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Fate of metal resistance genes in arable soil after manure application in a microcosm study

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ABSTRACT

Manure application contributes to the spread and persistence of metal resistance genes (MRGs) in the environment. We investigated the fate of copper (Cu) and zinc (Zn) resistance genes (*pcoA*, *pcoD* and *zntA*) in arable soil after Cu/Zn-containing manure application. Manure with or without addition of metals (Cu/Zn) was added in a soil microcosm over 2 months. Soil samples were collected for analysis on day 0, 30 and 60. The abundances of all MRGs (*pcoA*, *pcoD* and *zntA*) in manure group were significantly higher than those in untreated soil and manure+metals groups. All MRGs dissipated 1.2–1.3 times faster in manure group (from $-90\pm8\%$ to $-93\pm7\%$) than those in manure+metals group (from $-68\pm8\%$ to $-78\pm5\%$). The results indicated that manure from healthy pigs contributed to the occurrence of metals (Cu/Zn) and MRGs (*pcoA*, *pcoD* and *zntA*) in arable soil. The significant effects of manure application on the accumulation of *pcoA*, *pcoD* and *zntA* lasted for 1–2 months. Cu/Zn can slow down the dissipation of *pcoA*, *pcoD* and *zntA* after manure application. This is the first report to investigate the fate of MRGs in soil after manure application.

1. Introduction

Heavy metals from the polluted environment are hazardous to public health. Copper and zinc (Cu/Zn) are widely distributed in the environment and are often found in human managing components such as waste water treatment plants and farm lands. Cu/Zn are required in trace amounts for organisms' growth, but they are toxic when they are in excess. Evidence suggests that Cu/Zn pollution is promoted by anthropogenic activities, such as smelting, mining, and agricultural practices, including the usage of Cu/Zn-based pesticides and preservatives (Clausen et al., 2011; Percival and Outridge, 2013). It is a common practice that high pharmacologically effective concentrations of Cu/Zn (up to 250 and 3000 mg kg^{-1}, respectively) (Hill et al., 2000; Pérez et al., 2011) are added in animal diets for growth promotion and disease control. The practice discharges large amounts of Cu/Zn into the environment through manure application. High concentrations of Cu/Zn in manure (22–3388 and 93–8239 mg kg^{-1}, respectively), manure-amended soils (37–50 and 76–263 mg kg^{-1}, respectively) and even in edible crops (47 and 10 mg kg^{-1}, respectively) have been found (Hölzel et al., 2012; Ji et al., 2012; Legros et al., 2013), which poses a direct risk to public health via various exposure pathways, including consumption of food. Cu/Zn also pose a risk of damage to the ecosystem; e.g., they perturb soil bacterial structure and function. What's more, Cu/Zn promote occurrence and spread of resistance to metals, cross-resistance to antibiotics, as well as resistance to multidrug (Bednorz et al., 2013; Jacob et al., 2010; Li and Ramakrishna, 2011).

There is a need for better understanding of the fate of metal resistance genes (MRGs) in human-impacted environments. Cu/Zn contamination poses a long-standing effect on micro-organisms (Giller et al., 2009), including selective pressure for resistance. Bacterial resistance to Cu/Zn has been identified in isolates of animals (Cavaco et al., 2010; Mazaheri Nezhad Fard et al., 2011) and in the environment (Moskot et al., 2012). *Escherichia coli* (*E. coli*) is one of the key gastrointestinal microbiota with pathogenicity and commensalism, and is also ubiquitous in the environment. Metal resistance mechanisms such as *pcoAD* (plasmid-borne Cu resistance) (Bondarczuk and Piotrowska-Seget, 2013) and *zntA* (conferring resistance to Zn) (Rensing et al., 1997) were used by *E. coli* against the adverse effects from Cu/Zn. *pcoA* encodes a periplasmic multi-copper oxidase, while *pcoD* (a putative internal membrane protein) interacts with *pcoC* in Cu translocation into the cytoplasm. *zntA* has been proved to be responsible for Zn tolerance in *E. coli* (Beard et al., 1997; Rensing et al., 1997).

Abbreviations: MRGs, metal resistance genes; Cu/Zn, copper/zinc; *E. coli*, *Escherichia coli*; FAAS, Flame Atomic Absorption Spectrometry; ARGs, antibiotic resistance genes

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Increased resistance to Cu is often closely correlated with elevated resistance to Zn (Díaz-Raviña et al., 1994). Although those resistance genes conferring Cu/Zn-resistance are often found in bacteria, little is known about the distribution and fate of those genes via manure into arable soils. Up to 99% of soil bacteria can't be cultivated by conventional culture-based methods. Culture-independent method such as quantitative PCR allows sensitive quantization of bacterial resistance genes in recent studies (Fahrenfeld et al., 2014; Zhu et al., 2013).

The aim of the present study was to investigate the fate of MRGs (*pcoA*, *pcoD* and *zntA*) introduced by Cu/Zn-containing manure in arable soils over 2 months in a microcosm study. We used a culture-independent method to quantify the relative abundance of *pcoA*, *pcoD* and *zntA*.

2. Materials and methods

2.1. Microcosm set-up and treatment

Manure was collected from healthy, mature pigs at a typical pig farm. Arable soil was collected from farm lands at South China Agriculture University, Guangzhou, China. The soil type was loam with pH 7.13 and particle diameter (<0.01 mm) 51.7%. 1500 g of 2-mm-sieved soil and 60 g of manure (4%, w/w) (Heuer et al., 2011) were mixed and added in each pot in a microcosm. Three treatments were performed in three replicates: untreated soil, manure and manure+metals (Cu and Zn). Metals (Cu 100 mg kg^{-1} and Zn 300 mg kg^{-1}) were spiked in the soil in solution forms. All pots were incubated in the dark at approximately 20 °C. Moisture content was maintained at approximately 55% of the soil's water holding capacity. Water was added to the soil surface twice a week to compensate for the weigh loss of the microcosm. Soil samples were taken at the time points of day 0 (at 24 h), 30 and 60.

2.2. Metal analysis

0.25 g of soil subsample was digested in H_2O_2/HF/HNO_3 mixture (1: 2: 5 ml) by using a microwave digester (Milestone, USA). Digestion conditions were as follows: 180 °C for 7 min; 200 °C for 7 min; 220 °C for 14 min. The digested solution was heated to dryness and adjusted to 50 ml with HNO_3: water (2‰). The concentrations of Cu and Zn were simultaneously detected by using Flame Atomic Absorption Spectrometry (FAAS, Varian 220 FS, Palo Alto, USA).

2.3. DNA extraction and PCR

DNA of soil samples was extracted by using Power Soil DNA Kit (MO BIO) according to the manufacturer's instruction. The presence of MRGs (*pcoA*, *pcoD* and *zntA*) and *16S rRNA* gene was determined by PCR. The primers for PCR are shown in Table 1.

Table 1
Primers for PCR and real-time qPCR.

Target genes	Primers	Amplicon size (bp)	References
pcoA	F: GCTGCAGATGGCCAGTATGTAAA R:CCCTCGAGCGTAACCGGTCC	147	This study
pcoD	F:ATAACTTCAAGCCGGGGACCCAG R:AATGCACAGAGCGTCATTGT	245	This study
zntA	F:GGTCGGGTCTGGCATTGAAG R:TTGCAGCATCGGCGCGCAGGGTA	197	This study
16S rRNA gene	F:GGTAGTCYAYGCMSTAAACG R:GACARCCATGCASCACCTG	263	(Bach et al., 2002)

Conserved sequences of *pcoA*, *pcoD* and *zntA* were assigned by BLAST similarity with homologs in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). PRIMER 5.0 software was used to design the primers, and primer quality was evaluated by Oligo 7.0 software. The 25 μL of PCR mixture consisted of 1 μL DNA template, 0.5 μL of each primer (10 μM), 2.5 μL 10 × Ex Taq Buffer (Mg^{2+} Plus), 0.125 μL TaKaPa Ex Taq (5 U μL^{-1}), 2 μL dNTP Mixture (2.5 mM), and 18.375 μL ddH_2O. PCR procedure was as follows: initial 94 °C denaturation for 5 min, followed by 35 cycles consisting of denaturation (94 °C for 30 s), annealing for 30 s (56.3 °C for *pcoA* and *pcoD*, 58.6 °C for *zntA* and 62 °C for *16S rRNA* gene, respectively), extension (72 °C for 45 s), and a final extension step (72 °C for 10 min).

2.4. Real-time qPCR

Real-time qPCR using corresponding primers (Table 1) was conducted on a Bio-Rad IQ5 instrument (Bio-Rad Company, USA). PCR products were cloned into plasmids to generate standard curves for qPCR. To minimize the inhibition of sample matrix, 1/100 dilution of extracted DNA was used for quantification. The 20 μL of qPCR mixture consisted of 10 μL SYBR® Premix Ex Taq II (TaKaRa, Dalian, China), 1 μL template DNA, 0.8 μL of each primer (10 μM), and 7.4 μL molecular biology-grade water. qPCR procedure consisted of initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing at 60 °C for 30 s, then at 72 °C for 45 s with reading plate after each cycle. The positive control had the DNA from PCR products, and negative control had nuclease-free water. The specificity of the qPCR amplifications was checked by melting curves and gel electrophoresis. To minimize the variance caused by overall extraction efficiencies, total bacterial community, and possible sample degradation, the relative abundance of MRGs was obtained by normalizing their copy numbers to those of the *16S rRNA* genes.

2.5. Statistical analysis

All statistical analyses were performed with SPSS software (version 18.0, SPSS Inc., Chicago, IL, USA). Significant differences of relative abundance of MRGs at $p < 0.05$ between treatments were calculated by one-way ANOVA/LSD post-hoc test. Copies of MRGs were normalized (copies of MRGs/ copies of *16S rRNA* gene) and then log transformed for linear-regression (regression coefficient: estimates and model fit). The statistical significance of dissipation rate was analyzed by general linear model- Univatiate procedure.

3. Results

3.1. Concentrations of Cu and Zn

Initial concentrations of Cu and Zn in different treatments are shown in Table 2. The concentrations of Cu and Zn (11.3 ± 0.5 mg kg^{-1} and 34.6 ± 1.0 mg kg^{-1}, respectively) in manure group were higher than those (3.0 ± 0.2 mg kg^{-1} and 19.4 ± 0.5 mg kg^{-1}, respectively) in untreated soil group.

Table 2
Initial concentrations of Cu and Zn (day 0).

Treatments	Concentrations (mg kg^{-1})	
	Cu	Zn
Untreated soil	3.0 ± 0.2	19.4 ± 0.5
Manure	11.3 ± 0.5	34.6 ± 1.0
Manure+Metals	111.6 ± 4.5	340.5 ± 7.9