiotics on denitrification rates in soils (Banerjee and D'Angelo, 2013; DeVries et al., 2015), and antibiotics, even at ultralow levels (1, 10 and 1000 ng kg⁻¹), could significantly alter the nitrate dissipation rates (DeVries et al., 2015). The paddy fields are different from the riverine environments in terms of fertilization, drying-wetting alteration, and rice growth, although the waterlogged conditions of paddy fields are similar to that of riverine sediments. Knowledge of how antibiotics influence denitrification and N₂O release paddy soils are still limited. In addition to denitrification, anammox and DNRA may also be affected by antibiotics; however, there are no publications about the influence of antibiotics on anammox and DNRA activities, and their relative contributions to nitrate removal in paddy soils.

China is the largest antibiotics producer and consumer in the world, with a total estimated production and usage of antibiotics of 248000 and 162000 tons, respectively, in 2013 (Zhang et al., 2015). Tetracyclines, sulfonamides, fluoroquinolones, macrolides and β -lactams are the most widely used antibiotics in China (Luo et al., 2011; Zhang et al., 2015; Zhang et al., 2010), among which tetracyclines and sulfonamides are frequently detected in soils at relatively high residual concentrations (up to 1000 μ g kg⁻¹) (Ji et al., 2012; Wu et al., 2010).

In the present study, tetracycline and sulfamethazine were selected as representatives of tetracyclines and sulfonamides to investigate their single and combined effects on dissimilatory nitrate reduction processes and N₂O release in paddy soil. We hypothesized that antibiotics may reduce the rate of dissimilatory nitrate reduction processes and alter the N₂O release rate in paddy soil by inhibitory effects on nitrate-reducing microbes. The sorption and degradation behavior of the two antibiotics, the abundance of nitrate-reducing functional genes, and the expression of antibiotic resistance genes were investigated to elucidate the microbial mechanisms of the antibiotics' effects. This study provides comprehensive information on the responses of dissimilatory nitrate reduction processes and N₂O release to antibiotic exposure,

which is useful for antibiotic pollution control and N management in paddy fields.

2. Materials and methods

2.1. Soil and chemicals

A gleyic hydric Anthrosol soil, which is derived from a silty loam deposit, was collected from a rice paddy field at the Changshu Experimental Station of the Chinese Academy of Sciences in Jiangsu Province, China. Soils were sampled in July 2016 when the fields were still flooded and grown with rice. Soil samples (0–20 cm) were placed in sterile plastic bags, surrounded by ice packs and transported to the laboratory as soon as possible. The soil samples were divided into two subsamples: one subsample was air-dried and sieved (<2 mm) for the analysis of physicochemical properties and sorption experiments; another subsample was briefly stored at 4 °C and then was processed for ¹⁵N soil slurry incubation, N₂O release and antibiotic degradation experiments. The physicochemical properties of the soil were determined according to the methods in Shan et al. (2016); the soil had a pH (0.01 M CaCl₂) of 7.30 and contained 22.67 g kg⁻¹ total carbon (C), 1.58 g kg⁻¹ total N and 18.91 g kg⁻¹ soil organic carbon (SOC). The concentrations of tetracycline and sulfamethazine in the native soils were in the range of 3.3–7.9, and 3.6–8.4 μ g kg⁻¹ dry weight soil. To remove the ambient tetracycline and sulfamethazine, soils were pre-incubated at 25 °C for one-week, after which both tetracycline and sulfamethazine were below the detection limits.

Tetracycline and sulfamethazine with purities >99% were purchased from Sigma (Shanghai, China) and were used in the experiments. Selected physicochemical properties of these antibiotics are listed in Table S1.

2.2. Denitrification, anammox and DNRA rates

The potential rates of denitrification, anammox, and DNRA were determined by the ¹⁵N isotope pairing technique (¹⁵N-IPT) and ¹⁵NH₄⁺ oxidation technique combined with membrane inlet mass spectrometry (MIMS) measurement of ²⁹N₂ and ³⁰N₂ in the slurry of the soil (Deng et al., 2015; Shan et al., 2016; Yin et al., 2014). The soil slurry incubation experiments included three treatments: exposure to only tetracycline (TET), exposure to only sulfamethazine (SMZ), and coexposure to tetracycline and sulfamethazine (TET & SMZ). All the treatments were performed in 12 mL borosilicate glass vials (Exetainer; Labco, U.K.) with butyl rubber septa. Briefly, soil slurries were prepared by thoroughly mixing fresh soil with helium (He)-purged water at a ratio of 1:7 in the 12 mL glass vials. Then, all the vials were preincubated at 25 °C for one week to deplete the background nitrate and dissolved oxygen and to create favorable conditions for anammox. It should be noted that other forms of N generated from the preincubation may exert potential effects on the activities of denitrification and anammox; however, it is currently not possible to account for these effects due to the methodological limitations of ¹⁵N-IPT (Risgaard-Petersen et al., 2003).

After preincubation, the vials were spiked with 100 μ L of a He-purged stock solution of ¹⁵NO₃⁻ (99.2% ¹⁵N), resulting in a final concentration of 100 μ M ¹⁵N in each vial. The amount of ¹⁵NO₃⁻ amended was selected based on the actual application rates of chemical N fertilizer in the paddy field where the soil samples were collected (about 225 kg N ha⁻¹). Meanwhile, stock solutions of tetracycline and sulfamethazine were singly or jointly injected into the respective vials at specific volumes to achieve various antibiotic concentrations (0.6, 6, 60, 600, and 6000 μ g kg⁻¹ soil dry weight) in the TET, SMZ and TET & SMZ treatments, respectively.

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