



# Effect of long-term manure slurry application on the occurrence of antibiotic resistance genes in arable purple soil (entisol)



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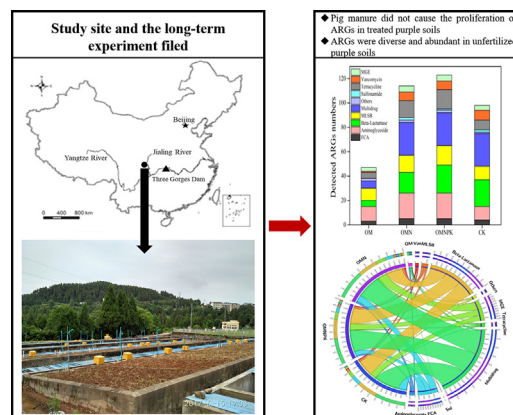
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## HIGHLIGHTS

- The profiles of ARGs in manured calcareous purple soil were characterized.
- Long-term manure treatment did not result in the dispersal of ARGs in purple soil.
- ARGs were surprisingly diverse and abundant in unfertilized purple soil.

## GRAPHICAL ABSTRACT



## ARTICLE INFO

### Article history:

Received 22 May 2018

Received in revised form 29 July 2018

Accepted 3 August 2018

Available online 03 August 2018

Editor: Jay Gan

### Keywords:

Long-term manure amendment

Antibiotic resistance genes

Horizontal gene transfer

Co-occurrence

Purple soil

## ABSTRACT

The application of animal manure is a highly recommended traditional agricultural practice for soils of relatively low fertility. However, for the farmland purple soils that are widely distributed in the upper Yangtze River region, little knowledge has been established in previous studies about the changes in the antibiotic resistome upon manure amendment. In the present study, the impact of long-term pig manure slurry application on the occurrence of antibiotic resistance genes (ARGs) and bacterial community was assessed in arable calcareous purple soil using high-throughput quantitative polymerase chain reaction and Illumina sequencing. Four treatments, including a non-fertilization control (CK) and pig manure (OM), OM plus mineral N fertilizer (OMN) and OM plus mineral NPK fertilizer (OMNPK) treatments were investigated. Across all the soil samples receiving different treatments, a total of 139 unique ARGs and 6 mobile genetic element genes were detected, with multidrug and beta-lactam the two most dominant types of ARGs. The results of the principal coordinate analysis (PCoA) suggest that the profiles of soil ARGs in the two treatments of OM combined with mineral fertilizer(s) (i.e., OMN and OMNPK) were similar to those in the control treatment, while the soil receiving only pig manure application had a different pattern of ARGs from the soils in the other three treatments. A clear reduction of soil ARGs was observed in the OM treatment. Significant and positive relationships were found not only among ARGs but also between mobile genetic elements (MGEs) and ARGs. However, no significant relationships were detected between ARG patterns and bacterial community composition. These results imply that the long-term application of pig manure slurry to

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purple soil does not lead to the prevalence of ARGs; however, the potential for the horizontal transfer of ARGs in calcareous purple soil should not be ignored.

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## 1. Introduction

With immunity to multiple antibiotics, emerging 'superbugs' has raised increasing concern worldwide over antibiotic resistance due to the decreased effectiveness of modern medicines and the risks to human health (Pruden et al., 2012). Due to the incomplete metabolism and weak absorption of antibiotics by livestock, the extensive use of these drugs in animal husbandry has often resulted in high levels of antibiotic residues, ARGs and MGEs in manures, which may exert positive selective pressure on antibiotic resistance (Zhu et al., 2013). These manures contain abundant nutrients, and their application as fertilizer is therefore a highly recommended agricultural practice for arable soils of low fertility. Moreover, it has been shown that ARGs can be readily shared, even among distantly related bacteria, through horizontal gene transfer (HGT) via MGEs, such as integrons, transposons and plasmids (Su et al., 2015). Land application of animal manures may also facilitate the enrichment and dissemination of ARGs in soil environments.

Numerous studies have examined the effects of the application of various manures on the diversity and abundance of ARGs in different soils. For example, the patterns of ARGs in samples of manure-treated soil from three large-scale swine farms differed significantly from the control soil sample, with an average enrichment of nearly 100-fold; further, the abundance of ARGs was correlated with antibiotic and metal/metalloid (i.e., Cu, Zn and As) concentrations (Zhu et al., 2013). Similarly, Chen et al. (2016) found that the application of chicken manure resulted in a remarkable increase in the abundance of soil tetracycline and multiple drugs resistance genes. In addition, Su et al. (2014) revealed that ARGs introduced by manure application accounted for approximately 70% of the total ARGs in soil. However, manure applications did not always lead to elevated abundances of ARGs. Manure applications increased ARGs in paddy soils at three (i.e., Jiaying, Yingtan and Nanchang sites) of four long-term experimental sites but not at the other site (i.e., Changsha site) in southern China (Tang et al., 2015). In a field-scale mass balance investigation, no marked variations in soil tetracycline resistance genes, *tetG*, *tetO*, and *tetW*, were observed after dairy manure application, despite the prevalence of these genes in applied manure (Fahrenfeld et al., 2014). Four (*tetM*, *tetB*, *tetD*, and *tetY*) of thirteen *tet* genes found in both fresh and composted pig manures were not detected in manure-treated paddy soils at the Changshu site in eastern China (Peng et al., 2015). Similarly, the application of different manures containing tylosin or chlortetracycline did not increase the abundance of culturable chlortetracycline-resistant bacteria in soil (Ghosh and Lapara, 2007). These findings suggest that the effect of animal manure application on the prevalence of the antibiotic resistome in amended soils remains inconclusive (Chen et al., 2016). Moreover, most of the previous studies used qPCR to assess the effect of manure application and only targeted a limited number of well-studied resistance genes (Lin et al., 2016; Peng et al., 2017; Tang et al., 2015). Therefore, the fragmentary ARG profiles observed in these studies cannot provide a full understanding of the impact of manure amendment on soil ARGs because not all types of ARGs were enriched after manure application (Fahrenfeld et al., 2014) with some actually decreasing (Chen et al., 2016). An improved understanding of the occurrence of a broad spectrum of ARGs in long-term manured fields is critical to comprehensively evaluate the effect of manure amendment on soil ARGs and elucidate the real-world fate of ARGs.

China is the largest antibiotic producer and consumer in the world. For 36 selected target antibiotics, 84.3% of the annual total consumption was used in livestock husbandry, and half of veterinary antibiotics were consumed by pigs (Zhang et al., 2015). A nationwide survey for

sulfonamide and tetracycline resistance genes in agricultural soils across China reported that the abundance of soil *tet* genes was positively related to soil properties (i.e., organic matter and pH) and swine manure production. Sichuan province, which is located in the upper reaches of the Yangtze River, was the greatest pig manure producer (22.2 million tons) in China (Y.T. Zhou et al., 2017). A vast hilly region (160,000 km<sup>2</sup>) of Sichuan is characterized by purple soils, which have low organic matter content (usually an average of <1.5%) and poor structure with abundant macropores (Li et al., 1991; Zhu et al., 2009). Land application of manures is often practised to improve not only soil fertility but also soil structure. Nevertheless, the potential adverse impact of manure application on the occurrence of ARGs in farmland soils of Sichuan is largely unknown. A recent investigation in the Three Gorges Reservoir (TGR) region of the upper Yangtze River, which is located to the east of Sichuan Province, revealed that two sulfonamide resistance genes (*sul1* and *sul2*), five *tet* genes (*tetA*, *tetB*, *tetM*, *tetQ*, and *tetG*) and class 1 integron (*int1*) were present in all surface water, soil (near the river bank) and sediment (on the riverbed) samples (Yan et al., 2018). These results indicate the potential export of ARGs from manured farmlands via runoff carrying eroded soil and subsequent delivery through the river channel system towards the TGR. Moreover, the contribution of manure application to the abundance of soil ARGs was reported to be regionally different (Munir and Xagorarakis, 2011; Tang et al., 2015) and dependent on soil type (Fang et al., 2015; Heuer and Smalla, 2007) and irrigation water sources (Knapp et al., 2010). For example, two sites (Wieringerwerf and Sloodorp) in the Netherlands generally had increased relative ARG levels after manure application, while, at another site (Heino), an increase in manure amendment by >10 times did not result in increased soil ARGs (Knapp et al., 2010). This phenomenon was attributed to the difference in soil types (well-drained sandy soil at Heino and marine silty loam soils at the other two sites) and irrigation water sources (uncontaminated irrigation water at Heino and heavily contaminated lake water at the other two sites). Purple soils are also well-drained due to the presence of abundant macropores, and almost all farmlands in this region are rain-fed (Zhu et al., 2009). Therefore, it is likely that the effect of manure application on the profile of ARGs in amended purple soils may be different from that in other soil types in China. Overall, there is clearly a need to explore the occurrence of ARGs in purple soils following long-term manure application.

Hence, we hypothesized that the long-term application of pig manure slurry does not alter ARG profiles in purple soils. In the present study, by combining high-throughput quantitative polymerase chain reaction (HT-qPCR) and Illumina sequencing, we aimed to (1) assess the effect of long-term manure application on the abundance and diversity of ARGs in the surface layer of farmland purple soils; (2) examine the effect of different fertilizations on the prevalence of the soil antibiotic resistome; and (3) address the shift in the structure of the bacterial community and soil properties to identify the key factors driving ARGs variability.

## 2. Materials and methods

### 2.1. Experiment site

The field experiment was carried out in Yanting Agro-Ecological Experimental Station of Purple Soil (31°16' N, 105°28' E), the Chinese Academy of Sciences, which is located within a headwater catchment of the Jialing River (a major tributary of the Yangtze River flowing into the TGR) in hilly central Sichuan, southeastern China. The site has a

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