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Increased occurrence of heavy metals, antibiotics and resistance genes in surface soil after long-term application of manure

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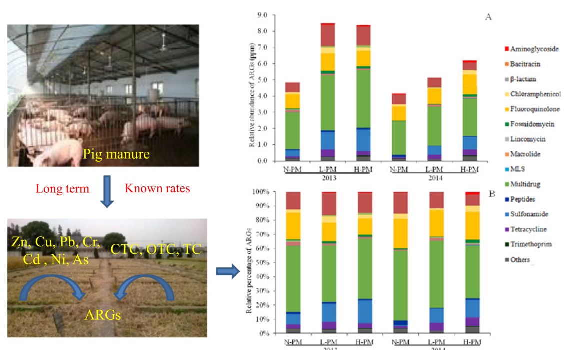
HIGHLIGHTS

- Metal concentrations in soil with manure application were higher than that without application.
- Chlortetracycline was the predominant antibiotic among three tetracyclines, while sulfonamides were not detected.
- The abundance of most *tet* and *sul* genes of sites with manure application was significantly higher than that of site without application.
- The abundance of ARGs positively correlated with soil organic matter, antibiotics, Cu, As and Zn levels.

GRAPHICAL ABSTRACT



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ABSTRACT

The purpose of this study was to investigate the impact of long-term application of pig manure on the accumulation of heavy metals, antibiotics and ARGs in surface soil sampled from the Jiaxing long-term field experimental site with three manure treatments, N-PM (0 kg/ha/y, dw), L-PM (7720 kg/ha/y, dw), and H-PM (11,580 kg/ha/y, dw), in 2013 and 2014. The results showed that most serious metal pollution of Zn and Cu was recorded in all manured samples in both years, and their contents exceeded the soil quality standards. Among the three tetracyclines, chlortetracycline was the predominant antibiotic detected with a range of 3.04–98.03 $\mu g \cdot kg^{-1}$ in 2013 and 28.67–344.74 $\mu g \cdot kg^{-1}$ in 2014 after long-term pig manure application. Q-PCR results showed that the average accumulation of ribosomal protection protein genes (*tet*M, *tet*O, *tet*Q and *tet*W) was lower than most of the efflux pump genes (*tet*A and *tet*G). The abundance of *tet* and *sul* genes of those sites with manure application was significantly higher than that of sites without manure application in both years. Metagenomics analysis of ARGs revealed that the abundance of multidrug resistance genes was the most abundant subtype, followed by fluoroquinolone, bacitracin, sulfonamide and tetracycline. There was a positive correlation between the levels of ARGs; soil organic matter, antibiotics, Cu, As, and Zn levels in both years. These results may shed light on the mechanism underlining the effects of long-term manure application on the occurrence and dissemination of ARGs in surface soil.

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1. Introduction

A large amount of livestock waste, especially pig manure, was produced every year in China and worldwide due to the emerging livestock

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industry production (Cheng et al., 2013; Peng et al., 2015). Manure application to agriculture fields is believed to be a good and inexpensive way to treat livestock waste and has been numerously reported in Germany (Heuer et al., 2011), the Netherland (Schmitt et al., 2006) and China (Ji et al., 2012; Cheng et al., 2013; Peng et al., 2015). As manure is a reservoir of resistant bacteria and antibiotic compounds (Ji et al., 2012), its application to agricultural soils is assumed to significantly increase the ARGs and resistant bacterial populations in soil (Fahrenfeld et al., 2014; Peng et al., 2015).

A large amount of metals and antibiotics are annually used in concentrated animal feeding operations (CAFOs) worldwide to treat animal diseases and promote animal growth, and for prophylactic, metaphylactic, and therapeutic purposes in animal husbandry (Hamscher et al., 2005). The use of antibiotics has been banned in the European Union since 2006, and recently its use for non-therapeutic purposes in the US has been stopped. However, antibiotics are still used in some parts of China. A previous report revealed that approximately 97,000 tons of antibiotics were used in the animal industry in China (Collignon and Voss, 2015). Most of these pollutants cannot be absorbed by animals and are released in the feces and urine, which then persist and accumulate in soils after manure application, causing a concerned problem (Ji et al., 2012). Surface soil is considered as a pool of residual antibiotics (Negreanu et al., 2012). Tetracyclines and sulfonamides have been frequently measured in soil and manure (Fang et al., 2015; X. Zhang et al., 2016, H. Zhang et al., 2016; Pan and Chu, 2017). Once added to the soil, antibiotics interact with the soil solid phase and are prone to microbial transformation. This biotransformation may result in a retransformation of metabolites into the parent compound similarly as in manure (Foerster et al., 2009; Zarfl et al., 2009). Meanwhile, low and elevated levels of antibiotics in the environment trigger the development of antibiotic-resistant microbial populations (Boxall et al., 2003). These antibiotics may even cause serious allergies or may be toxic to humans at significant concentrations (Kumar et al., 2005). However, surface runoff and particle-facilitated transport may disperse all antibiotics in the environment (Larsbo et al., 2008; Joy et al., 2013; Popova et al., 2013).

Zn and Cu were used as feed additives for animal growth promotion through antimicrobial activity mechanisms, and the highest accumulations found in animal manures were 4333.8 and 730.1 $mg\cdot kg^{-1}$, respectively (Ji et al., 2012). Anjum et al. (2011) detected Cr, Zn, Ni, Fe, Cu and Cd concentrations of 36.2, 42.5, 43.2, 241, 13.3, and 11.20 $mg\cdot kg^{-1}$, respectively, in cultivated soils. In the research of Ogiyama et al., the reported concentration of Zn and Cu in a manure-amended arable field ranged from 72 $mg\cdot kg^{-1}$ to 170 $mg\cdot kg^{-1}$, and 18 $mg\cdot kg^{-1}$ to 109 $mg\cdot kg^{-1}$, respectively (Ogiyama et al., 2005). In addition to emerging nontraditional ARGs contaminants, anthropogenic-derived sources of metals represent a major source of contamination in agricultural soils (Stepanauskas et al., 2005). Different from antibiotics, metals are not subject to degradation and can represent a long-term selection pressure. However, most studies of the effects of manure amendment on the occurrence of metals and antibiotics employ the investigation of grab samples or short-term laboratory studies. Meanwhile, most studies have focused on the total heavy metals with less attention to the bioavailable heavy metals.

Antibiotic resistance genes (ARGs) are considered as new emerging contaminants (Pruden et al., 2006). The diversity and abundance of various ARGs have been investigated in soils with manure application (Ji et al., 2012; Zhu et al., 2013). Hong and colleagues (Hong et al., 2013) observed an increased abundance of tetracycline resistance genes in soil after pig manure injection, and these genes remained elevated for up to 16 months. Antibiotics can be divided into nine categories, and in general, aminoglycosides, tetracyclines, sulfonamide, florfenicol, and quaternary ammonium compound resistance genes are measured. Sulfonamide and tetracycline resistance genes are frequently detected in soils from many sites (Schmitt et al., 2006; Walsh et al., 2011). Zhou et al. (2017) detected the spatial distribution of eight major genes (*tet*O, *tet*Q, *tet*W, *tet*M, *tet*B, *tet*T, *sul*I, *sul*II) in agricultural soil across China finding that the northeast region of China was a hot spot of sulfonamide resistance genes. *tet* and *sul* genes have also been reported as the most frequently detected ARGs in animal manures and livestock lagoons (Ji et al., 2012). The top three ARGs in the fecal samples from Chinese dairy farms are *cfx*A, *tet*Q and *tet*W (Zhou et al., 2016). At least 40 different tetracycline resistance (*tet*) genes have been characterized (Roberts, 2005), and three mechanisms have been identified: antibiotic efflux pumps, target modification with ribosomal protection protein, and antibiotic inactivation (Lambert, 2005). Instead of ARGs detection through qPCR, several other studies focused on the metagenomic approaches to study ARGs, because it provides detailed insights into ARGs information. Metagenomic techniques have been used to study ARGs for estuary, deep ocean sediments (Chen et al., 2013), activated sludge, coastal sediments (Cai and Zhang, 2013), domestic wastewater (Christgen et al., 2015), and long-term field application of sewage sludge (Chen et al., 2016).

Soil becomes the primary sink of metals and antibiotics because some classes of antibiotics, such as tetracyclines (TCs), can be absorbed to soil particles strongly and are resistant to biodegradation (Ji et al., 2012), which may cause the more serious problem of ARGs in soil and the surrounding environment (Pruden et al., 2013). Recently, an increasing number of reports have suggested that the increasing abundance of ARGs may be due to waste releases of heavy metals; there are known links between heavy metals and antibiotic resistance maintenance and proliferation (Stepanauskas et al., 2006; Knapp, 2011; Ji et al., 2012). *sul*I and *sul*II are strongly correlated with the levels of Cu, Zn and Hg. Many ARGs are positively correlated with soil Cu levels, with approximately half being highly significant ($P < 0.05$). Moreover, Cr, Ni, Pb and Fe are also significantly correlated with specific ARGs (Knapp et al., 2011). However, some other studies have demonstrated that the presence of ARGs is relatively independent of their respective antibiotic inducer (Ji et al., 2012).

Overall, an understanding of the occurrence of metals, antibiotics, and ARGs in soil in relation to manure application at known rates is important in the development of manure management practices. In the present study, we determined the impact of long-term (9 years) pig manure application on (1) the residual amounts of different heavy metals and classes of antibiotics; (2) the diversity and abundance of ARGs; (3) the correlations between antibiotic residues, ARGs, soil properties, and heavy metals. The results of the study will provide a comprehensive understanding of the ecological risk caused by long-term pig manure application.

2. Materials and method

2.1. General description of long-term field experiment site and sampling

The field experimental site in Jiaxing, China (30°50′19.78″ N, 120°43′4.59″ E), operated since 2005, with the cropping system of rice–rape rotation, was selected in the present study. Rice-growing season runs from June to November and rape-growing season runs from November to May of the second year. The soil type is gleyed paddy soil with a weakly alkalescent pH. The mean annual precipitation in this site is 1200 mm and the average annual temperature is 15.7 °C. Three treatments, namely, N-PM, L-PM, and H-PM representing no manure, low amount of manure, and high amount of manure applied, respectively, were arranged in a completely randomized block design, each with three replicates. The manure was applied into the soil surface with a large bucket and a small shovel and was ploughed for incorporation with the soil, similar to the local farmers' procedures. Generally, manure was applied twice a year, and total applied manure was 7720 and 11,580 kg/ha/y dw in the L-PM and H-PM treatments, respectively. Concentrations of heavy metals and antibiotics in the manure are given in Table S3. Detailed information of the soil texture, properties, and manure applications is given in Table 1. Surface soils were collected at a depth of 0–5 cm after crops were harvested in November 2013 and

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