Ecotoxicology and Environmental Safety 130 (2016) 279–288

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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

Impacts of supplementing chemical fertilizers with organic fertilizers manufactured using pig manure as a substrate on the spread of tetracycline resistance genes in soil

Yijun Kang [a,b], Yangyang Hao [a], Min Shen [b], Qingxin Zhao [b], Qing Li [a], Jian Hu [a,*]

[a] Environmental Science & Engineering, Yangzhou University, Yangzhou, Jiangsu, PR China
[b] College of Marine and Bio-engineering, Yancheng Teachers University, Yancheng, Jiangsu, PR China

ARTICLE INFO

Article history:
Received 30 October 2015
Received in revised form
15 April 2016
Accepted 23 April 2016
Available online 3 May 2016

Keywords:
Bio-organic fertilizer
Organic fertilizer
Pig manure
Tetracycline resistance genes (TRGs)
Tetracycline-resistant bacteria (TRB)

ABSTRACT

Using pig manure (PM) compost as a partial substitute for the conventional chemical fertilizers (CFs) is considered an effective approach in sustainable agricultural systems. This study aimed to analyze the impacts of supplementing CF with organic fertilizers (OFs) manufactured using pig manure as a substrate on the spread of tetracycline resistance genes (TRGs) as well as the community structures and diversities of tetracycline-resistant bacteria (TRB) in bulk and cucumber rhizosphere soils. In this study, three organic fertilizers manufactured using the PM as a substrate, namely fresh PM, common OF, and bio-organic fertilizer (BF), were supplemented with a CF. Composted manures combined with a CF did not significantly increase TRB compared with the CF alone, but PM treatment resulted in the long-term survival of TRB in soil. The use of CF+PM also increased the risk of spreading TRGs in soil. As beneficial microorganisms in BF may function as reservoirs for the spread of antibiotic resistance genes, care should be taken when adding them to the OF matrix. The PM treatment significantly altered the community structures and increased the species diversity of TRB, especially in the rhizosphere soil. BF treatment caused insignificant changes in the community structure of TRB compared with CF treatment, yet it reduced the species diversities of TRB in soil. Thus, the partial use of fresh PM as a substitute for CF could increase the risk of spread of TRGs. Apart from plant growth promotion, BF was a promising fertilizer owing to its potential ability to control TRGs.

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1. Introduction

The use of fertilizers, especially chemical fertilizer (CF), in China is more intensive and wide-spread than in any other country (Smith and Siciliano, 2015). Although the contribution of CF inputs to increased grain productivity since 1978 is non-negligible, it has resulted in problems related to water pollution mainly due to nutrient losses (Norse, 2005), greenhouse gas emissions (Liu et al., 2011; Yan et al., 2015), and degradation of soil structure (Cui et al., 2014). Therefore, partial reduction of a CF by adding organic components (Li et al., 2013; Tester, 1990) or plant growth-promoting rhizobacteria (PGPR) (Adesemoye et al., 2008; Kumar et al., 2009), or by precision fertilization (Bongiovanni and Lowenberg-Deboer, 2004; Matson et al., 1997) is assumed to be effective. Animal manure, as a plentiful renewable resource, has been used as an organic fertilizer (OF) for a long time in China, and has played important roles in agricultural production. Recent reports have shown that the application of pig manure (PM) compost as a partial substitute for the conventional CF urea can serve as a mitigation strategy for reducing greenhouse gas emissions, increasing grain yield, and controlling plant disease (Tao et al., 2015; Yan et al., 2015; Yang et al., 2015). Also, supplementing CFs with animal manures had much stronger effects on the abundance of nitrogen cycle genes compared with CF treatments (Sun et al., 2015). This indicated that the combined application of animal manures and CFs was a better approach to increase and sustain soil fertility and crop yields as well as reduce greenhouse gas emissions compared with the use of CF alone (Bandyopadhyay et al., 2010; Bokhtiar and Sakurai, 2005).

In most villages in China, however, animal manures are widely applied to land without keeping an adequate account of nutrient contents applied or risks incurred (David et al., 2015; Spear et al., 2004). Antibiotic resistance genes (ARGs) in animal manures, as emerging contaminants, are derived from the overuse of antibiotics in livestock as both therapeutic drugs and growth promoters (Chopra and Roberts, 2001; Gao et al., 2015; Sengeløv et al.,

Abbreviations: PM, Pig manure; CF, Chemical fertilizer; OF, Organic fertilizer; BF, Bio-organic fertilizer; TCs, Tetracyclines; TRG, Tetracycline resistance gene; TRB, tetracycline-resistant bacteria

* Corresponding author.
E-mail address: huj@yzu.edu.cn (J. Hu).

http://dx.doi.org/10.1016/j.ecoenv.2016.04.028
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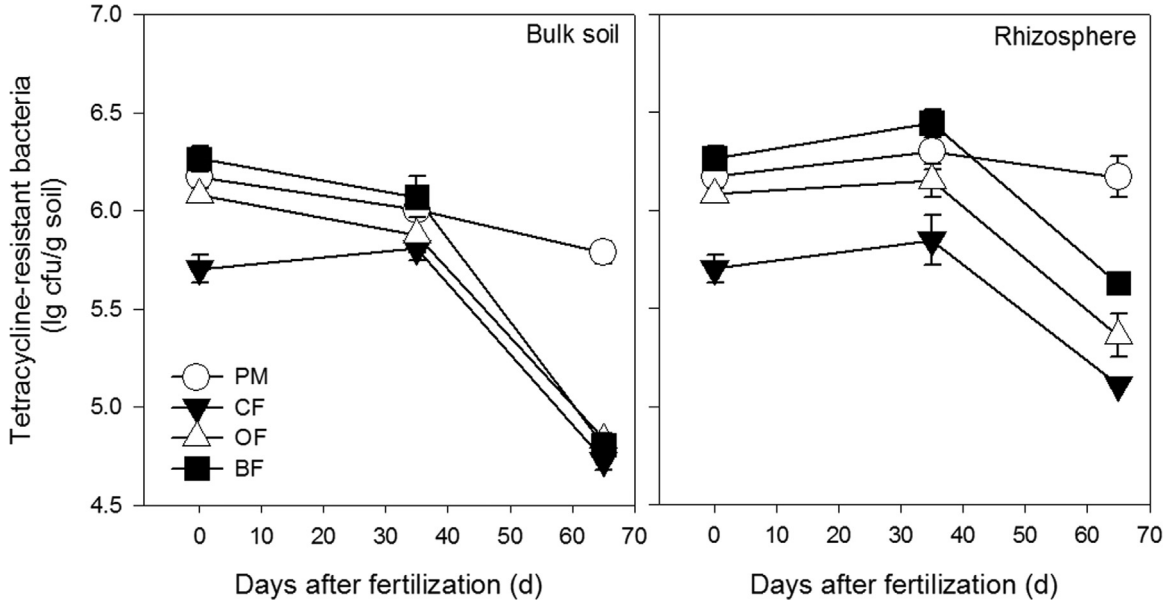

Fig. 1. Time courses of abundance of total culturable tetracycline-resistant bacteria (TRB) in bulk and cucumber rhizosphere soils. TRB are defined as those growing on plates containing 16 μg mL^{-1} of tetracycline. CF, PM, OF, and BF represent chemical fertilizer (NPK), chemical fertilizer (20% off)+pig manure, chemical fertilizer (20% off)+common organic fertilizer (composted pig manure), and chemical fertilizer (20% off)+bio-organic fertilizer (fermented with composted pig manure and some beneficial microorganisms), respectively.

Table 1
Three-way ANOVA analyzing the influence of fertilizer types, time, and compartment (bulk soil and rhizosphere) and respective correlations.

Parameter		TRB	*tetC*	*tetZ*
Type	Bulk soil	2.228	1.848	10.293***
	Rhizosphere	2.783	2.604	2.798*
	Time (0 d)	0.841	1.432	5.043**
	Time (35 d)	0.948	1.469	4.029*
	Time (65 d)	6.011**	2.254	2.761
Compartment	Time (0 d)	ND	ND	ND
	Time (35 d)	1.617	6.482*	2.404
	Time (65 d)	7.483*	1.656	0.023
	Type (PM)	0.168	2.744	2.418
	Type (CF)	0.523	4.546*	0.947
	Type (OF)	0.531	2.382	5.773*
	Type (BF)	1.088	0.229	11.524**
Time	Bulk soil	36.086***	60.642***	17.742***
	Rhizosphere	13.884***	14.871***	7.814**
	Type (PM)	0.576	8.347*	1.478
	Type (CF)	15.605***	36.123***	8.203**
	Type (OF)	15.961***	21.014***	12.264***
	Type (BF)	17.698***	15.476***	7.615**

F values are shown in the table, and significance is indicated by asterisks, with respectively. ND stands for "not determined". CF, PM, OF, and BF represent chemical fertilizer (NPK), chemical fertilizer (20% off)+pig manure, chemical fertilizer (20% off)+common organic fertilizer (composted pig manure), and chemical fertilizer (20% off)+bio-organic fertilizer (fermented with composted pig manure and some beneficial microorganisms), respectively.

* $P < 0.05$.
** $P < 0.005$.
*** $P < 0.0001$.

2003; Thiele-Bruhn, 2003; Zhu et al., 2013). ARGs have gained attention for their high-frequency spread in the environment through a horizontal gene transfer, in which genes code for resistance through mobile plasmids, transposons, conjugative transposons, and integrons (Recchia and Hall, 1995; Roberts, 1996; Witte, 1998). As a result, the use of animal manures with residual antibiotics and ARGs as an OF leads to the decreases in soil and water microbes, biomasses, enzymatic activities, and plant growth (Hammesfahr et al., 2008; Heuer et al., 2011; Kong et al., 2006; Ma et al., 2014; Thiele-Bruhn, 2003; Thiele-Bruhn and Beck, 2005; Wei et al., 2009). Some recent studies have shown that the total abundance of *tet* genes was not significantly reduced by fertilizing with composted versus fresh manure (Peng et al., 2015); even autoclaving of PM did not significantly reduce tetracycline resistance genes (TRGs) (Kang et al., 2016). However, little information is available about the impacts of supplementing a CF with various types of OFs manufactured using animal manures as substrates, especially the bio-organic fertilizer (BF) combined with a common OF and beneficial microorganisms, on the spread of ARGs in soil compared with the use of CF alone.

In this study, a greenhouse experiment with cucumber (a vegetable that is often eaten raw) was conducted to test the impacts of supplementing a CF (NPK) with the PM and a commercial OF as well as a BF on the spread of TRGs in soil compared with the use of CF alone. Also, the differences in spreading characteristics between bulk and rhizosphere soils were compared. Simultaneously, the community structures and diversities of tetracycline-resistant bacteria (TRB) within treatments were discussed.

2. Materials and methods

2.1. Collection and preparation of fertilizers

Four kinds of fertilizers, namely, fresh PM, CF (NPK), common commercial OF, and BF were collected or purchased for the greenhouse experiment. The PM was collected from a pig farm with a 10-year feeding history located in Qinfeng Town, Yangzhou City, which yielded about 1000 pigs every year. The composition of the feed was 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.) containing 215 ± 16.33 μg kg^{-1} of TCs was also used for feeding pigs at the dose of 2.5 kg per pig daily according to the manufacturer's recommendation. Sampling was done after obtaining permission from Mr. Dalin Zhou, the owner of the farm. With a 45-day aerobic composting process of the PM, the OF was obtained. The BF was a mixture of the aforementioned OF and some beneficial bacteria including *Bacillus subtilis* (SQR-9), *Paenibacillus polymyxa* (SQR-21),

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