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Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil

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ABSTRACT

Sewage sludge and manure are common soil amendments in crop production; however, their impact on the abundance and diversity of the antibiotic resistome in soil remains elusive. In this study, by using high-throughput sequencing and high-throughput quantitative PCR, the patterns of bacterial community and antibiotic resistance genes (ARGs) in a long-term field experiment were investigated to gain insights into these impacts. A total of 130 unique ARGs and 5 mobile genetic elements (MGEs) were detected and the long-term application of sewage sludge and chicken manure significantly increased the abundance and diversity of ARGs in the soil. Genes conferring resistance to beta-lactams, tetracyclines, and multiple drugs were dominant in the samples. Sewage sludge or chicken manure applications caused significant enrichment of 108 unique ARGs and MGEs with a maximum enrichment of up to 3845 folds for *mexF*. The enrichment of MGEs suggested that the application of sewage sludge or manure may accelerate the dissemination of ARGs in soil through horizontal gene transfer (HGT). Based on the co-occurrence pattern of ARGs subtypes revealed by network analysis, *aacC*, *oprD* and *mphA-02*, were proposed to be potential indicators for quantitative estimation of the co-occurring ARGs subtypes abundance by power functions. The application of sewage sludge and manure resulted in significant increase of bacterial diversity in soil, *Proteobacteria*, *Acidobacteria*, *Actinobacteria* and *Chloroflexi* were the dominant phyla (>10% in each sample). Five bacterial phyla (Chloroflexi, Planctomycetes, Firmicutes, Gemmatimonadetes and Bacteroidetes) were found to be significantly correlated with the ARGs in soil. Mantel test and variation partitioning analysis (VPA) suggested that bacterial community shifts, rather than MGEs, is the major driver shaping the antibiotic resistome. Additionally, the co-occurrence pattern between ARGs and microbial taxa revealed by network analysis indicated that four bacterial families might be potential hosts of ARGs. These results may shed light on the mechanism underlining the effects of amendments of sewage sludge or manure on the occurrence and dissemination of ARGs in soil.

1. Introduction

The persistence and spread of antibiotic resistance genes (ARGs) in the environment are promoted by anthropogenic activities such as sewage sludge applications in arable land (Threedeach et al., 2012). Municipal wastewater appears to be a significant reservoir of ARGs (Lachmayr et al., 2009), however, most ARGs cannot be effectively removed by wastewater treatment processes. ARGs have been detected at all stages of the municipal wastewater treatment process (Lachmayr et al., 2009; Munir et al., 2011), and the vast majority of ARGs are discharged from the municipal wastewater treatment process together with sewage sludge (Bondarczuk et al., 2016). Land application is one of the key management approaches of sewage sludge from wastewater treatment plants (Kim and Aga, 2007). This practice is cost effective with added benefits from its residual nutrients (Candela et al., 2007), but it also represents a major pathway of ARGs spread onto farmlands (Chen and Zhang, 2013). Land application of sewage sludge may result in the spread of ARGs in soil and to underground water (Threedeach et al., 2012). More importantly, bacteria have been shown to readily share genetic information by HGT via mobile genetic elements (MGEs) including plasmids, transposons, and integrons (Zhu et al., 2013; Pruden et al., 2006), allowing the transfer of resistance genes from sewage sludge microorganisms to indigenous environmental bacteria (Gaze et al., 2011). Sewage sludge has been shown to be a hotspot for bacteria carrying ARGs and MGEs (Su et al., 2015) and its application may enhance the HGT of ARGs in soil, although the bacterial populations of sewage sludge and soil may be quite distinct (Hammesfahr et al.,

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2008). Nevertheless, the fate of ARGs in arable soils following the long-term application of sewage sludge still remains poorly understood (Burch et al., 2014).

Animal manure represents a major source of antibiotics and ARGs in the environment (Wang et al., 2011; Binh et al., 2008, Binh et al., 2009). Manure disposal may transfer ARGs into the environment through a variety of pathways connected with common practice — land application (Joy et al., 2013; Wu et al., 2010). As a common practice, manure application introduces not only nutrients and organic matter for crop growth, but also antimicrobial agents into arable soil (Xiong et al., 2015; Cheng et al., 2013). Several studies have examined the effects of manure application on antibiotic resistance in soil from various perspectives, showing a stimulation effect of agricultural usage of manure containing antibiotics on the dissemination of antibiotic resistance in soil bacterial communities (Jechalke et al., 2013; Tang et al., 2015; Kyselková et al., 2015).

In general, for minimizing the effects of fluctuation of soil environmental conditions on microbial community, laboratory microcosm experiments have been commonly used in many studies (Chen et al., 2014; Chen et al., 2015). For instance, some studies have investigated the impacts of the application of sewage sludge on soil ARGs in a microcosm incubation experiment (Burch et al., 2014). However, due to the different conditions between laboratory and field experiments (Rahube et al., 2014), the results of previous studies may not completely reflect the impacts in field. In this study, we compared the impact of the application of sewage sludge with chicken manure (served as a positive control) and chemical fertilizer (with no obvious antibiotic-related properties) on soil resistome. To the best of our knowledge, there are only a few studies that systematically evaluate the fate and transport of ARGs in soil following the long-term application of sewage sludge. Therefore, by using Illumina sequencing and high throughput quantitative PCR (HT-qPCR) including 295 primer sets targeting almost all major classes of ARGs, the objectives of this study were to (1) evaluate the effects of long-term application of sewage sludge and manure on the abundance and diversity of ARGs, bacterial communities structure and potential bacterial pathogens in soil; (2) using network analysis to explore the indicator ARGs for the quick estimation of ARGs in environmental samples; (3) explore the co-occurrence patterns among ARGs subtypes, and between ARGs and microbial populations.

2. Materials and methods

2.1. Sampling site and sample collection

The field experiment was set up in a fluvo-aquic soil in the long-term experiment station of the Chinese Academy of Agricultural Sciences (CAAS), in Dezhou of Shandong Province, China (37°20′ N, 116°38′ E). The experiment began in 2006, aimed at investigating the N and P input-output balances and soil P accumulation when sewage sludge and chicken manure were applied to a soil. Totally, eight treatments (i.e. CK, 0.5N, 1N, 0.5SS, 1SS, 2SS, 4SS, 1CM) were carried out and the detailed information about the design of this field experiment is shown in Figure S1 and Table 1. The basic information about the soil, sewage sludge and chicken manure is listed in Table S1. We collected 24 samples (surface soil 0–15 cm) covering all treatments with triplicate in August 2015 (about ten months after the application of sewage sludge and chicken manure). The soils were immediately frozen on dry ice, transported to laboratory within 24 h and stored at −80 °C before analysis.

2.2. DNA extraction

DNA was extracted from 0.5 g frozen samples using a FastDNA® Spin Kit for soil (MP Biomedical, Santa Ana, California, USA) according to the manufacturer's instructions. The quality of the DNA was checked by spectrophotometric analysis using NanoDrop ND-1000 (Nanodrop, USA) and the concentration of DNA was determined using QuantiFluor® dsDNA system (Promega, Madison, Wisconsin, USA) using fluorometric analysis with a microplate reader (Spectramax M5, USA). Soil DNA was stored at −20 °C until use.

Table 1
The application rates of sewage sludge, chicken manure and chemical fertilizers in different treatments.

Treatment	Sewage sludge[a]	Chicken manure	Urea	Superphosphate	Potassium sulphate
	$t \cdot hm^{-2}$	$t \cdot hm^{-2}$	$kg \cdot hm^{-2}$	$kg \cdot hm^{-2}$	$kg \cdot hm^{-2}$
CK	0	0	0	600	240
0.5N	0	0	65.25	600	240
1N	0	0	130.50	600	240
0.5SS	4.5	0	65.25	600	240
1SS	9	0	65.25	600	240
2SS	18	0	65.25	600	240
4SS	36	0	65.25	600	240
1CM	0	10	65.25	600	240

[a] Before wheat sowing every year (mid of October), the air-dried sewage sludge was applied as the basic fertilizer incorporated into the soil.

2.3. High-throughput quantitative PCR

High-throughput qPCR reactions were performed using the Wafergen SmartChip Real-time PCR system. This system can be used for large scale gene expression studies, which can process 5184 nanowell reactions per run (Wang et al., 2014). A total of 296 primer sets were used, including 295 primer sets targeting almost all major classes of ARGs and MGEs and 1 16S rRNA gene (Su et al., 2015). After the initial enzyme activation at 95 °C for 10 min, 40 cycles of the following program were used for amplification: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s. The melting process was automatically generated by Wafergen software. After that, results were analyzed with SmartChip qPCR Software excluding the wells with multiple melting peaks or amplification efficiency beyond the range (90%–110%) and then screened with conditions that (1) a threshold cycle (C_T) must be <31 and (2) positive samples should have three replicates simultaneously. Relative copy number was calculated referring to a previous study (Eq.1) (Ou-yang et al., 2015). Besides, a comparative C_T method was used to calculate the ARGs' fold change (FC value) of amended samples compared to the control (Eq.2) (Schmittgen and Livak, 2008). The detection limit C_T (31) was taken as a replacement for the genes with no amplification.

$$\text{Gene copy number} = 10^{(31-C_T)/(10/3)} \quad (1)$$

$$\begin{aligned} \Delta C_T &= C_{T(ARG)} - C_{T(16S)} \\ \Delta\Delta C_T &= C_{T(Target)} - \Delta C_{T(Ref)} \\ FC &= 2^{(-\Delta\Delta C_T)} \end{aligned} \quad (2)$$

Where C_T is the threshold cycle, ARG is one of the 295 antibiotic resistance gene assays, 16S is the 16S rRNA gene assay, Target is the amended sample, Ref is the control sample.

2.4. Illumina sequencing and analyses of 16S rRNA gene

The V4-V5 region of bacterial 16S rRNA was selected for amplification with primers F515: GTGCCAGCMGCCGCGG and R907: CCGTCAAT TCMTTTRAGTTT. To pool all samples for one run of Illumina sequencing, the reverse primers were designed with 24 unique barcodes. After the initial enzyme activation at 95 °C for 5 min, 35 cycles of the following program were used for amplification: 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Then, the amplification products were purified and submitted to Illumina Hiseq2000 platform (Novogene, Beijing, China) for sequencing. QIIME quality filters filtered the raw reads. The generated