Environmental Pollution 211 (2016) 332–337

Contents lists available at ScienceDirect

Environmental Pollution

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

Effects of manure and mineral fertilization strategies on soil antibiotic resistance gene levels and microbial community in a paddy–upland rotation system☆

Hui Lin [a, *], Wanchun Sun [a], Zulin Zhang [b], Stephen J. Chapman [b], Thomas E. Freitag [b], Jianrong Fu [a], Xin Zhang [c], Junwei Ma [a]

[a] The Institute of Environmental Resource and Soil Fertilizers, Zhejiang Academy of Agricultural Sciences, Hangzhou 310021, PR China
[b] The James Hutton Institute, Craigiebuckler, Aberdeen AB15 8QH, Scotland, United Kingdom
[c] College of Forest and Biotechnology, Zhejiang Agricultural and Forestry University, Lin'an 311300, PR China

ARTICLE INFO

Article history:
Received 13 October 2015
Received in revised form
4 January 2016
Accepted 4 January 2016
Available online 15 January 2016

Keywords:
Antibiotic resistance genes
Manure
Fertilization
Paddy–upland rotation
Soil microbial community

ABSTRACT

This work investigated the responses of antibiotic resistance genes (ARGs) and the soil microbial community in a paddy–upland rotation system to mineral fertilizer (NPK) and different application dosages of manure combined with NPK. The occurrence of five tetracycline ARGs (*tetA*, *tetB*, *tetC*, *tetG* and *tetW*), two sulfonamide ARGs (*sul1* and *sul2*) and one genetic element (*IntI*1) was quantified. NPK application showed only slight or no impact on soil ARGs abundances compared with the control without fertilizer. Soil ARGs abundances could be increased by manure-NPK application but was related to manure dosage (2250–9000 kg ha^{-1}). Principal component analysis (PCA) showed that the soil ARG profile of the treatment with 9000 kg ha^{-1} manure separated clearly from the other treatments; the ARGs that contributed most to the discrimination of this treatment were *tetA*, *tetG*, *tetW*, *sul1*, *sul2* and *IntI*1. Community level physiological profile (CLPP) analysis showed that increasing manure dosage from 4500 kg ha^{-1} to 9000 kg ha^{-1} induced a sharp increase in almost all of the detected ARGs but would not change the microbial community at large. However, 9000 kg ha^{-1} manure application produced a decline in soil microbial activity. Determination of antibiotics and heavy metals in soils suggested that the observed bloom of soil ARGs might associate closely with the accumulation of copper and zinc in soil.

1. Introduction

The bloom of antibiotic resistance genes (ARGs) has been a major concern from antibiotic contamination of the environment, which might contribute to the emergence of multi-resistant human pathogens and ultimately reduce the efficiency of antibiotic therapy (Martinez, 2008). The increasing prevalence of ARGs, among not only clinically important pathogens but also environmental bacteria, would pose a global threat to human health (Forsberg et al., 2012; Marti et al., 2013). As one of the ancient evolutionary origins of antibiotic resistance, the soil microbiota has been proposed as a reservoir of ARGs available for exchange with clinical pathogens (Forsberg et al., 2012).

Land use of manure has a long-term agricultural history and is being promoted because of the arising mineral fertilizer cost, a concern for sustainable soil productivity and ecological stability (Zhen et al., 2014) as well as the necessity of unwanted wastes disposal. Unfortunately, many studies have suggested that the livestock manure today serves as a reservoir of inorganic and organic pollutants (e.g. heavy metals; veterinary antibiotics) (Sarmah et al., 2006), antibiotic resistance bacteria, ARGs and pathogens (Zhu et al., 2013; Wichmann et al., 2014). As a consequence, there is increasing concern about the possible environmental consequences of land application of manure, particularly its potential contribution to soil resistance expansion. Several studies have examined the effect of manure amendment on the fate and transport of ARGs in soil (Heuer et al., 2011; Fahrenfeld et al., 2014; Peng et al., 2015), where the use of manure with residual antibiotics universally increases soil ARGs. However, not all types of soil ARGs increase following manure amendment despite the presence of these ARGs in manure, especially in field-scale experiments

☆ This paper has been recommended for acceptance by Jay Gan.
* Corresponding author.
E-mail address: linhui@mail.zaas.ac.cn (H. Lin).

http://dx.doi.org/10.1016/j.envpol.2016.01.007
0269-7491/

(Fahrenfeld et al., 2014; Peng et al., 2015). There is still uncertainty if there are differences in ARGs pools of the microbial communities depending on soil type and ecological management, which may be responsible for the variations in the impact of fertilization on soils (Munir and Xagoraraki, 2011). A broad research approach across a range of soil types, habitats, climatic regions and management schemes is necessary. Since most current studies have focused on grab samples or soil microcosms, more future works could be carried out under field conditions where real-world factors influence ARG fates.

Paddy–upland rotation is the most important cropping system in southern and eastern Asian countries, which is unique from other wetland or upland cropping system because of its association with frequent cycling between wetting and drying under anaerobic and aerobic conditions (Zhou et al., 2014). This type of rotation has many different sequences, where numerous grain and industrial crops could be rotated with paddy rice. Therefore, the main objective of this study was to assess the responses of some typical ARGs (tetracyclines and sulfonamides) to mineral fertilizer (NPK) alone and different application rates of manure combined with NPK in a rice-wheat rotation field located in South China. Also, additional information on how soil microorganism might respond to fertilization was provided. This study represents an example of the assessment of ARGs occurrence and fate in the paddy–upland rotation system.

2. Methods and materials

2.1. Study site and manure

The field experiment was conducted at an experimental station located in Shaoxing City (120.7E, 30.8N), Zhejiang Province, P.R. China. The soil was classified as a clay loam with 17.7% sand, 39.8% silt and 42.5% clay. The background soil sampled at 2013 before the field experiment showed a pH_{H2O} of 5.75, an electrical conductivity (EC) of 84.0 μs cm^{-1} and an organic matter content of 3.65%. The climate of the region was a sub-tropical monsoon climate with an annual average temperature of 18.1 °C and an average annual rainfall of 1200–1400 mm. The manure sample was a compost product of chicken manure with an average N of 1.6%, P of 2.0% (as a weight % P_2O_5) and K of 2.0% (as a weight % K_2O), which was purchased from a local organic fertilizer-producing factory in Shaoxing City. A preliminary survey of heavy metals (ICP-AES/ICP-MS) and veterinary antibiotics (HPLC–MS/MS) showed that the manure product contained 131.2 mg kg^{-1} Cu, 309.5 mg kg^{-1} Zn, 122.3 mg kg^{-1} Pb, 42.2 mg kg^{-1} As, 24.27 mg kg^{-1} chlortetracycline, 0.24 mg kg^{-1} sulfadimidine and 0.26 mg kg^{-1} trimethoprim.

2.2. Field experimental design

The experiment consisted of five treatments: (CK) no fertilizer applied, (CF) mineral fertilizer containing 225 kg N ha^{-1}, 90 kg P_2O_5 ha^{-1} and 180 kg K_2O ha^{-1}, (M1) 2250 kg ha^{-1} of manure combined with mineral fertilizer containing 189 kg N ha^{-1}, 45 kg P_2O_5 ha^{-1} and 135 kg K_2O ha^{-1}, (M2) 4500 kg ha^{-1} of manure combined with mineral fertilizer containing 153 kg N ha^{-1} and 90 kg K_2O ha^{-1}, and (M3) 9000 kg ha^{-1} of manure combined with mineral fertilizer containing 81 kg N ha^{-1}. All manure treatments contained equivalent contents of NPK to the CF treatment. Fifteen experimental plots (18 m^2; 5 treatments × 3 replicates per treatment) were arranged randomly. All treatments received the same crop rotation. Manure was incorporated into the topsoil as a basal fertilizer before seeding. Also, half of the NPK was supplied with manure and half at the tillering stage as a top-dressing. The first application of basal fertilizers in M1, M2 or M3 treatment was carried out in Jul 2013 before the sowing of paddy rice (*Oryza sativa* L., Shaojing 18). The paddy rice was harvested in Dec 2013. After that, the same rate of basal fertilizer was applied in Dec 2013, and wheat (*Triticum aestivum* L., Yangmai 18) was cultivated after a second basal fertilization. The third basal fertilization was performed in Jun 2014, after the wheat was harvested. Subsequently, paddy rice was cultured and harvested in Dec 2014. For each plot, more than three soil cores (5 cm in diameter and 20 cm deep) were collected at random and pooled. The soil samples collected in Dec 2014 were subjected to further investigation. The soil samples for DNA extraction and ARG analysis were stored at −20 °C. The MicroResp™ analysis was done immediately after the soils were sampled from the field.

2.3. Detection and quantification of target genes

DNA was extracted from a 0.25-g sample of soil and 0.1-g sample of manure with a PowerSoil® DNA Isolation Kit (Mo Bio, Cambio in the UK) according to the manufacturer's instructions. End-point PCR assays were carried out to test the ARGs using the specific primer pairs listed in Table S1. Seven tetracycline resistance genes (*tetA*, *tetB*, *tetC*, *tetG*, *tetM*, *tetQ* and *tetW*), two sulfonamide resistance genes (*sul1* and *sul2*) and one genetic element (Class 1 integron, *IntI*1) were determined in soil and manure. Bacterial 16S rRNA gene abundances were also determined to estimate the quantities of bacterial populations. The PCR amplicons were ligated into a vector using the pMD™19-T Vector Cloning Kit (Takara), and the recombinant plasmids were transformed into *Escherichia coli* TOP10 competent cells. The positive clones with the target gene insert were sequenced, and the plasmid DNA was extracted from the correct clone. After the concentration was measured, the purified plasmid DNA was serially diluted and analysed using real-time PCR to generate an external standard curve. Real-time PCR (qPCR) was performed with the BioRad CFX96 Real-Time PCR System with SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad) with the optimal annealing temperatures shown in Table S1. The specificity of the amplified products was confirmed by the melting temperature and the dissociation curve in each run. The abundance of each ARG was expressed as a proportion of the soil bacterial 16S rRNA. Each sample was analysed in triplicate with a standard curve, and a negative control included in each run. The PCR efficiencies (93.7%–110.4%) were examined to test for inhibition, and R^2 values were higher than 0.990 for all standard curves (Table S2).

2.4. CLPP determination using MicroResp™

MicroResp™ is a colourimetric method based on the colour change of a pH indicator dye due to the release of CO_2 by a heterotrophic community, which allows soil respiration to be determined. Since the individual species within the soil microbial community have different abilities to respire different substrates, adding different substrates leads to a CLPP or catabolic fingerprint of the community which is known to be effective at distinguishing changes in microbial community (Campbell et al., 2003; Chen et al., 2008). In this work, CLPPs was obtained using MicroResp™ technology according to the directions provided by the manufacturer with a total of fifteen substrates recommended by Campbell et al. (2003). The soil samples showed moisture contents in the range of 26.26%–28.59% (Table S3). Prior to soil being used, the moisture contents of all soil samples were adjusted to 30% and incubated at 25 °C for 5 days. Substrates were added to deep well plates with the soil samples to measure the substrate induced respiration (SIR) at 30 mg C g^{-1} soil water for L-cysteine HCl, citric acid, D-fructose, D-galactose, D-glucose, gamma amino butyric acid, L-lysine, L-alanine,