Canadian Journal of Microbiology
Revue canadienne de microbiologie

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2016-0767.R1
Manuscript Type:	Article
Date Submitted by the Author:	07-Feb-2017
Complete List of Authors:	Kang, Yijun; Yancheng Teachers University Hao, Yangyang; Yangzhou University Xia, Dan; Yancheng Teachers University Shen, Min; Yancheng Teachers University Li, Qing; Yangzhou University Hu, Jian; Yangzhou University
Keyword:	Pig manure, Tetracycline-resistant bacteria (TRB), Tetracycline resistance genes (TRGs), <i>E. coli</i> , <i>Pseudomonas</i> spp.

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Title

The effects of pig manure application on the spread of tetracycline resistance in bulk and cucumber rhizosphere soils: a greenhouse experiment

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Running title

Spread of TRGs in bulk and rhizosphere soils

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Abstract

Understanding the dynamics of tetracycline-resistant bacteria (TRB) and tetracycline resistance genes (TRGs) in bulk and rhizosphere soils is important to evaluate the spread of TRGs from pig manure to human. In this work, a greenhouse experiment was conducted to investigate the difference in abundance of TRB, tetracycline-resistant *E. coli* (TRE), tetracycline-resistant *Pseudomonas* spp. (TRP), as well as TRGs between bulk and cucumber rhizosphere soils. The application of pig manure resulted in the long-term persistence of TRB, TRE, TRP and TRGs in bulk soil and rhizosphere of cucumber for at least 65 days. Pig manure application dose was the major driving force in altering the abundances of TRB and TRE, whereas TRP was disturbed mainly by compartment (bulk soil or rhizosphere). Both TRE and the percentage of TRE in bulk and rhizosphere soil were increased linearly along with the increase in dose of pig manure. The exponential relationships between pig manure dose and TRP along with TRP percentage were also noted. There were significant differences of relative abundances of TRGs between bulk and cucumber rhizosphere soils, suggesting the use of pig manure exerted a more lasting impact on the spread of TRGs in the rhizosphere than in the bulk soil.

Keywords: Pig manure; Tetracycline-resistant bacteria (TRB); Tetracycline resistance genes (TRGs); *E. coli*; *Pseudomonas* spp.

INTRODUCTION

Tetracyclines (TCs), a class of broad-spectrum agents, have been used for long time against a wide range of gram-positive and gram-negative bacteria in both humans and animals (Chopra and Roberts 2001). TCs are used mainly for treatment and prophylaxis in the livestock than human (Sengeløv et al. 2003; Thiele-Bruhn 2003). This may result in the selection of resistant pathogens and of mutants in the bacterial genome through a horizontal gene transfer (HGT) (Recchia and Hall 1995; Roberts 1996; Witte 1998). Antibiotic abuse or misuse in China is a serious concern as reflected in a report, where the average antibiotic consumption per person is nearly 10 times more than in the United States (Li 2014). Study by Hu and colleagues has reported that Chinese individuals have the highest number of antibiotic resistance genes (ARGs), especially tetracycline resistance genes (Hu et al. 2013). Manure excreted by animals is one of the major source of antibiotic pollution in the environment (Gao et al. 2015; Zhu et al. 2013), and pig manures are applied in rural China as organic fertilizer, directly to the soil without any treatment. Thus, the use of sewage sludge and manure containing antibiotic-resistant bacteria (ARB) or even with dead ARB (Kang et al. 2016a) lead to the large-scale pollution of soil and water (Heuer et al. 2011; Thiele-Bruhn 2003; Thiele-Bruhn and Beck 2005).

TCs have great impact on aquatic environments because these environments serve as a source of exposure to humans (Pruden et al. 2013). They may not only percolate down to the water body with rainfall, but can also spread to crops and thus contaminate foods consumed by humans (Chee-Sanford et al. 2001). The rhizosphere is the direct route through which tetracycline-resistant bacteria (TRB) and TRGs pass. It is very important to reveal the differences between bulk and rhizosphere soils when assessing the risks associated with the spread of antibiotic resistance

(Jechalke et al. 2013). Studies have demonstrated that plant roots influence the soil environment and its microbiota by exudation of growth substrates. It amplifies the effect of manure application on the abundance of ARB and ARGs in rhizosphere compared to those in bulk soil (Jechalke et al. 2014; Jechalke et al. 2013; Li et al. 2014). However, it was found that *sul1* and *sul2* genes coding for dihydropteroate synthases were less abundant in the rhizosphere compared to bulk soil (Kopmann et al. 2013). These suggested that the spread of ARGs in rhizosphere may be associated with different types of antibiotic pollutants. Understanding the dynamics of TRB and TRGs in bulk and rhizosphere soils are important to evaluate the ecological consequences of the transfer of these genes from livestock to human.

In this present study, a greenhouse experiment with cucumber was conducted to test the effects of pig manure application with different doses on the spread and abundance of TRB and TRGs in bulk and cucumber rhizosphere soils. Besides, *Escherichia coli* and *Pseudomonas* spp. were predominant tetracycline-resistant species in pig manure (Sawant et al. 2007) and common opportunistic human pathogens in soil (Silby et al. 2011; Tashiro et al. 2012). Thus, the abundances of tetracycline-resistant *E. coli* (TRE) and *Pseudomonas* spp. (TRP) were also determined to assess the potential bio-indicators for management of manure application.

MATERIALS AND METHODS

Pig manure application

The method for collection of pig manure is similar to that described previously (Kang et al. 2016a; Kang et al. 2016b). Briefly, pig manure with a ten year feeding history located in Qinfeng Town, Yangzhou City, which yielded about 1,000 pigs every year with TCs contents $925.3 \pm 78.7 \mu\text{g kg}^{-1}$, was collected from a pig farm. The composition of feed is maize (51.9%), wheat (24%), soybean meal (15%), grass meal (3%), fish meal (4.3%), bone meal (1.3%), and common salt (0.5%). The fattening pan age (Loushi Co. Ltd.) with $21.57 \pm 16.33 \mu\text{g kg}^{-1}$ of TCs was also used for feeding pigs at 2.5 kg per pig daily rate. The liquid pig manures were homogeneously mixed through the soil in one day.

Greenhouse experiment

With almost the same methods for experimental design and sampling as our previous work (Kang et al. 2016b), a greenhouse experiment was conducted between March to May 2014. With a completely randomized block design, a total of fifteen plots ($2.5 \text{ m} \times 6 \text{ m}$) with an equal row spacing of 0.8 m were established. Top soils of each plot were fertilized evenly with five different doses of pig manure, namely 0 kg per plot (blank control), 4 kg per plot (referred to as PM4), 8 kg per plot (usual doses, around $5,300 \text{ kg ha}^{-1}$, which is equal to 100 kg per ha compound fertilizer containing 15%-16% N, referred to as PM8), 16 kg per plot (referred to as PM16), and 32 kg per plot (referred to as PM32). Twelve cucumbers (*Cucumis sativus* L.) seedlings (Nongda14) at trefoil stage were transplanted in each plot on Mar 18, 2014. No other fertilizers were applied in the later growth period. The daily average temperatures of the greenhouse were 25°C. From the upper 0-10 cm soil layers, soil samples were collected with five-spot sampling method on days 0, 35

(full-bloom stage), and 65 (harvesting stage) after fertilization. Rhizosphere soil was obtained by collecting the remaining tightly adhering soil after shaking off the loosely adhering soil, and was only sampled on days 35 and 65. Data of 0 day were also used when comparison among treatments for the analysis of rhizosphere effects.

Enumeration of the populations of TRB, TRE and TRP

Ten-gram samples of soil (wet weight) were added to 90 ml of sterile distilled H₂O, subsequently shaken at 120 rpm for 20 min, followed by serial 10-fold dilutions with distilled H₂O. From the proper serial dilutions, one hundred microliters were transferred and plated on LB-TC agar media, which was comprised of 1/10-strength Luria-Bertani agar (Silby et al. 2011) supplemented with 16 µg ml⁻¹ of TC, to grow culturable TRB according to the Clinical and Laboratory Standards Institute (CLSI) document M100-S16 (Clinical and Laboratory Standards Institute 2011). Dilutions were plated on 1/10-strength LB agar without TC to enumerate total culturable bacteria (TCB). Agar plates were incubated at 37°C for 24 h, followed by counting and calculation of colony-forming units (cfu) (Sengeløv et al. 2003). For an enumeration of TRE and TRP, the culture media were substituted with Eosin Methylene Blue agar (Levine 1918) and Kings' B agar (King et al. 1954). Strains of TRE and TRP were also confirmed preliminary by morphology. Known wet weight soils were also dried at 105°C to constant weight to determine the moisture content. The data were presented as the mean cfu g⁻¹ of dry soil of three replicate samples.

Q-PCR analysis of TRGs

Soil genomic DNA was extracted according to the protocol of the Power-Soil™ DNA Isolation Kit (MO BIO Laboratories Inc., Carlsbad, CA) and amplified for *tetC* and *tetZ* genes, encoding an efflux pump, which were predominantly found in gram-negative and -positive bacteria (Aminov et al. 2002; Benacer et al. 2010; O'Neill et al. 2013; Tauch et al. 2000). Q-PCR assay for the detection

of TRGs was the same as that described previously (Kang et al. 2016b). Briefly, a 20 μ L reaction mixture contained 10 μ L of iTaq Universal SYBR Green Supermix, 0.4 mM of each primer, and 10 ng of template DNA. The temperature cycle consisted of: 95°C for 1 min, followed by 40 cycles of 94°C for 10 s, 68°C or 61°C for 45 s (for *tetC* or *tetZ*), and the subsequent disassociation analysis. The primers for *tetC* and *tetZ* genes were *tetC*-FW (5'-GCGGGATATCGTCCATTCCG-3') and *tetC*-RV (5'-GCGTAGAGGATCCACAGGACG-3'), and *tetZ*-FW (5'-CCTTCTCGACCAGGTCGG-3'), and *tetZ*-RV (5'-ACCCACAGCGTGTCCGTC-3'), respectively. The PCR was conducted using Bio-Rad MiniOpticon (Bio-Rad Laboratories, CA, USA) with SYBR Green I detection for estimating the copy numbers of TRGs. The copies of 16S rRNA genes representing total bacterial cell abundance from each sample were also quantified for normalization purposes with the primer set 341F (5'-CCTACGGGNGGCWGCAG-3') and 515R (5'-ATTCCGCGGCTGGCA-3') (He et al. 2007). The standard curves were prepared in triplicate from linearized plasmid serial dilutions containing between 10^9 and 10^3 16S rRNA gene copies (10^7 and 10^2 for TRGs). It was calculated directly from the concentration of extracting plasmid carrying target genes from soil samples according to the standard method (Nathani et al. 2013). The amplification efficiency (E) was estimated from the slope of the standard curve using the following formula: $E = (10^{-1/\text{slope}}) - 1$. The efficiency of PCR between 95% and 105% was used for further analysis (Yu et al. 2014).

Statistical analysis

The data of culturable TRB in bulk and rhizosphere soils were expressed as cfu g^{-1} of dry soil over time. The proportion of the TRB, TRE and TRP to the total culturable bacteria, *E. coli* and *Pseudomonas* spp. were also calculated for each treatment. Means and standard errors (SE) were imported into SPSS for Windows (version 19.0, Armonk, NY), and differences between treatments

were tested by analysis of variance with Duncan's multiple range test. The significance level was set at a p value of 0.05.

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RESULTS

Effects of pig manure application on TRB abundance in bulk and cucumber rhizosphere soils

The effects of pig manure application on TRB in bulk and cucumber rhizosphere soils were shown in Fig. 1. Compared with total culturable bacteria in bulk soil and rhizosphere of cucumber (Fig. 1A, 1D), TRB abundances were decreased by about 2 lg units at each measurement point. Similar to the control treatment, TRB in PM4 treatment increased on day 35 followed by a decline. TRB remained almost unchanged in PM8 and PM16 treatments, whereas TRB decreases in PM32 treatments. There were positive correlations between total culturable bacteria and TRB in bulk soil and rhizosphere of cucumber (Fig. 1C, 1F).

The statistical evaluation revealed that the abundances of TRB were significantly influenced by the various factors like: time, pig manure application dose, and soil compartment. However, the significant interaction effects showed differences between the above factors (data not shown); simple effect analyses were conducted to pinpoint the differences. As shown in Table 1, the abundances of TRB were significantly influenced by pig manure application dose both at the levels of soil compartment and time. TRB were not significantly influenced by soil compartment at each level of time and dose, but significantly influenced by the time both in bulk soil and rhizosphere of cucumber. This indicated that TRB was sensitive over time in a different soil compartment. Apart from PM0, differences of TRB in pig manure application treatments were not significant. It is suggested that pig manure application may result in long-term persistence of TRB in bulk soil and rhizosphere of cucumber for 65 days. Moreover, it can be concluded that pig manure application dose was the major driving force in altering the abundances of TRB; however, these were hardly

affected by the soil compartment factor.

Effects of pig manure application on abundance of TRE to total culturable *E. coli* populations in bulk and cucumber rhizosphere soils

Percentages of TRE to total culturable *E. coli* populations in bulk and cucumber rhizosphere soils were similarly affected by the pig manure treatments (Fig. 2). TRE percentages to total culturable *E. coli* populations sharply decreased in bulk soils for PM8, PM16, and PM32 treatments over time. The differences among treatments on day 65 were narrowed down compared to day 0 and day 35, which were more obvious in the rhizosphere of cucumber. This indicated that the application of pig manure can result in a bloom of TRE in bulk and rhizosphere of cucumber in less than 65 days. TRE in bulk and rhizosphere of cucumber were positively correlated with total culturable TRE (Fig. 2B, 2D).

The percentages of TRE were significantly influenced in bulk soil and rhizosphere of cucumber by pig manure application dose at the levels of day 0 and day 35, apart from day 65 (Table 1). This indicated that the differences between treatments were narrowed on day 65 through some unknown path. Similar to TRB, percentages of TRE were not affected by compartment, except at the level of day 35. The time significantly influenced the percentages of TRE both in bulk soil and rhizosphere of cucumber levels. The increasing application of pig manure intensified the influences of time on the percentages of TRE.

Effects of pig manure application on percentages of TRP to total culturable *Pseudomonas* spp. populations in bulk and cucumber rhizosphere soils

Percentages of TRP were increased on day 35 compared to day 0, followed by a decrease on day 65 (Fig. 3). Differences of percentages of TRP in bulk soil treatments were relatively stable at all three

test points, while these differences in rhizosphere soil treatments were broadened over time. It suggested the long-term risk of spread of TRP to cucumber through rhizosphere. In both bulk and rhizosphere soils, TRP abundances were significantly correlated with total culturable TRB (Fig. 3B, 3D).

Percentages of TRP to total culturable *Pseudomonas* spp. populations in rhizosphere of cucumber were significantly affected by the pig manure application dose, but this trend was not same in bulk soil (Table 1). This suggested that the spread of TRP was far easier in rhizosphere soil than in bulk soil, indicating that accumulation of TRP in rhizosphere may aggravate the risk of spread of TRG to plant. The percentage of TRP was significantly influenced by pig manure application dose at the level of day 35. Compartment also significantly affected the percentage of TRP at the levels of day 35 and day 65. With increasing use of pig manure, the percentages of TRP were not significantly affected by compartment except at the level of PM16. Apart from the percentage of TRP in bulk soil, the percentage of TRP was significantly influenced by time in both bulk and cucumber rhizosphere soils. At the application levels of PM16 and PM32, the percentage of TRP was significantly influenced by the time at the levels of all the pig manure application doses except at the level of PM0. The data showed that time may amplify the effect of pig manure application dose on accumulation of TRP in plough layer.

Effects of pig manure application on gene copies and relative abundances of *tetC* and *tetZ* in bulk and cucumber rhizosphere soils

TetC and *tetZ* gene copies of control treatment (without pig manure application) were maintained at around 10^3 and 10^5 to 10^6 copies per gram soil (Fig. 4). The use of pig manure significantly increased the *tetC* gene copies by 3 to 4 lg units on day 0 in bulk soil, followed by decreased on day 35 and day 65. *TetC* copies in rhizosphere showed the similar trend compared to the bulk soil. The

pig manure application also increased *tetZ* gene copies from 1 to 2.5 lg units on day 0 in bulk and cucumber rhizosphere soils. There were no drastic changes in PM4 treatments in both bulk and rhizosphere soils over time, but the decreases were also seen in PM8, PM16, and PM32 (Fig. 4).

The relative abundances of *tetC* were significantly increased with the increasing dose of pig manure in bulk soil, except PM4 and PM8 (Fig. 5). In rhizosphere soil, there were no significant differences among PM0, PM4, PM8 and PM16, all of which were significantly lower than PM32 treatment. Similar trends were observed on day 65. Generally, the relative abundances of *tetC* and *tetZ* were significantly influenced by pig manure dose in the bulk but not in the cucumber rhizosphere soils (Table 1). Interestingly, with time, differences were seen between bulk and rhizosphere soils. On day 35, the relative abundances of *tetC* in bulk soils were significantly higher than those in rhizosphere soils. Nevertheless, the relative abundances of *tetC* in bulk soils were significantly lower than those in rhizosphere soils, except PM4. These suggested that the use of pig manure could exert a lasting and growing impact on the spread of the *tetC* gene in rhizosphere compared to bulk soil. This may aggravate the risk of spreading of TRGs to cucumber. Relative abundances of *tetZ* in bulk soils were increased by pig manure application both on day 35 and day 65. However, there were no significant differences between PM4 and PM8 and between PM16 and PM32 on day 35, in between PM16 and PM32 on day 65. On day 35, no differences were seen among treatments in rhizosphere soils. While on day 65, there were no significant differences in between PM4 and PM8, PM8 and PM16, and PM16 and PM32. Despite the relative abundances of *tetZ* in bulk soils were higher than that in rhizosphere soils at two sampling points, the decreases of the relative abundances of *tetZ* with time in bulk soils was much faster than that in cucumber rhizosphere soils, which suggested that the use of pig manure could also result in a possibly lasting spread of *tetZ* gene in rhizosphere compared to bulk soil.

DISCUSSION

It was concluded that pig manure application dose was the major driving force in altering the abundances of TRB. This is in line to the previous published study (Zhu et al. 2013), suggesting that reduction of pig manure application was a possible way to decrease the risk of spread of TRGs in soil. Similarly, some researchers suggested that antibiotic resistance should be eliminated only when the selective pressure is released (Martinez 2009); antibiotic resistance in animals and its transfer to humans were clearly reduced through banning the use of some antibiotics in farming (Aarestrup et al. 2001). In this study, an exponential correlation between pig manure application dose and TRB was seen both in bulk and cucumber rhizosphere soils (Supplementary Fig. 1S). TRB abundance in bulk and rhizosphere soils reached a peak when pig manure application rate was at 16 kg per plot. The excessive use of pig manure may lead to reduction of bacterial populations and alteration of bacterial communities (Verma et al. 2014; Zhao et al. 2014); on the other hand, it could be explained as the limited recipient bacterial cells in the soil (Kang et al. 2016b). Despite the increasing use of pig manure resulted in the increasing accumulation of TCs in the soil. Our previous study demonstrated that once TRBs and TRGs were established in the soil, their levels were not dependent on TC content anymore (Kang et al. 2016a).

The abundances of coliform bacteria and *Pseudomonas* spp. were common indicators for monitoring soil and water qualities (Al-Badaii and Shuhaimi-Othman 2015; Mclean et al. 2013). In this regard, they also have the potential to be used as indicators for tetracycline contaminations. For this reason, we constructed the relationships between pig manure dose and TRE or TRP (Supplementary Fig. 2S, 3S). Both the abundance and percentage of TRE in bulk and rhizosphere soil increased linearly along with the increasing pig manure dose, which was similar to the

published result (Marti et al. 2013). The exponential relationships between pig manure dose and TRP and percentage of TRP were obtained. These indicated that the abundance of TRE or percentage of TRE, when used as bio-indicators, seemed more suitable than TRP. Coliform bacteria have proven to be relatively sensitive to environmental factors, including storing time and time variations (Duriez and Topp 2007; Walczak and Xu 2011), suggesting the possibly frequent transfer of TRGs to coliform bacterial cells in soil. Besides, despite the fact that abundances of the TRP were induced by the amendment of pig manure (Udikovic-Kolic et al. 2014), TRP abundances, similar to the TRB, became stable when soil received high rates of pig manure (Supplementary Fig. 3S), which may be due to the possible changes of bacterial communities.

With the time, the differences of percentages of TRE among treatments were decreasing, and contrary results were obtained for TRP (Fig. 2, 3). This would be explained as follows: (i) the spread of TRE in soil depth was faster than that of TRP, and (ii) the survival time of TRE in plough layer is shorter than TRP, especially in cucumber rhizosphere soil. A study found that multi-antibiotic resistant *E. coli* died off significantly faster than any of other antibiotic-sensitive strains (Duffy et al. 2006); once *Pseudomonas* spp. acquired antibiotic resistance genes, they became highly adapted to environmental stresses (Silby et al. 2011). As a result, from a long-term perspective, the spread of TRGs from TRP in cucumber rhizosphere soils are more serious than from TRE. On the other hand, TRGs derived from TRE could pollute underground water. Even so, considering that the spread of TRGs in soil can also be transferred from *E. coli* to *Pseudomonas* spp. (Gotz and Smalla 1997), the control of contamination of TRGs in soil seems different due to their hosts' cell-cell communications. Now that persistence of TRP in soil was longer than of TRE, it is also inferred that TRP colonizing on plant tissues even on fruits may be more abundant than TRE at harvest. A report showed that manuring increased the abundance of antibiotic-resistant coliform

bacteria in soil but had no effect on vegetables, which needs more investigations (Marti et al. 2013).

The *tetC* and *tetZ* genes, encoding an efflux pump, were predominantly found in gram-negative and -positive bacteria (Aminov et al. 2002; Benacer et al. 2010; O'Neill et al. 2013; Tauch et al. 2000). There were no significant differences between bulk and cucumber rhizosphere soils (Fig. 4), which was consistent with the result obtained by Li et al. (2014). Unlike other ARGs such as major facilitator super gene family (MFS), small multidrug resistance gene family, and β -lactamase genes, accumulations of TRGs in rhizosphere are less than that in bulk soil. To the best of our knowledge, though *tetC* gene has not been found in *E. coli* and *Pseudomonas* spp. (Vignaroli et al. 2012; Zhang et al. 2012), it has been detected in some pathogens including *Chlamydia suis* (Dugan et al. 2004) and *Salmonella* in China (Dai et al. 2005), which requires more concerns. A study stated that *tetM*, *tetZ* and *tetW* persisted in manure-amended soils for at least 3 weeks (Kyselková et al. 2013); however, it was found that both *tetC* and *tetZ* could persist in bulk and cucumber rhizosphere soils for at least 65 days, which further warns us of the risk of TRGs contamination. The time courses of TRB and TRGs were not identical (Fig. 1 and Fig. 4). This indicated that TRB carrying the two TRGs derived from pig manure may not well adapt to the soil environment, or they probably settle down to deep soil layers; whereas TRB, possibly indigenous microbial flora, were dependent on nutrition in soil, for they were relevant to total culturable bacteria (Fig. 1).

There were significant differences of relative abundances of TRGs between bulk and cucumber rhizosphere soils, suggesting that the use of pig manure exerted a more lasting impact on the spread of *tetC* and *tetZ* genes in rhizosphere than in bulk soil, which may aggravate the risk of spreading of TRGs to cucumber. Further studies should focus on (i) the spread of TRGs from pig manure to plant and underground in spatial scale, (ii) the mechanism of shuttle and succession of TRGs around rhizosphere, and (iii) effect of commercial bio-fertilizer on the spread of TRGs in the environment

as well.

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Acknowledgements

This work was supported by the National Natural Science Foundation of China (41501256 and 41101235), the Special Fund for Grain Bumper Science and Technology of National Key Technology Research and Development Program (2016YFD0300502), the National Science-technology Support Plan Projects (2015BAD01B03), the Special "Industry-University-Institute" Cooperation Project of Yangzhou (YZ2016264), the Agricultural Innovation Project of Yancheng (YK2015027), the "Qing Lan" Project Foundation of Jiangsu Province, the 333 Talents Project of Jiangsu Province, and the Spark Plan (2015GA690257).

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Figure Legends

Figure 1. Time course of abundances of total culturable bacteria (A, D), tetracycline-resistant bacteria (B, E) in bulk and rhizosphere soils, and relationship between total culturable bacteria and tetracycline-resistant bacteria (C, F). Tetracycline-resistant bacteria is defined as those growing on plates containing $16 \mu\text{g ml}^{-1}$ of tetracycline. PM4 to PM32 represent pig manure application doses ranged from 4 kg per plot to 32 kg per plot; Ctrl stands for non-manured treatment.

Figure 2. Time course of percentage of tetracycline-resistant *E. coli* (TRE) to total number of culturable *E. coli* (A, C) in bulk and rhizosphere soils, and relationships between total culturable bacteria and population of TRE (B, D). TRE is defined as those growing on Eosin Methylene Blue agar plates containing $16 \mu\text{g ml}^{-1}$ of tetracycline.

Figure 3. Time course of percentage of tetracycline-resistant *Pseudomonas* spp. (TRP) to total number of culturable *Pseudomonas* spp. (A, C) in bulk and rhizosphere soils, and relationships between total culturable bacteria and population of TRP (B, D). TRP is defined as those growing on Kings' B agar plates containing $16 \mu\text{g ml}^{-1}$ of tetracycline.

Figure 4. Time course of *tetC* (A) and *tetZ* (C) gene copies in bulk soils, as well as *tetC* (B) and *tetZ* (D) gene copies in cucumber rhizosphere soils. For the convenience of comparison, data from bulk soil samples on day 0 were still combined with data from rhizosphere samples.

Figure 5. Comparisons of relative abundances of *tetC* and *tetZ* between bulk and cucumber rhizosphere soils on day 35 and 65. Different lowercase letters represent significant differences among treatments ($p < 0.05$, Duncan's multiple range test).

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TABLE 1 Three-way ANOVA analyzing the influence of application dose, time, and compartment (bulk soil and rhizosphere) and respective correlations

Parameter		TRB	Percentage of TRE	Percentage of TRP	<i>tetC</i>	<i>tetZ</i>
Dose	Bulk soil	15.35***	4.23**	1.95	5.33**	2.85*
	Rhizosphere	17.49***	3.42*	6.52***	0.37	0.03
	Time (0 d)	55.84***	4.24**	0.35	ND	ND
	Time (35 d)	3.35*	6.21***	7.63***	9.56***	2.53
	Time (65 d)	19.53***	0.26	2.45	0.01	0.06
Compartment	Time (0 d)	ND	ND	ND	ND	ND
	Time (35 d)	0.51	0.44	5.96*	12.13**	46.06***
	Time (65 d)	0.03	0.06	9.13**	0.01	0.26
	Dose (PM0)	0.03	0.00	0.00	0.00	0.01
	Dose (PM4)	0.00	0.08	1.87	0.40	3.62
	Dose (PM8)	0.15	0.00	2.88	0.40	3.97
	Dose (PM16)	0.00	0.41	4.10*	0.98	9.34**
	Dose (PM32)	0.30	0.06	3.05	8.77**	9.87**
Time	Bulk soil	3.30*	33.56***	11.66***	24.03***	42.76***
	Rhizosphere	5.69**	29.39***	30.94***	1.05	0.13
	Dose (PM0)	13.53***	3.01	0.58	0.00	0.05
	Dose (PM4)	6.63**	15.31***	4.79*	0.78	3.27
	Dose (PM8)	0.40	14.74***	10.95***	2.04	3.62
	Dose (PM16)	0.24	21.76***	13.52***	4.05*	7.40*
	Dose (PM32)	1.33	32.49***	15.00***	31.22***	8.56**

F values are shown in the table, and significance is indicated by asterisks, with $P < 0.05$, $P < 0.005$, and $P < 0.0001$ indicated by *, **, and ***, respectively. PM, pig manure; ND stands for not determined.

250x144mm (150 x 150 DPI)

190x165mm (150 x 150 DPI)

195x165mm (150 x 150 DPI)

203x175mm (150 x 150 DPI)

200x186mm (150 x 150 DPI)

Figure 1S

Figure 2S

Figure 3S

Supplementary Fig. 1S. Correlation analyses between pig manure application dose and tetracycline-resistant bacteria in bulk soil (A), and in cucumber rhizosphere soil (B). Data from all the sampling points were grouped together for the correlation analyses.

Supplementary Fig. 2S. Correlation analyses between pig manure application dose and percentage of tetracycline-resistant *E. coli* in bulk soil (A) and in cucumber rhizosphere soil (B), and tetracycline-resistant *E. coli* population in bulk soil (C) and in cucumber rhizosphere soil (D). Data from all the sampling points were grouped together for the correlation analyses.

Supplementary Fig. 3S. Correlation analyses between pig manure application dose and percentage of tetracycline-resistant *Pseudomonas* spp. in bulk soil (A) and in cucumber rhizosphere soil (B) and tetracycline-resistant *Pseudomonas* spp. population in bulk soil (C) and in cucumber rhizosphere soil (D). Data from all the sampling points were grouped together for the correlation analyses.