Soil Biology & Biochemistry 114 (2017) 229–237

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

# Do manure-borne or indigenous soil microorganisms influence the spread of antibiotic resistance genes in manured soil?

Qing-Lin Chen [a, b], Xin-Li An [a, b], Hu Li [a], Yong-Guan Zhu [a, c], Jian-Qiang Su [a], Li Cui [a, *]

[a] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, 1799 Jimei Road, Xiamen 361021, China
[b] University of Chinese Academy of Sciences, 19A Yuquan Road, Beijing 100049, China
[c] State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

ARTICLE INFO

*Article history:*
Received 12 January 2017
Received in revised form
18 July 2017
Accepted 23 July 2017
Available online 29 July 2017

*Keywords:*
Antibiotic resistance genes
Spread and fate
Manure-borne microorganisms
Manured soil
HT-qPCR

ABSTRACT

Manure application is a common practice that not only adds nutrients and organic matter to arable soils for crop growth, but also introduces antibiotic resistance genes (ARGs), posing a potential risk to human health. To investigate the mechanisms underlying the spread of ARGs in manured soil, especially the impact of manure-borne and indigenous soil microorganisms, a microcosm experiment with four specially designed treatments over a period of two months was conducted, including soil, soil with irradiated pig manure, irradiated soil with pig manure, and soil with pig manure. A total of 240 unique ARGs were detected via a high-throughput quantitative PCR (HT-qPCR) targeting almost all major classes of ARGs. Manure application significantly increased the diversity and abundance of ARGs in soil ($P < 0.01$), and also markedly shifted the bacterial composition that was significantly correlated with ARGs profiles. Manure-borne microorganisms contributed largely to the elevation of ARGs due to both the addition of manure-borne antibiotic resistant bacteria (ARB) in soil and potential horizontal gene transfer (HGT) via mobile genetic elements (MGEs) from manure-borne ARB to indigenous soil microorganisms. In contrast, indigenous soil microorganisms were demonstrated to prevent the dissemination of ARGs from manure to soil. The reason could be due to that indigenous soil microorganisms prevented the invasion and establishment of manure-borne ARB in soil. The abundance of ARG in manured soil decreased over time, but was still higher than that in control soil, indicating the persistence of ARGs in manured soil. These findings may shed light on the mechanisms underlying the spread and fate of ARGs in manured soil and also clues for ARGs mitigation.



## 1. Introduction

Antibiotic resistance genes (ARGs) that occur in the environments such as soils are currently regarded as a serious global health concern (Pruden et al., 2006; Bush et al., 2011), because ARGs can be transmitted and shared between environmental bacteria and human pathogens (Forsberg et al., 2012). In addition, once ARGs are acquired by environmental bacteria, the probability of their maintenance in natural ecosystems is high, even without a high loading of antibiotics (Pallecchi et al., 2008). However, the mechanisms underlying the spread and fate of ARGs in environment are still not clear.

In recent years there has been increasing concern regarding ARGs in soils because soil is a receptor of released antibiotics and ARGs (Kemper, 2008), especially after anthropogenic activities such as manure application. Land application of animal manures is a common practice in agriculture and is regarded as a cost-saving and efficient way to recycle nutrients from animal wastes (Binh et al., 2008; Chee-Sanford et al., 2009). However, antibiotics are commonly used in concentrated animal feeding operations worldwide to treat animal diseases and promote animal growth (Sarmah et al., 2006; Karci and Balcioglu, 2009). The produced manures have been identified as reservoirs of high levels of antibiotics, heavy metals, antibiotic resistant bacteria (ARB), antibiotic resistant genes (ARGs), and mobile genetic elements (MGEs) (Binh et al., 2008; Pakpour et al., 2012; Zhu et al., 2013). Manure application has also been demonstrated to increases the occurrence and spread of ARGs in soils (Binh et al., 2008; Zhu et al., 2013; Chen et al., 2016). By using high-capacity quantitative PCR arrays, 149 unique resistance genes were detected, and the top 63 ARGs being

* Corresponding author.
*E-mail address:* lcui@iue.ac.cn (L. Cui).

http://dx.doi.org/10.1016/j.soilbio.2017.07.022
0038-0717/

enriched in soil amend with manure (Zhu et al., 2013). Furthermore, ARGs in soil have been demonstrated to be able to enter food chain and even act as potential source of ARGs in human pathogens (Forsberg et al., 2012) via contaminated crops or groundwater, and consequently impact human health (Koike et al., 2007; Marti et al., 2013; Zhu et al., 2017). In contrast to the concern that manure application enhanced the level of ARGs in soil, other studies focused on how to reduce the potential risk of ARGs and found that increases in ARG abundance following manure application was not maintained long-term, but decayed with time ranging from tens of days to six months (Fahrenfeld et al., 2014). Another study reported that a period of delay (such as 15 months) between sewage sludge application and crop harvest was sufficient to attenuate these organic fertilizer-borne ARGs (Rahube et al., 2014). To mitigate the potential risk of manure application, it is important to understand the processes and mechanisms affecting the acquisition and spread of ARGs in soils after manure application.

The increase of ARG levels in manured soils could be related to the considerable amount of resistant bacteria in manure, which can lead to a direct increase in ARGs after manure application (Jiang et al., 2002). Previous studies stated that shifts in microbial communities caused by organic fertilizer amendment (Zhen et al., 2014) can affect the occurrence and abundance of ARGs, and bacterial taxa were found to significantly correlate with the profile of ARGs by network analysis (Chen et al., 2016; Li et al., 2015). The ability of manure-borne microorganisms to adapt to and establish in the soil environment is likely a very important factor for the persistence and spread of ARGs. This depends on many factors, such as the different physicochemical properties between manure and soil, and the competitive or cooperative interaction between manure-borne and indigenous soil microorganisms. It has been reported that the ability of a microbial community to resist invasion by invading species is related to the diversity of the indigenous species (Kennedy et al., 2002; Wertz et al., 2006; van Elsas et al., 2007). Another study also suggested a negative correlation between the diversity of soil microorganisms and the survival of the invader, due to the competition for limiting resources (van Elsas et al., 2012). However, how such interactions between manure-borne and indigenous soil microorganisms affect the occurrence and abundance of ARGs in manured soils remains poorly understood. A comprehensive understanding of this impact is required and will benefit the development of strategies to mitigate the spread of ARGs from animal manures to agricultural soils.

To address the question of how manure-borne and indigenous soil microbial communities affect the spread and fate of ARGs in manured soil, a microcosm experiment with four treatments was conducted in the present work over a period of two months (Kyselková et al., 2015). The four treatments included soil without manure addition (Soil) used as control, soil with γ-irradiated pig manure (Soil + RM), γ-irradiated soil with pig manure (RS + M), and soil with pig manure (Soil + M). By using a high-throughput quantitative PCR (HT-qPCR) including 296 validated primer sets targeting almost all major classes of ARGs and Illumina sequencing, we evaluated the abundance and diversity of ARG in manured soil, the impact of manure-borne and indigenous soil microorganisms on the spread of ARG, and the temporal change of ARGs after manure application.

## 2. Materials and methods

### 2.1. Sampling of soil and collection of pig manure

Surface soil (0–20 cm) was collected from a cropland used for planting rice in Jiaxing, Zhejiang, south China (30°50′7.7″ N, 120°43′5.7″ E) in August 2015. The soil parent material was lacustrine deposits and the soil was free from manure applications for approximately ten years. Soil was air dried for 7 days, sieved (<2 mm), and then kept in a dark place at room temperature until use. Pig manure was obtained in August 2015, from a local commercial pig farm with a history of antibiotic use including tetracycline, macrolide lincosamide streptogramin B (MLSB), and sulfonamides. The manure was air dried for three days at room temperature and sieved (<2 mm). For the manure, bacterial population was $3.34 \times 10^{13}$ copies per gram solid; the absolute abundance of ARGs was $6.79 \times 10^{13}$ copies per gram solid; the normalized abundance of ARG was 2.03 copies per 16S rRNA genes. Detailed properties of soil and manure are listed in Table S1. Around 6 and 0.8 kg of soil and manure were treated by γ-irradiation (25 kGy) with a $^{60}$Co source in order to prepare the treatments used in the following microcosm experiments (Wertz et al., 2006; van Elsas et al., 2012; Philippot et al., 2013). We used a plate count procedure to determine if the irradiation had efficiently killed the indigenous bacteria (Yan et al., 2017). Briefly, 0.5 g of soil and manure was spread onto trypticase soy and potato dextrose agar plate respectively. No bacterial or fungal growth was detected on agar plates after 6 days of incubation at 37 °C. In addition, we estimated the number of PCR templates that could be detected in irradiated samples. Briefly, DNA was extracted from the irradiated soil and manure, and a SYBR® Green approach (Roche 480, Roche Inc., USA) was used to estimate the number of possible residual PCR templates in the irradiated samples (Ouyang et al., 2015). The number of 16S rRNA gene copies per gram detected in irradiated soil ($2.37 \times 10^5$) and manure ($1.46 \times 10^6$) was seven orders of magnitude lower than that in non-irradiated soil ($4.47 \times 10^{12}$) and manure ($3.34 \times 10^{13}$).

### 2.2. Microcosm experiment

Before amendment with manure, the air-dried soil was adjusted to 60% of water-holding capacity (WHC) using autoclaved deionized water and pre-incubated in the dark at 25 °C for two weeks to recover the microbial activity (Chen et al., 2015; Liu et al., 2016). After the soil pre-incubation, soil microcosms were set up in plastic boxes (radius 15 cm, height 5 cm) covered with parafilm to allow air access but reduce loss of moisture. Four treatments were prepared at the beginning with the equivalent of 300 g dry soil and different amendments, i.e., control (Soil) containing soil only; treatment (Soil + RM) containing soil and 15 g irradiated dry pig manure; treatment (RS + M) containing irradiated soil and 15 g dry pig manure; and treatment (Soil + M) containing soil and 15 g pig manure. The manure application ratio used in the present study corresponds roughly to a typical agricultural rate of 40 $m^3$ manure $ha^{-1}$. A total of 15 replicates were prepared for each treatment and used for destructive harvesting at five time points, so three replicates were collected for each time point. Soils were thoroughly mixed with manure and water content was adjusted to 60% of WHC using autoclaved deionized water. Incubations were performed at 25 °C in the dark and autoclaved deionized water was supplemented every two days to maintain their water content. Three replicates from each treatment were sampled immediately after mixing (day 0) and also after the following incubation time (day 10, 20, 30 and 60). The collected samples were then stored at −80 °C before DNA extraction.

### 2.3. DNA extraction

DNA was extracted from 0.5 g soil with a FastDNA Spin Kit for soil (MP Biomedical, Illkirch, France) according to the manufacturer's instructions. The quality of extracted DNA was analyzed with a spectrophotometer (Nanodrop ND-1000, Thermo Scientific,