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Effects of long-term pig manure application on antibiotics, abundance of antibiotic resistance genes (ARGs), anammox and denitrification rates in paddy soils[☆]

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ABSTRACT

Previous studies of long-term manure applications in paddy soil mostly focused on the effects on denitrification, occurrence of antibiotics and antibiotic resistance genes (ARGs) without considering the effects on anaerobic ammonium oxidation (anammox). Here, we investigated the potential rates of anammox and denitrification, occurrence of antibiotics and AGRs in response to three fertilization regimes (C, no fertilizer; N, mineral fertilizer; and NM, N plus pig manure) in six long-term paddy experiment sites across China. The potential rates of anammox (0.11–3.64 nmol N g^{-1} h^{-1}) and denitrification (1.5–29.05 nmol N g^{-1} h^{-1}) were correlated with the abundance of anammox genes (*hzsB*) and denitrification functional genes (*narG, nirK, nirS* and *nosZ*), respectively. The anammox and denitrification rates were affected by soil organic carbon (SOC) and significantly ($p < 0.05$) increased in NM treatments relative to those in N treatments. Although pig manure application increased antibiotic concentrations and abundance of ARGs compared with N treatments, the increased antibiotics did not directly affect the anammox and denitrification rates. Our results suggested that long-term pig manure application significantly increased antibiotic concentrations, abundance of ARGs, and rates of anammox and denitrification, and that the effects of pig manure-derived antibiotics on anammox and denitrification were marginal. This is the first report that investigates the effects of long-term pig manure application on anammox in paddy soils. More attention should be paid to the potential ecological risk of increased ARGs caused by pig manure application in paddy soils.

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1. Introduction

Rice serves as the staple food for more than half of the world's population. In China, 25% of the total cultivable land is used to cultivate rice (Liu et al., 2014). Manure and chemical fertilizer were widely applied to paddy soil to increase rice yield (Liu et al., 2011). Both applications of chemical fertilizer and organic manure can achieve high rice productivity with organic manure was more sustainable (Hao et al., 2008). However, the adverse effects of manure-derived antibiotics on soil microbial biodiversity, functions and burden of antibiotic resistance genes (ARGs) are a major issue of concern for human and animal health (Zhu et al., 2013). Recently, it was reported that the annual usage of antibiotics in China was approximately 210,000 tons with livestock industries accounting for 46.1% (Zhu et al., 2013). The maximum production of livestock manure is approximately 3.19 billion tons/year in China (Wang et al., 2006), and large amounts of manure are used in paddy soil to increase soil fertility and rice yield. Manure has become a reservoir of antibiotics because its application significantly increases the concentrations of antibiotics and the abundance of ARGs in soils (Heuer et al., 2011a; Kang et al., 2016; Luby et al., 2016; Tang et al., 2015).

Effects of long-term pig manure applications on denitrification have been intensively studied in paddy soils showing that

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denitrification activities were significantly increased due to that pig manure application increased the amount of total organic C and microbial biomass C (Dambreville et al., 2006; Müller et al., 2003; Rochette et al., 2000). Some studies reported that organic matter provided favorable micro-sites for denitrification, and applications of pig manure could generate favorable conditions for denitrification under both laboratory (Hénault et al., 2001) and field conditions (Dambreville et al., 2006). Before the discovery of anaerobic ammonium oxidation (anammox) where ammonium is oxidized to dinitrogen (N_2) using nitrite as an electron acceptor under anaerobic condition (Jetten, 2001), denitrification was considered to the major pathway of dissimilatory nitrate reduction processes converting fixed N to N_2 (Xu et al., 2013). It has been reported that anammox widely occurs in the paddy soils and contributes substantially to total N_2 production there in (Shan et al., 2016; Yang et al., 2015; Zhu et al., 2011). However, the effects of long-term pig manure application on anammox activities in paddy soils are still obscure.

Soil microbial communities related to nitrate reduction play a vital role in controlling rates of anammox and denitrification (Giles et al., 2012). Bacterial dissimilatory nitrate reduction functional genes, like *hzsB*, and *narG*, *nirS/nirK*, *norB*, and *nosZ* were commonly used for the detection of anammox and denitrifying bacteria community, which indirectly influenced the activities of nitrate reduction processes (Throback et al., 2004; Shen et al., 2015). In the native soil environment, antibiotics can potentially affect the microbial populations by acting as an ecological factor in the environment, and numerous studies have detected changes of microbial population structure by adding of antibiotics in soil (Ding and He, 2010). The effects of antibiotics on microbial population are dependent on the microbial groups (Hammesfahr et al., 2008), the original soil properties (Čermák et al., 2008), the chemical properties of antibiotics, and the dose of antibiotics (Zielezny et al., 2006). Selvam et al. (2012) demonstrated that the presence of antibiotics could affect the bacterial population at the higher concentrations (50 mg kg^{-1} OTC, 10 mg kg^{-1} SDZ, and 10 mg kg^{-1} CIP) as compared to low concentrations (5 mg kg^{-1} OTC, 1 mg kg^{-1} SDZ, and 1 mg kg^{-1} CIP). In addition, antibiotics were also shown to be harmful to microbial activities and could alter the microbial community composition resulting in the disturbance of the related biogeochemical processes (Roose-Amsaleg and Laverman, 2016). It was reported that denitrification rates were influenced by antibiotics at ultralow doses (1, 10, and 1000 ng L^{-1}) in soil (DeVries et al., 2015), and a synergistic inhibition effect was observed for multiple antibiotic exposures in the Yangtze Estuary sediments where five antibiotics (sulfamethazine, thiamphenicol, oxytetracycline, erythromycin, and norfloxacin) were used and their concentrations ranged from 0 to 89.1, 0 to 62.9, 0 to 22.5, 0 to 14.2 and 0–45.4 ng L^{-1}, respectively (Yin et al., 2017). Other studies have also shown that denitrification rates were reduced in response to various antibiotics in aquatic system (1.0 mg L^{-1} erythromycin, clarithromycin, and amoxycillin) (Costanzo et al., 2005), in groundwater environment (0.01 mg L^{-1} sulfamethazine and 1.0 mg L^{-1} chlortetracycline) (Ahmad et al., 2014), and in estuarine and coastal sediments (0.01, 0.1, 1, 10 mg L^{-1} thiamphenicol) (Yin et al., 2016). Until now, little has been reported about the effects of pig manure-derived antibiotic residues on anammox and denitrification rates in soils. Furthermore, the relationships between increased antibiotics and anammox or denitrification rates in the paddy soil are still obscure.

Although the direct determination of anammox and denitrification rates in soils is nearly impossible because of the high atmospheric background of dinitrogen (N_2), by using a ^{15}N tracer technique and a slurry experiment, the potential rates of anammox and denitrification can be determined in paddy soil (Zhao et al., 2015; Shan et al., 2016). In this study, a laboratory slurry-based ^{15}N tracer technique combined with membrane inlet mass spectrometry (MIMS) was applied to measure potential rates of anammox and denitrification in paddy soils from six long-term paddy experiment sites with three fertilization regimes (C, no fertilizers; N, mineral fertilizer; and NM, N plus pig manure). The aims were to i) investigate the effects of long-term pig manure application on the occurrence of antibiotics, abundance of ARGs, anammox and denitrification functional genes, and rates of anammox and denitrification ii) to explore the potential relationship between the manure-derived antibiotics and rates (anammox and denitrification) from different paddy soils in China after long-term pig manure application.

2. Materials and methods

2.1. Soil sampling

Soil samples were collected from the following six experimental sites with long-term manure applications in China: Jiangxi Jinxian (JXJX), Hunan Ningxiang (HNNX), Sichuang Suining (SCSN), Hunan Changde (HNCD), Zhejiang Haining (ZJHN) and Jiangxi Yingtan (JXYT). Soil samples were taken at surface 0–20 cm depth from each treatment with at least three replicates (ca. 2 to 4 Kg from each subsample). The experimental locations, fertilizer, input of manure applications and other information are listed in detail in Table 1 (Bi et al., 2009; Hao et al., 2008; Fan et al., 2015; Zhang et al., 2008; Liang et al., 2013; Liu et al., 2011). The experiments were continued for 34 years (JXJX site), 29 years (HNNX), 11 years (ZJHN), 34 years (SCSN site), 27 years (JXYT site) and 25 years (HNCD site) with treatments (C, no fertilizers; N, mineral fertilizer; and NM, N plus pig manure) arranged in a randomized complete block design with three replicates. Composted manure was used and mixed with soil by plowing twice per year, as local farmers had done before cultivation of crops in the long-term experimental sites. The amount of pig manure used in the experimental field was 22500 kg ha^{-1} y^{-1} dm in the JXJX site, 35333 kg ha^{-1} y^{-1} dm in the HNNX site, 15000 kg ha^{-1} y^{-1} dm in the SCSN site, 15000 kg ha^{-1} y^{-1} dm in the HNCD site, 12800 kg ha^{-1} y^{-1} dm in the ZJHN site, and 4500 kg ha^{-1} y^{-1} dm in the JXYT site (Table 1). The rotation of crops was rice\rice twice per year for the sites JXJX, HNCD, ZJHN and JXYT. While, the rotation of crops for the sites HNCD and SCSN was rice/corn and rice/wheat, respectively. Soil sampling time was from October to November 2015 after the harvest of rice. After collection of soil samples, they were divided into four portions, one of which was stored at 4 °C for measuring potential denitrification rates. A second portion was air-dried at room temperature for the analyses of soil chemical and physical properties as described below. A third portion was dried and sieved to <100 mesh for determination of the antibiotics and a fourth portion was frozen at −80 °C for molecular analysis.

2.2. Determination of chemical properties of soil

Chemical properties of soil samples were determined according to Shan et al. (2016). In brief, total N and C of the soils were measured by using an elemental analyzer (Elementar Cario EL III; Elementar Analysensysteme GmbH, Germany). Ammonium, nitrate and sulfate in the soil samples were measured on a continuous flow analyzer (SA1000; Skalar Analytical, Breda, the Netherlands). The soil organic carbon (SOC) content was determined via the $K_2Cr_2O_7$ oxidation method, and the dissolved organic carbon (DOC) content was determined by a total organic carbon analyzer (TOC-VCS/CP, Shimadzu, Japan). Soil pH was determined in a $CaCl_2$ solution at a

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