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Soil types influence the fate of antibiotic-resistant bacteria and antibiotic resistance genes following the land application of sludge composts

Junya Zhang, Qianwen Sui, Juan Tong, Hui Zhong, Yawei Wang, Meixue Chen, Yuansong Wei*

State Key Joint Laboratory of Environmental Simulation and Pollution Control, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

Department of Water Pollution Control Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

University of Chinese Academy of Sciences, Beijing 100049, China

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ABSTRACT

Sewage sludge was generally considered a significant reservoir of antibiotic resistance genes (ARGs) and could enter agricultural systems as fertilizer after composting. Soil types and the discrepancy of sludge composts could have influenced the fate of antibiotic-resistant bacteria (ARB) following the land application of sludge composts, which deserved to be clarified. Thus, the fate of ARB and ARGs following the land application of three types of sludge composts (A, B, and C) to three different soils (red soil, loess, and black soil) was investigated. The results showed that *tetX*, which was enriched the most during composting, did not affect the soil resistome, whereas *tetG* did. Soil types influenced the dynamics of ARB and ARGs significantly, whereas no significant difference was observed among compost types. The advantage of reducing ARGs during the composting process in compost B did not extend to land application. Land application of composts influenced the microbial community significantly at the early stage, but the microbial community returned to the control pattern gradually. Changes in the microbial community contributed more to the dynamics of ARGs in red and black soil compared with other factors, including co-selection from heavy metals, horizontal gene transfer, biomass and environmental factors, whereas horizontal gene transfer, reflected by *intI1* levels, contributed the most in loess.

1. Introduction

In a world without effective antibiotics, an estimated loss of 11 million people and a reduction in global economy size by 0.1–3.1% are expected to occur by 2050 (Fitchett and Atun, 2016). Generally, antibiotics and their metabolites are discharged into the sewage system after usage (Wellington et al., 2013), and thus sewage sludge has become one of the major contributors to the increased environmental burden of antibiotic resistance (Mao et al., 2015; Munir et al., 2011). Sludge-associated antibiotics and antibiotic-resistant bacteria (ARB) and genes (ARGs) enter agricultural systems when sludge is used as fertilizer, which boosts the worldwide dissemination and further development of antibiotic resistance (Bondarczuk et al., 2016; Xie et al., 2016).

Municipal wastewater treatment plants (WWTPs) and their possible link to the global threat of antibiotic resistance have been studied widely (Kumaraswamy et al., 2014; Zhang et al., 2016a), where sewage sludge contributes more than effluent to the release of ARGs into the environment (Mao et al., 2015; Munir et al., 2011). In China, sludge production grew by approximately 13% annually from 2007 to 2013 and 6.25 million tons of sewage sludge (dry solids) were produced in 2013 (Yang et al., 2015). The large amounts of sewage sludge produced annually pose a challenge regarding the environmental burden of an antibiotic resistome, which refers to the collection of ARGs in an ecological niche (Calero-Caceres et al., 2014). Because sewage sludge contains valuable nutrients and organic matter, it has been frequently used as soil fertilizer, representing another major pathway for ARGs to spread into farmlands (Bondarczuk et al., 2016; Burch et al., 2014; Munir and Xagoraraki, 2007; Singer et al., 2016). Sewage sludge produced in the European Union is expected to reach 13 million tons in 2020 and approximately 44% is expected to be recycled for land application (Calero-Caceres et al., 2014). Nevertheless, information regarding expansion of antibiotic resistance in the environment due to the land application of sewage sludge products remains scarce (Bondarczuk et al., 2016; Maria and Husman, 2016).

Composting is an effective and widely used method for sludge treatment, where sewage sludge is transformed into mature products, such as humus, for soil amendment or fertilization (Wei et al., 2000).

* Corresponding author at: State Key Joint Laboratory of Environmental Simulation and Pollution Control, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.

E-mail addresses: jyzhang@rcees.ac.cn (J. Zhang), yswei@rcees.ac.cn (Y. Wei).

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Although composting has been demonstrated to enrich ARGs in sludge, ARGs decrease in abundance after the addition of natural zeolite (Su et al., 2015; Zhang et al., 2016a). Concerning the fate of ARB and ARGs introduced through the land application of sewage sludge, various studies have focused on bio-solids after anaerobic digestion, storage, or lime stabilization (Burch et al., 2014; Q. Chen et al., 2016; L. Chen et al., 2016; Munir and Xagoraraki, 2007; Xie et al., 2016). Nevertheless, there is insufficient information on the land application of sludge compost, thus emphasizing the urgent need to broaden our knowledge in this field (Bondarczuk et al., 2016). Additionally, inconsistent results have been obtained from different studies. For instance, one research group demonstrated that long-term application of sewage sludge increases the abundance of ARGs in soil (Q. Chen et al., 2016; L. Chen et al., 2016), whereas another study found elevated levels of ARGs in amended soil samples in one site, with no significant increase in another site (Munir and Xagoraraki, 2007). These observations may be due to the differences in soil types analyzed between these studies, as the microbial community, soil texture and environmental factors vary between soil types, which could have influenced the fate of ARGs. Moreover, the mature degree and microbial community varies between different sludge compost products, which could have also contributed to the changes of ARGs. Therefore, determining the influence of soil types and the discrepancy between different sludge composts on the development of antibiotic resistance should also be emphasized, and performing pot experiments would be an excellent method of controlling causality.

Moreover, sewage sludge is generally considered a typical hotspot of co-contamination with antibiotics and heavy metals (Pruden et al., 2013). The ability of heavy metals to act as co-selection or cross-selection agents of antibiotic resistance has been widely demonstrated (Di et al., 2016; Hu et al., 2016; Lloyd et al., 2016; Pal et al., 2015; Yu et al., 2017; Zhang et al., 2016a; Zhao et al., 2017), as amounts of heavy metals enter the soil with the land application of sludge composts. Nonetheless, the effects of heavy metals on the evolution of antibiotic resistance following the land application of sludge composts are still unclear.

A previous report suggested that increased efforts should be devoted to reducing the abundance of ARGs in sewage sludge before land application (Q. Chen et al., 2016; L. Chen et al., 2016). However, whether the advantages of reducing ARG levels after sludge treatment could be extended to land application remains to be elucidated. Thus, pot experiments simulating the land application of three different sludge composts to three soil types were employed to investigate the dynamics of ARB and ARGs, to evaluate and distinguish the effects of soil and compost types on these dynamics, to elucidate whether the benefits of reducing ARG levels during sludge composting extends to land application, and to determine the fate of heavy metal resistance and changes in microbial community to further clarify ARG profiles.

2. Materials and methods

2.1. Pot experiments

Pot experiments were carried out with three soil types fertilized by three different sludge composts. To provide integrated soil agro-habitats, radish was planted. The dose was determined by the requirements for radish planting with N at 150 mg/kg dry weight (DW), P_2O_5 at 150 mg/kg DW and K_2O at 100 mg/kg DW. Soil types were red soil from Jiangxi Province (S1), loess from Shanxi Province (S2), and black soil from Jilin Province (S3) in China, whereas the three different sludge composts were the control (A), compost with addition of natural zeolite (B), and compost with addition of nitrification inhibitor (C) that were manufactured as previously reported (Zhang et al., 2016a). Compost maturity was different, with B being the highest followed by A and then C. Through the addition of natural zeolite (B), sludge composting led to reduced ARG levels (Zhang et al., 2016a, 2016b). S1 was collected from a site at E116°, N28.7° and classified as humic acrisol with a soil texture of loamy clay, S2 (E109°, N36.8°) was classified as calcaric cambisols and sandy loam, while S3 (E125.4°, N43.8°) was classified as histosol and clay loam. The soils were air dried and then crushed to sieve through a 2-mm mesh before use; sludge composts were also crushed before filtering through the mesh. Detailed information regarding sludge composts and soils is listed in Table S1. Sludge composts were added once at the beginning, whereas there were two rounds of radish planting. Soil without sludge compost was regarded as the control group and the group with the addition of chemicals, including urea, $NaH_2PO_4{\cdot}2H_2O$, and K_2SO_4, was designated as the NPK group. The volume of each pot was approximately 2.5 L with a diameter of 15 cm and 2.5 kg of soil was loaded into each pot. Each treatment was conducted in triplicate, and thus a total of 45 ($3 \times 5 \times 3$) pots were used. Milli-Q water was used for irrigation throughout the experiments to avoid introducing ARGs.

The experiment lasted for 172 days and samples were collected on days 1, 6, 12, 22, 43, 82, 117, and 172. Sampling was conducted at a depth of 10–15 cm and three random sites were chosen from each pot. The soils were mixed thoroughly and approximately 5 g of soil was collected from each pot. Soils from triplicate pots for each treatment were then mixed as a representative sample. There were 120 samples collected in total and then pH, total organic carbon (TOC), moisture content, volatile solids (VS), heavy metals and the C/N ratio were determined as previously described (Zhang et al., 2016c, 2015). The results are shown in Table S2, S3, and S4 for samples from S1, S2, and S3, respectively.

2.2. DNA extraction and microbial community analysis

Total genomic DNA was extracted in triplicate from 0.5 g of sample using the FastDNA Spin Kit for soil (MP Biomedical, France). The triplicate DNA extracts were then combined for further analysis. Extracted genomic DNA was detected and quantified by electrophoresis using a 1% agarose gel and a NanoDrop 2000 (Thermo Scientific, USA), respectively, and then stored at −20 °C for further analysis. PCR primers 515F/806R targeting the bacterial and archaeal 16S V4 genomic region were selected for microbial community analysis (Caporaso et al., 2010). The Beijing Genomics Institute (BGI) provided small-fragment library construction and pair-end sequencing using an Illumina MiSeq sequencing system (Illumina, USA). Pairs of reads from the original DNA fragments were merged using FLASH (Magoč and Salzberg, 2011) and then filtered using QIIME quality filters. PCR chimeras were filtered out in UCHIME (Edgar et al., 2011) and then the sequences were uploaded to MG-RAST (http://metagenomics.anl.gov) under the project numbers of 17,760, 17,764, 17,767, 17,768, and 17,776 for NPK, control, A, B, and C samples, respectively. Taxonomic classification of the sequences in each sample was performed individually using the Ribosomal Database Project (RDP) Classifier where sequences of different taxonomy levels were assigned a bootstrap cutoff value of 50% as suggested by the RDP (Wang et al., 2007). Then, operational taxonomic units (OTUs) were denoised using package ade4 of R software and OTUs with an abundance below 0.01% were removed.

2.3. Quantitative PCR (qPCR)

Seven frequently detected ARGs (*tetG*, *tetM*, *tetX*, *ermB*, *ermF*, *ereA* and bla_{TEM}), the integron I gene (*intI1*), four heavy metal resistance genes (MRGs; *arsC*, resistance to As; *merA*, resistance to Hg; *czcA*, resistance to Co/Zn/Cd; *pcoA*, resistance to Cu), and 16S rRNA were quantified. *intI1* and MRGs were selected to represent the potential of horizontal gene transfer (HGT) and co-selection with heavy metals, respectively (Casellas et al., 2014; Pal et al., 2015). Plasmids containing these specific genes served as standards to construct qPCR standard curves. The reaction system and conditions are described in detail elsewhere (Zhang et al., 2016a) and the reactions were conducted on an