Journal of Hazardous Materials 299 (2015) 215–221

Contents lists available at ScienceDirect

Journal of Hazardous Materials

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

Antibiotic resistance genes in manure-amended soil and vegetables at harvest

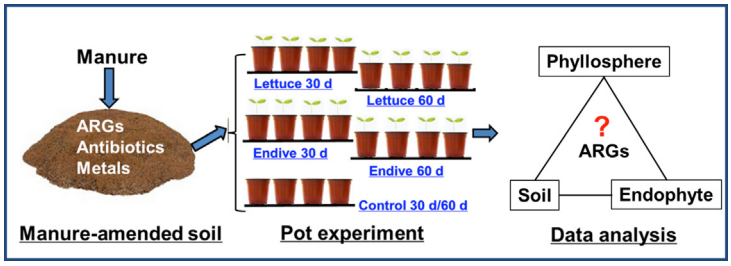
Feng-Hua Wang[a,b], Min Qiao[a,*], Zheng Chen[c], Jian-Qiang Su[d], Yong-Guan Zhu[a,d]

[a] State Key Lab of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China
[c] Department of Environmental Science, Xi'an Jiaotong-Liverpool University, Suzhou 215123, PR China
[d] Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, PR China

HIGHLIGHTS

- High residual of antibiotics and ARGs existed in manure-amended soil, which could persist in soil for a long time.
- Planting had an effect on the distribution of ARGs in manure-amended soil.
- ARGs were also detected on harvested vegetables grown in manure-amended soil, including root endophytes, leaf endophytes, and phyllosphere microorganisms.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form 23 April 2015
Accepted 16 May 2015
Available online 9 June 2015

Keywords:
Manure-amended soil
Antibiotic resistance genes
Integrase gene
Vegetables

ABSTRACT

Lettuce and endive, which can be eaten raw, were planted on the manure-amended soil in order to explore the influence of plants on the abundance of antibiotic resistance genes (ARGs) in bulk soil and rhizosphere soil, and the occurrence of ARGs on harvested vegetables. Twelve ARGs and one integrase gene (*int*I1) were detected in all soil samples. Five ARGs (*sul*I, *tet*G, *tet*C, *tet*A, and *tet*M) showed lower abundance in the soil with plants than those without. ARGs and *int*I1 gene were also detected on harvested vegetables grown in manure-amended soil, including endophytes and phyllosphere microorganisms. The results demonstrated that planting had an effect on the distribution of ARGs in manure-amended soil, and ARGs were detected on harvested vegetables after growing in manure-amended soil, which had potential threat to human health.

1. Introduction

The spread and aggregation of antibiotic resistance genes (ARGs) in multidrug-resistant pathogens is one of the most intractable clinical challenges [1,2]. Large amount of antibiotics are poorly absorbed by livestock [3], and high concentrations of residual antibiotics have been commonly detected in animal manures [4,5,6]. Manure has also been proved to be a reservoir of bacteria carrying ARGs and MGEs (Mobile Genetic Elements) such as plasmids, transposons, and integrons [7,8].

China produced an estimated 1900 million tons of livestock manure annually [9]. When manure was used as fertilizer, residual antibiotics and ARGs could disperse into agricultural soils, which may exert selection pressure on antibiotic resistance [10]. In addition, the horizontal transfer of ARGs from manure to soil bacteria is an important factor in ARGs dissemination, which could medi-

* Corresponding author.
E-mail address: minqiao@rcees.ac.cn (M. Qiao).

http://dx.doi.org/10.1016/j.jhazmat.2015.05.028
0304-3894/

ate persistence of ARGs in soil as bacteria from manure may not be well adapted to the soil environment [11]. Manure has been shown to promote the horizontal transfer of ARGs in soils [12]. Furthermore, the rhizosphere of plant is also known as a hot spot of gene transfer [13,14]. The rhizosphere soil is quite different from the bulk soil in their properties, microbial composition, and activity. Previous study had demonstrated that antibiotic effects can be enhanced in rhizosphere by exudation of nutrients and root growth [15]. Therefore, antibiotic resistance could be different between bulk and rhizosphere microbial communities. More importantly, the enrichment of ARGs in manure-amended soil can potentially disseminate resistance to vegetables, particularly those are eaten raw or subjected to minimal processing, which representing an important vehicle for ARGs transmission into human and posing potential threat to human health [11,16,17].

However, only few studies have explored the influence of plants on the abundance of ARGs in bulk soil or rhizosphere soil, and the occurrence of ARGs on harvested vegetables so far [17,18]. In this study, we sampled manure-amended soil and conducted a pot experiment with or without vegetables to indicate the fate of specific ARGs and *int*I1 gene in soil and the vegetables at harvest. The results will provide important information for an integrative risk assessment of soils receiving manure application.

2. Material and methods

2.1. Experimental design

Soil used for the pot experiments was collected from a vegetable field, which had been receiving manure for more than 3 years, and the most recent application was 5 d before sampling. The soil properties are as follows: pH of 7.0, organic matter content of 38.6 g kg^{-1}, total carbon of 24.0 g kg^{-1}, total nitrogen of 2.8 g kg^{-1}, Cu of 107.6 mg kg^{-1}, Zn of 383.8 mg kg^{-1}, and Pb of 35.7 mg kg^{-1}. The collected soil were air-dried and passed through a 2 mm mesh sieve.

Each plastic pot contains 1.0 kg soil (14.5 cm in diameter and 12 cm in height) and a nylon mesh bag (24 μm-mesh size, height of 8 cm; diameter of 6.5 cm; containing 150 g soil) placed in the central. Because the mesh only allowed the transport of water and nutrition and stopped the roots extension, the soil in or out of the bag was taken as rhizosphere soil or non-rhizosphere soil (bulk soil), respectively. The soils were thoroughly watered and pre-incubated overnight. Lettuce and endive seeds were sown in the nylon bags next day, and harvested at day 30 and day 60, respectively. Each treatment had four replicates and the controls are the pots without plants (4 replicates). All pots were incubated in a climate-controlled room, which is 25 °C in daylight (14 h, 300 μmole m^{-2} s^{-1}) and 20 °C in dark (10 h) (60% humidity). The pots were watered every two days with deionized water to maintain at 70% of water-holding capacity.

2.2. Soil and vegetable sampling

Soil samples were taken at 30 d and 60 d, respectively. Control soil, bulk soil, and rhizosphere soil were collected from each pot using a medicine spoon sterilized with 70% ethanol between sampling. Soil samples were stored at −80 °C before DNA extraction and chemical analysis. The edible portion of each plant were harvested at 30 d and 60 d, and carefully washed in running water to remove adhering soil and debris, and then packed individually. Plant roots were also separated and washed in sterile water to remove soil particles. All vegetable samples were stored at −80 °C before DNA extraction.

2.3. Pretreatment of vegetable samples

The vegetable samples were divided into three parts, that is, root endophytes, leaf endophytes, and phyllosphere microorganisms. Before extracting the DNA of endophytes, the plants (leaf and root) were processed following the method as described by Miller [19]. The plants were surface sterilized by immersion in 30% hydrogen peroxide for 30 min, followed by rinsing in sterile Milli-Q filtered water (Millipore) (3 min × 3 times). Then, washed with 70% ethanol for 1 min and rinsed as above. Surface-sterilized samples were dried using sterilized filter papers. To confirm the surface disinfection process was successful, 0.1 ml water used for the final washing step was added into the TSB medium, and incubated at 30 °C, 180 rpm for 7 d. Samples with no bacterial growth were considered successfully sterilized.

2.4. Extraction of DNA from soil and vegetables

For soil samples, 0.5 g soil was used for extraction with the FastDNA SPIN Kit (Bio 101 Inc., Vista, CA). For the root and leaf samples, 0.5 g samples were cut with a scalpel into pieces and carefully mixed before extraction. For the phyllosphere bacteria gathering, around 1.0 g leaf were transferred into a 50 ml centrifuge tubes containing 45 ml autoclaved 1 × phosphate buffered saline supplemented with 0.02% Tween 20, and shook at 200 rpm at 30 °C in a shaking incubator for 2 h. The washing solutions were filtered with nylon nets and centrifuged at 9000 rpm for 30 min. The supernatants were discarded and the pellets were preserved in the sodium phosphate buffer of the FastDNA SPIN Kit for Soil (Bio 101 Inc., Vista, CA). The concentration of the extracted DNA was determined by spectrophotometer analysis (NanoDrop ND-1000, NanoDrop Technologies, Willmington, DE).

2.5. Detection and quantification of target genes

Polymerase chain reaction (PCR) detection assays were used for broad-scale screening of the presence/absence of fifteen tetracycline genes (seven efflux pump genes (*tet*A, *tet*C, *tet*E, *tet*K, *tet*L, *tet*A/P, *tet*G), seven ribosomal protection proteins (RPPs) genes (*tet*M, *tet*O, *tet*Q, *tet*S, *tet*T, *tet*W, *tet*B/P), and one enzymatic modification gene (*tet*X)), four beta-lactamases genes (bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{ampC}), three sulfonamide genes (*sul*I, *sul*II, *sul*III), three quinolone genes (*qnr*A, *qnr*B, *qnr*S), three erythromycin genes (*ere*A, *ere*B, *mph*A), and the integrase gene of class I integrons (*int*I1). PCR detection assays were performed as previously described [20]. Primers and annealing temperatures are described in Table S1. The ARGs that were frequently detected and *int*I1 gene were quantified by quantitative PCR (Q-PCR) using a SYBR Green approach. Eubacterial 16S rRNA genes were quantified according to the TaqMan Q-PCR method [21]. All Q-PCR analyses were performed using an iCycler IQ5 Thermocycler (Bio-Rad, Hercules, CA).

2.6. Antibiotic analysis

The extraction procedures for tetracyclines, quinolones, sulfonamides, and their degradation products in soils followed methods described before with some modification [20]. LC-MS/MS was used to separate and detect the antibiotics [22]. Five target tetracyclines, including tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), methacycline (MTC), and doxycycline (DXC), and nine degradation products, including anhydrotetracycline (ATC), 4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC), 4-epioxytetracycline (EOTC), α-apo-oxytetracycline (α-apo-OTC), β-apo-oxytetracycline (β-apo-OTC), isochlortetracycline (ICTC), 4-epianhydrochlortetracycline (EACTC), demethylchlortetracycline (DMCTC), four sulfonamides, including sulfadimethoxine (SDM),