Front. Environ. Sci. Eng. 2020, 14(1): 1
https://doi.org/10.1007/s11783-019-1180-x

RESEARCH ARTICLE

Increasing prevalence of antibiotic resistance genes in manured agricultural soils in northern China

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HIGHLIGHTS

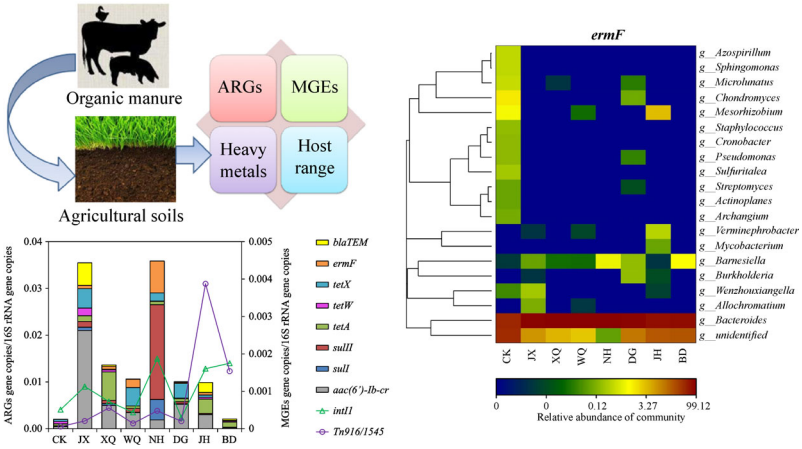
- Manure application increased the abundances of ARGs and MGEs in agricultural soils.
- Five classes of ARGs and two MGEs were prevalent in manured and unfertilized soils.
- Genera *Pseudomonas* and *Bacteroidetes* might be the dominant hosts of *intI1* and *ermF*.
- The abundances of ARGs positively correlated with TC, TN, OM, Cu, Zn, Pb and MGEs.

ARTICLE INFO

Article history:
Received 17 May 2019
Revised 10 August 2019
Accepted 16 August 2019
Available online 18 October 2019

Keywords:
Antibiotic resistance
Mobile genetic elements
Soil
Manure
Heavy metals

GRAPHIC ABSTRACT



ABSTRACT

Land application of manure tends to result in the dissemination of antibiotic resistance in the environment. In this study, the influence of long-term manure application on the enrichment of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in agricultural soils was investigated. All the analyzed eight ARGs (*tetA*, *tetW*, *tetX*, *sulI*, *sulII*, *ermF*, *aac(6')-Ib-cr* and bla_{TEM}) and two MGEs (*intI1* and *Tn916/1545*) were detected in both the manured and control soils, with relative abundances ranging from 10^{-6} to 10^{-2}. Compared with the control soil, the relative abundances of ARGs and MGEs in manured soils were enriched 1.0–18.1 fold and 0.6–69.1 fold, respectively. High-throughput sequencing analysis suggested that at the phylum level, the bacteria carrying *intI1* and *ermF* might be mainly affiliated with *Proteobacteria* and *Bacteroides*, respectively. The dominant genera carrying *intI1* and *ermF* could be *Pseudomonas* and *Bacteroides*, independent of manure application. Correlation analysis revealed that ARGs had strong links with soil physicochemical properties (TC, TN, and OM), heavy metals (Cu, Zn and Pb) and MGEs, indicating that the profile and spread of ARGs might be driven by the combined impacts of multiple factors. In contrast, soil pH and C/N exhibited no significant relationships with ARGs. Our findings provide evidence that long-term manure application could enhance the prevalence and stimulate the propagation of antibiotic resistance in agricultural soils.

1 Introduction

Elevated frequencies of antibiotic resistance genes (ARGs) in the environmental bacteria and their potential acquisition by human bacterial pathogens have been recognized as a worldwide public health issue (Pruden et al., 2006;

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Sharma et al., 2016; Sharma et al., 2019); the problem is exacerbated by the overuse of antibiotics in medicine and livestock systems. In the livestock and poultry industry, antibiotics are frequently used for disease control and growth promotion (Wu et al., 2010). China is the largest producer and consumer of antibiotics in the world, and nearly half of the 210000 tons of antibiotics each year are applied in livestock industries (Hvistendahl, 2012). The extensive use of antibiotics has caused high concentrations of residual antibiotics and high abundances of ARGs in animal manure, which serves as an essential reservoir of ARGs, antibiotic-resistant bacteria (collectively known as the "resistome"), and pathogens (Zhu et al., 2013; Xu et al., 2015; Zhou et al., 2017).

Manure application can remarkably improve soil fertility and crop yields; however, it can also cause a number of environmental problems, including the spread of antibiotic resistance (Liu et al., 2017). Previous studies have revealed the dissemination of manure-derived ARGs in agro-ecosystems after animal manure application (Guo et al., 2018; Xie et al., 2018), even in cases where the source animals had never been treated with antibiotics (Udikovic-Kolic et al., 2014; Zhang et al., 2017). Peng et al. (2017) observed that the abundances of ARGs in soils had significantly increased after continual application of pig manure for 30 years. Furthermore, bacteria are known to readily share genetic information through horizontal gene transfer (HGT) via mobile genetic elements (MGEs), such as integrons, transposons and plasmids, allowing the transfer of ARGs from manure microorganisms to indigenous environmental bacteria. HGT is recognized as the primary factor driving soil resistome alteration following manure application (Xie et al., 2018), owing to the high abundances of MGEs in manure (Zhu et al., 2013; Zhang et al., 2017). Class 1 integron is highly involved in the horizontal transfer of antibiotic resistance and is commonly measured as a proxy of anthropogenic pollution (Gillings et al., 2015). Transposases could be greatly enriched in animal manures and exhibited positive relationships with ARGs abundances (Zhu et al., 2013).

Additionally, there are associations between antibiotic resistance proliferation and heavy metals (Nguyen et al., 2019). The co-selection and cross-selection of antibiotic and heavy metal resistances caused by heavy metals, particularly Cu and Zn, have been reported to frequently occur in soils (Ji et al., 2012; Zhu et al., 2013; Zhao et al., 2017). Cu and Zn are often used as animal feed additives, and their contents in manure could reach 730.1 and 4333.8 mg/kg, respectively (Ji et al., 2012). Unlike antibiotics, heavy metals did not degrade readily and may exert continued selective pressure (Guo et al., 2018).

Manure-derived ARGs could be recruited by indigenous bacteria in soils and thereby contribute to the antibiotic resistome (Xie et al., 2018). Moreover, common ARGs shared between soil microorganisms and human pathogens have been identified through functional metagenomics, suggesting the clinical significance of soil antibiotic resistance (Forsberg et al., 2012). In addition, manure-derived ARGs are likely to enter the food chain, posing potential risks to human health when vegetables growing in manure-amended soils are consumed by humans (Zhu et al., 2017). Therefore, it is imperative to explore the prevalence and fate of ARGs in agricultural soils induced by manure application.

This study is intended to investigate the influences of long-term manure application on the prevalence and propagation of antibiotic resistance in agricultural soils in northern China. Eight ARGs were chosen as target genes, including three tetracycline resistance genes (*tetA*, *tetW*, and *tetX*), two sulfonamide resistance genes (*sulI* and *sulII*), one macrolide gene (*ermF*), one quinolone resistance gene (*aac(6')-Ib-cr*), and one β-lactam resistance gene (bla_{TEM}). These genes were selected because they have been reported to frequently occur in various environments and are representative genes conferring resistance to the major classes of antibiotics. To explore the mechanisms underlying HGT-related ARGs selection, two representative MGEs were also examined: class 1 integron gene (*intI1*) and conjugative transposon *Tn916/1545*. Moreover, the host ranges for *intI1* and *ermF* genes were investigated by high-throughput sequencing analysis. Finally, correlation analysis among the abundances of ARGs and MGEs, the levels of heavy metals and soil physicochemical properties was performed.

2 Materials and methods

2.1 Sampling sites and sample collection

Surface soils were sampled from seven agricultural fields where organic manures had been applied for over 3 years before sampling. The manured soils were collected between March and May 2016 from Jixian, Xiqing, Wuqing, Ninghe, Dagang, Jinghai and Baodi Districts (referred to as JX, XQ, WQ, NH, DG, JH and BD, respectively) in Tianjin, China. Another soil sample collected from an agricultural field in Jixian District in Tianjin (without manure application) was used as the control (CK) sample. The fertilized and control soils were all fluvo-aquic soils. The detailed information on the sampling sites is shown in Table 1. For each site, four soil subsamples (0–10 cm) were mixed thoroughly to form one sample. Soil samples were stored at −80°C until further analysis.

2.2 Soil physicochemical properties and heavy metal analysis

The soil characteristics were measured according to the standard methods (Lu, 2000). Soil moisture was determined by oven-drying soil samples at 105°C for 24 h. Soil

pH was measured with a water-to-soil ratio of 2.5:1. Total carbon (TC) was detected by a TOC analyzer (TOC-VCPH, Shimadzu, Japan), and organic matter (OM) was detected by the $K_2Cr_2O_7$ oxidation method. Total nitrogen (TN) was analyzed by the Kjeldahl procedure. The soil properties are listed in Table 1.

Seven types of heavy metals (Cr, Ni, Cu, Zn, As, Pb and Cd) were analyzed. Soil samples were digested with a mixture of concentrated acids (HNO_3, $HClO_4$, and HF). The resulting solutions were diluted, filtered, and stored at 4°C before analysis. The metal concentrations were then quantified by ICP-MS (iCAP-Q, Thermo Scientific, USA). Data were expressed on a dry weight (DW) basis.

2.3 DNA extraction

DNA was extracted from 500 mg moist soil using FastDNA SPIN kit for Soil (MP Biomedical, LLC, France). The concentration and purity of extracted DNA were analyzed by a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA).

2.4 ARGs and MGEs analyses by qPCR

PCR assays were used to detect the presence/absence of ARGs and MGEs and performed with a C1000 Touch™ Thermal Cycler (Bio-Rad, CA, USA). PCR products were purified via EasyPure Quick Gel Extraction Kit (TransGen Biotech, Beijing, China), ligated into pGEM-T Easy vector (Promega, Madison, WI, USA) and cloned into *Escherichia coli* DH5α (Tiangen, Beijing, China). Clones carrying target genes were sequenced and verified using BLAST alignment tool. Plasmids containing target genes were extracted using Plasmid Kit (TaKaRa, Japan).

Eight ARGs (*tetA*, *tetW*, *tetX*, *sulI*, *sulII*, *ermF*, *aac(6')-Ib-cr* and bla_{TEM}) and two MGEs (*intI1* and *Tn916/1545*) were determined by real-time quantitative PCR (qPCR) using SYBR-Green approach. The 16S rRNA genes were determined by TaqMan qPCR method (Suzuki et al., 2000). Table 2 summarizes the primers and annealing temperatures. The qPCR reaction mixtures comprised 1.6 μL of template DNA, 0.3 μL of each primer at 20 μmol/L (ShengGong, Shanghai, China), 10 μL of SYBR Premix Ex Taq (TaKaRa, Japan) and 7.8 μL of ddH_2O. The qPCR procedure was performed with a Bio-Rad IQ5 thermocycler (Bio-Rad, CA, USA), under the following protocol: 1) 95°C for 45 s, 2) 40 cycles of 95°C for 10 s, and 3) annealing temperatures for 30 s. Melting curve analysis (55°C–95°C, 0.5°C per read) was used to confirm product specificity. Each gene was quantified in triplicate. The R^2 values of all calibration curves were greater than 0.99.

2.5 High-throughput sequencing analysis

The high-throughput sequencing analysis of *intI1* and *ermF* was conducted on an Illumina MiSeq PE300 platform (Allwegene Co., Ltd., Beijing, China). To amplify *intI1* and *ermF* genes, specific primers and annealing temperatures were used (Table 2). The sequencing data were subjected to bioinformatic analysis. The raw data were filtered using the criteria and methods published previously (Luo et al., 2018). The sequences were assembled using FLASH. The assembled sequence set was clustered into operational taxonomic units (OTUs) under a threshold of 97% similarity using UCLUST software. Representative sequences from each OTU were annotated through BLAST in the NCBI NR database.

2.6 Data analysis

Averages and standard deviations were calculated with Microsoft Excel, 2010. One-way ANOVA (statistical significance at $P < 0.05$), *t*-tests and Spearman correlation analyses were conducted using SPSS software (version 19.0).

3 Results and discussion

3.1 Heavy metal contents

Table 3 presents the concentrations of heavy metals in soils together with the soil quality standards of China. Six of the

Table 1 Sampling sites and physicochemical properties of soils

Samples	Fertilizers	Amount of fertilizer applied (kg/hm^2)	Soil texture	Moisture (%)	pH	TC (g/kg)	OM (%)	TN (g/kg)	C/N
CK	Unfertilized	0	Clay loam	6.14	8.38	6.04	1.37	0.51	11.81
JX	Swine manure	30000	Clay loam	17.87	7.51	19.59	4.46	0.99	19.89
XQ	Chicken manure	31250	Clay loam	21.15	7.38	28.88	3.79	1.85	15.61
WQ	Swine manure	27000	Clay loam	30.13	7.86	26.80	3.12	2.06	13.01
NH	Swine manure	33250	Clay loam	34.52	6.94	50.67	8.81	2.83	17.91
DG	Swine manure	25000	Clay loam	7.67	8.14	25.48	3.75	1.79	14.26
JH	Swine manure	19000	Clay loam	7.84	7.66	3.53	0.80	0.50	7.00
BD	Chicken manure	28000	Clay loam	22.46	7.22	20.92	4.71	0.79	26.43

Table 2 Primer sequences of the ARGs, MGEs, and 16S rRNA

Target Genes	Primer	Sequences (5′—3′)	Amplicon size (bp)	Annealing temp (°C)	Ref.
tetA	*tetA*-FW	GCTACATCCTGCTTGCCTTC	210	55	Ng et al. (2001)
	tetA-RV	CATAGATCGCCGTGAAGAGG			
tetW	*tetW*-FW	GAGAGCCTGCTATATGCCAGC	168	60	Aminov et al. (2001)
	tetW-RV	GGGCGTATCCACAATGTTAAC			
tetX	*tetX*-FW	CAATAATTGGTGGTGGACCC	468	58	Ng et al. (2001)
	tetX-RV	TTCTTACCTTGGACATCCCG			
sulI	*sulI*-FW	CGCACCGGAAACATCGCTGCAC	163	56	Negreanu et al. (2012)
	sulI-RV	TGAAGTTCCGCCGCAAGGCTCG			
sulII	*sulII*-FW	TCCGGTGGAGGCCGGTATCTGG	191	60	Negreanu et al. (2012)
	sulII-RV	CGGGAATGCCATCTGCCTTGAG			
ermF	*ermF*-FW	CGACACAGCTTTGGTTGAAC	309	56	Chen et al. (2007)
	ermF-RV	GGACCTACCTCATAGACAAG			
acc(6')-Ib-cr	*acc(6')*-FW	TTGCGATGCTCTATGAGTGGCTA	482	55	Zhang et al. (2016)
	acc(6')-RV	CTCGAATGCCTGGCGTGTTT			
bla_{TEM}	bla_{TEM}-FW	ATCAGCAATAAACCAGC	516	55	Zhang et al. (2016)
	bla_{TEM}-RV	CCCCGAAGAACGTTTTC			
intI1	HS463a	CTGGATTTCGATCACGGCACG	473	55	Hardwick et al. (2008)
	HS464	GGWTACCTTGTTACGACTT			
Tn916/1545	*Tn916/1545*-FW	GACAGTATTAAGCCATCAGAC	142	41	Zhang et al. (2016)
	Tn916/1545-RV	TCTTCCGAACACAATCATCT			
16S rRNA	*1369F*	CGGTGAATACGTTCYCGG	123	56	Suzuki et al. (2000)
	1492R	GGWTACCTTGTTACGACTT			

Table 3 Heavy metal concentrations in surface soils from different sites (mg/kg DW)

Samples	Cr	Ni	Cu	Zn	As	Pb	Cd
CK	77.50±1.68	43.15±0.47	39.39±0.79	32.93±0.86	17.47±0.27	23.84±0.12	ND
JX	123.18±11.49**	47.46±1.36**	25.03±1.32	13.61±1.01	8.33±0.04	10.29±0.63	ND
XQ	82.28±3.54	47.89±0.40**	74.59±1.06**	104.78±0.95**	19.86±0.17**	24.23±0.49	0.10±0.01
WQ	62.02±2.14	42.28±0.21	36.61±1.22	39.20±0.46**	13.45±0.22	23.43±0.31	0.10±0.01
NH	79.94±2.52	37.63±0.53	147.13±3.15**	205.51±1.42**	10.42±0.13	40.15±0.89**	0.35±0.02
DG	60.22±1.63	38.15±0.33	36.52±0.34	28.17±0.25	11.82±0.09	17.29±0.50	ND
JH	97.59±1.24*	45.98±0.52*	26.70±0.26	10.39±0.41	8.36±0.20	10.75±0.07	ND
BD	129.47±8.01**	53.63±0.77**	46.49±1.40**	20.98±0.43	13.69±0.09	21.86±0.26	ND

Notes: * Denotes heavy metal concentrations significantly higher ($P < 0.05$) in manured soils than in CK, ** represents statistical significance with $P < 0.01$. ND: not detected. The heavy metal concentrations were compared with standards from national environmental quality standards for soils (GB15618-2018).

heavy metals (Cr, Ni, Cu, Zn, As and Pb; all except Cd) were observed in both the manured and control samples. Among the regions, only NH had heavy metal contents exceeding the soil quality standards; specifically, Cu (147.13 mg/kg) and Cd (0.35 mg/kg) were above the standards. This result might have been related to the fact that the highest amount of manure was applied in NH (Table 1). Guo et al. (2018) reported that pig manure application resulted in significantly higher heavy metal contents than those observed in control soil, implying that long-term manure land application might bring about heavy metal pollution.

High concentrations of the heavy metals Cr, Cu and Zn were observed in some of the manured soil samples. The concentrations of Cr, Cu and Zn likely represent environmental risk after the long-term land application of organic manures. Zhang et al. (2005) reported that the contents of Cu, Zn and Cr in animal manures from representative intensive feedlots in China were in the ranges of 1017–1591, 7113–8710, and 0–688 mg/kg, respectively. High

accumulations of Cu and Zn in animal manures most likely result from the use of feed additives to promote animal growth. Elevated levels of Cu and Zn are poorly absorbed by the animal gut and are excreted as feces (Ji et al., 2012). However, in this study, not all of the manured soils exhibited significantly higher concentrations of heavy metals than the CK sample, e.g., As and Pb (Table 3), suggesting that manure amendment did not significantly induce the accumulation of these metals in soils. This result might be attributed to the low concentrations of these metals in manures, or to the quick uptake of metals in manures by plants. Overall, the long-term application of organic manures could lead to the accumulation of certain types of heavy metals in soils.

3.2 Diversity and abundances of ARGs and MGEs

All eight ARGs (*tetA*, *tetW*, *tetX*, *sulI*, *sulII*, *ermF*, *aac(6')-Ib-cr* and bla_{TEM}) and two MGEs (*intI1* and *Tn916/1545*) were detected in both the manured and control samples. Absolute gene copy numbers of ARGs and MGEs in soils are presented in Fig. 1 and Table S1. The absolute abundances of the eight ARGs varied considerably (10^4 to 10^9 copies per g dry soil) among different samples. The total absolute abundances of the eight ARGs in manured soils ranged from 3.86×10^7 to 1.63×10^{10} copies per g dry soil, which were remarkably higher than the abundance in CK (1.16×10^7 copies per g dry soil). Similarly, manured soils had much higher prevalences of *intI1* and *Tn916/1545* (ranging from 4.68×10^6 to 8.51×10^8 copies per g dry soil) than did the control soil (2.97×10^6 and 3.28×10^5 copies per g dry soil for *intI1* and *Tn916/1545*, respectively). The absolute abundances of 16S rRNA genes ranged from 10^9 to 10^{11} copies per g dry soil among different sites (Fig. 1).

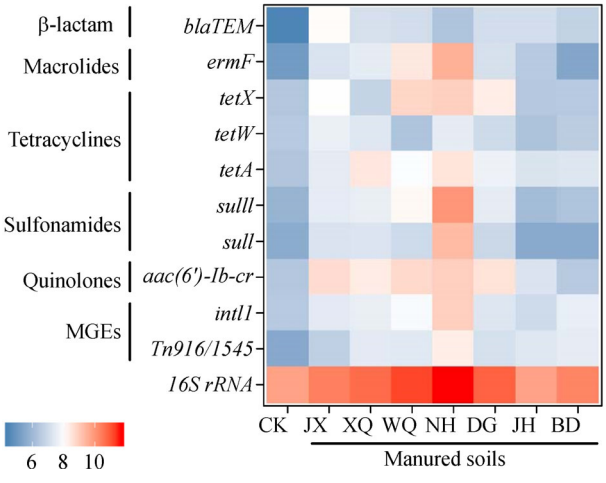

Fig. 1 Absolute abundances of ARGs, MGEs and 16S rRNA in soils from different sites. Plotted values are log10-transformed numbers of gene copies per g dry soil.

Absolute gene copies of ARGs and MGEs were normalized to those of ambient 16S rRNA genes (Fig. 2). The relative abundances of ARGs in soils exhibited obvious variation among the sampling sites, ranging between 10^{-6} and 10^{-2}. The *aac(6')-Ib-cr* (1.76×10^{-4} to 2.10×10^{-2}) and *sulII* (1.10×10^{-4} to 2.03×10^{-2}) were the two most dominant ARGs in soils, followed by *tetA* and *tetX* with relative abundances between 10^{-4} and 10^{-3}. The relative abundances of other ARGs, including *tetW*, *sulI*, *ermF* and bla_{TEM}, were generally in the range of 10^{-6} to 10^{-3}. The abundances of the ARGs are generally consistent with those reported in previous studies conducted on soils applied with chicken or swine manures (Zhou et al., 2017; Guo et al., 2018), or soils adjacent to feedlots (Wu et al., 2010; Ji et al., 2012). For example, the reported abundances of *tetA*, *tetW* and *tetX* ranged from approximately 10^{-5} to 10^{-2}, and those of *sulI* and *sulII* ranged from approximately 10^{-5} to 10^{-1}.

The *aac(6')-Ib-cr*, a common type of plasmid-mediated quinolone resistance (PMQR) gene, was observed at high abundances in this study. Manure application has been reported to contribute to the propagation of (fluoro) quinolones and PMQR genes in the environment and to alter the indigenous bacterial community (Xiong et al., 2015). Similarly, an unexpectedly high prevalence of *aac(6')-Ib-cr* was observed in soils receiving swine manure for 11 years (Xu et al., 2015), which implied the prevalence of *aac(6')-Ib-cr* in the environment and its robust adaptability. In the present study, the abundances of *sulII* were greater than those of another sulfonamide resistance gene, *sulI*, at all sampling sites, which coincides with the observations in an aquaculture environment in Tianjin (Gao et al., 2012). The *sul* genes have been found to be frequently accompanied by integrons, which might facilitate their persistence (Wang et al., 2014). The tetracycline resistance genes *tetA*, *tetX* and *tetW*, which encode efflux pump proteins, inactivating enzymes, and ribosomal protection proteins (RPPs), respectively, are ubiquitous in soils and in fresh and composted manures (Wu et al., 2010; Cui et al., 2016). The *tetW* and other RPPs genes have been found to be predominant in the gastrointestinal tracts of pigs and steers (Aminov et al., 2001), contributing to their frequencies in animal manures. In addition, *tetA*, *tetX*, *tetW* and six other tetracycline resistance genes were reported to be the most abundant *tet* genes in manures or compost-treated field soils (Zhu et al., 2013), suggesting the resilience of these genes. Furthermore, bla_{TEM} was identified as the most abundant β-lactam resistance gene subtype in soils of swine farms (Yang et al., 2019). Genes encoding β-lactam, including bla_{TEM}, have been reported as belonging to the highest risk category, which merits substantial attention (Zhang et al., 2016). The widespread distribution of *ermF* in various environments could be explained by the mechanism of *erm* genes action. The *erm* proteins are responsible for the methylation of an adenine residue in 23S rRNA, and this region is the target of

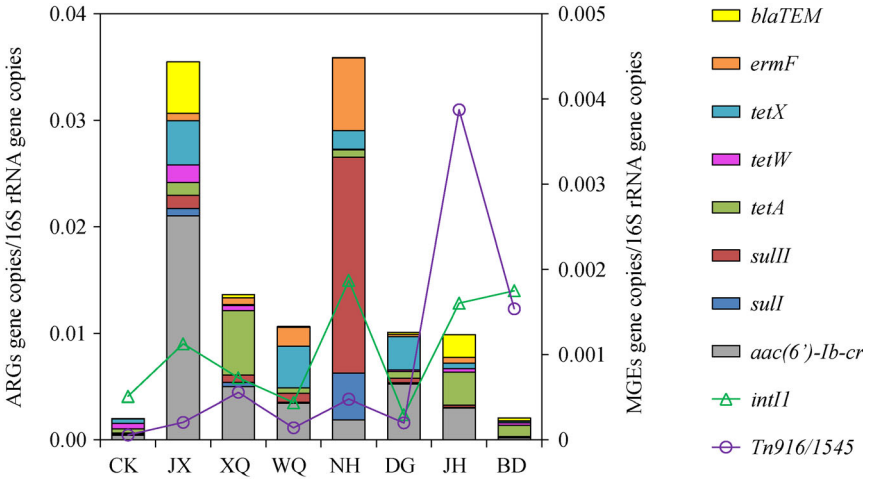

Fig. 2 Relative abundances of ARGs and MGEs in soils from different sites.

macrolides, lincosamides, and streptogramins B (MLSB) that can result in *erm* genes propagation (Brenciani et al., 2011).

With respect to MGEs in soils, the abundances of *intI1* and *Tn916/1545* ranged from 10^{-5} to 10^{-3}, which were comparable to those of previous studies (Wang et al., 2014; Zhang et al., 2016). As one of the most prevalent MGEs, *intI1* is an important vector for ARGs dissemination and is often used as an indicator of HGT. More than 80 different gene cassettes of *intI1* have been described and shown to confer resistance to a broad range of antibiotics (Zhang et al., 2009; Zhang et al., 2018a). *Tn916/1545* is one of conjugative MGEs and could transfer among microorganisms through cell-to-cell contact (Zhang et al., 2016). The *Tn916-1545* transposon family is widely related to various antibiotic resistance determinants, and its members have a broad host range (Guo et al., 2017).

Furthermore, the total relative abundances of the eight ARGs in manured soils were 5.0–18.1 fold greater than the relative abundance in CK, except BD where the abundance was comparable to that in CK (Fig. 2). In most cases, the manured soils contained strikingly more abundant ARGs, e.g., *tetA*, *sulII*, bla_{TEM}, *ermF*, and *aac(6')-Ib-cr*, than did CK (Fig. S1). Besides, the manured soils had much higher prevalence of *Tn916/1545* (2.5–69.1 times) than that in CK (Fig. S1). However, the relative abundances of *intI1* in manured soils were not always greater (0.6–3.7 fold) than those in the control sample; specifically, they were lower in WQ and DG than in CK (Fig. S1). Nevertheless, the increased levels of antibiotic resistance in manured soils were strongly related to the long-term manure application. Previous studies have suggested that animal manure has become a crucial reservoir of antibiotic resistance, and its application to agricultural soils has resulted in marked increases in ARGs and MGEs abundances in soils (Heuer et al., 2011; Zhu et al., 2013; Peng et al., 2017). Our results confirm that long-term manure application could stimulate the propagation of antibiotic resistance in agricultural soils.

3.3 High-throughput sequencing of *intI1* and *ermF*

To further study the host bacterial diversity of functional genes involved in HGT mechanism and antibiotic resistance, high-throughput sequencing of *intI1* and *ermF* was performed. The sequences of *intI1* and *ermF* could be used to speculate their respective bacterial taxonomy. The *intI1* and *ermF* genes were chosen as representatives of MGEs and ARGs given their prevalence and high abundances in various environments (Wang et al., 2014; Pu et al., 2018). A total of 14516–97845 high-quality sequencing tags were obtained for *intI1* and *ermF* in the eight samples, and the number of OTUs ranged from 22 to 591 across all samples (Table 4). The Shannon index values of *intI1* were generally higher than those of *ermF*, suggesting the possibility of a more diverse host bacterial community for *intI1* than for *ermF* in soils. It is not surprising that the host range of *intI1* (as one type of MGEs) was wider than that of *ermF* due to *intI1*'s strong ability to transfer among species. Moreover, the Shannon index value of *ermF* in CK was 3.87, which was much higher than the corresponding values in manured soils, revealing that manure application might reduce the host bacteria diversity of *ermF*. The situation for *intI1* was relatively different, and the Shannon index value of *intI1* was higher in CK than in all of the manured samples except those from JH and BD.

Figure 3 reveals the potential host bacteria composition for *intI1* and *ermF* at the phylum level. As for *intI1* (Fig. 3(a)), seven dominating phyla were speculated and the most detected bacteria across all samples belonged to *Proteobacteria* (19.1%–91.6%). The *intI1* genes seem to be widely disseminated among the *Proteobacteria* and

Table 4 Bacterial diversity index values of *intI1* and *ermF* in soil samples

Samples	Tags		OTUs		Chao1		Coverage		Shannon	
	intI1	*ermF*	*intI1*	*ermF*	*intI1*	*ermF*	*intI1*	*ermF*	*intI1*	*ermF*
CK	36726	97845	339	230	340	236	0.9960	0.9996	2.89	3.87
JX	14516	62790	441	233	466	244	0.9940	0.9994	1.82	0.85
XQ	20182	64383	309	48	334	59	0.9929	0.9998	1.86	0.37
WQ	23260	58540	250	187	319	200	0.9919	0.9994	1.26	0.44
NH	33810	56807	336	22	359	29	0.9908	0.9998	1.12	0.10
DG	16180	60648	152	261	179	265	0.9963	0.9997	1.92	1.48
JH	32667	65265	591	129	586	235	0.9918	0.9990	5.06	1.79
BD	36797	61476	432	17	419	17	0.9955	0.9999	6.18	1.43

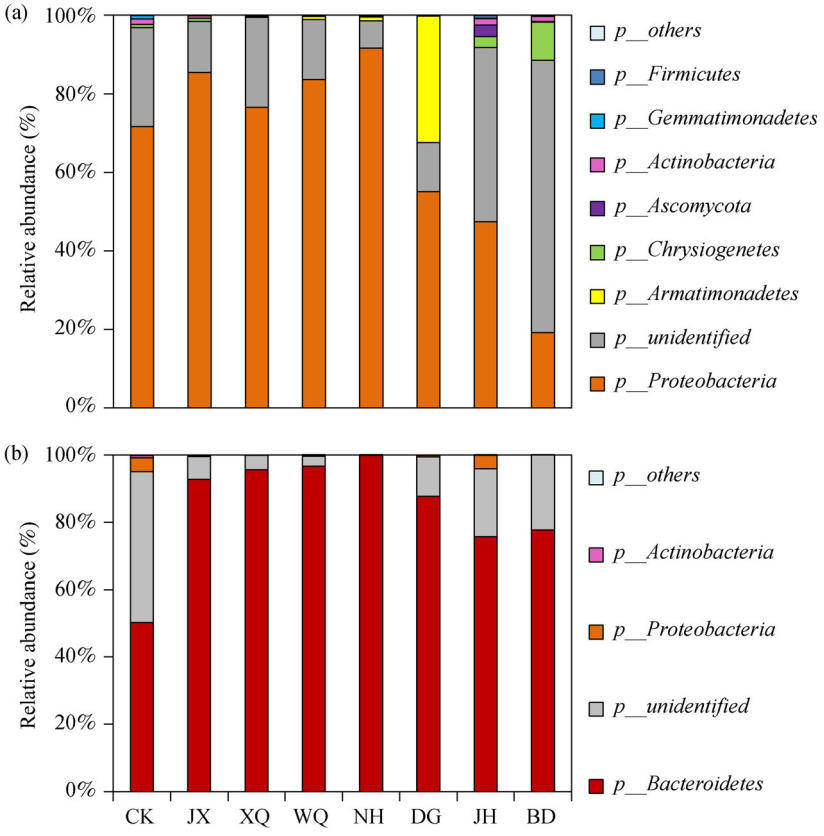

Fig. 3 The host bacteria composition for (a) *intI1* and (b) *ermF* at the phylum level (Phyla with relative abundances less than 0.5% are pooled into the category "others").

play crucial roles in the ARGs propagation (Rosewarne et al., 2010). Wang et al. (2014) constructed the clone libraries of *intI1* in soils and found that the most identified bacteria belonged to *Proteobacteria*. A recent study explored *intI1* in over 73000 currently available complete and draft bacterial genomes, and reported that the host species of *intI1* were highly conserved within 96% in *Gammaproteobacteria* class belonging to the phylum *Proteobacteria* (Zhang et al., 2018a). In CK, low frequencies of *Actinobacteria* (1.4%) and *Gemmatimonadetes* (0.9%) were also found. In manured soils, the remaining predominant members varied distinctly among different sampling sites. A considerable amount of *Armatimonadetes* (32.2%) were observed in DG, but few *Armatimonadetes* were detected in other manured soils. In addition, *Chrysiogenetes* (9.7%) and *Ascomycota* (2.9%) became the second most dominant populations in BD and JH, respectively.

As for *ermF* (Fig. 3(b)), three dominant phyla were speculated. *Bacteroides* (50.3%–99.9%) accounted for the largest proportion of host bacteria across all samples, followed by *Proteobacteria* (up to 4.1%) and *Actinobacteria* (up to 0.7%). Previous studies reported the high prevalence of *ermF* in *Bacteroides* isolates (Chung et al., 1999; Eitel et al., 2013), which is consistent with our findings. Overall, *Proteobacteria* and *Bacteroidetes* were probably the main host bacteria for *intI1* and *ermF* in this study and have been found in other manure-soil matrices (Wang et al., 2018; Xie et al., 2018), indicating that they could survive well in the eutrophic environment (Wang et al., 2018).

Figure 4 illustrates the possible host bacteria composition for *intI1* at the genus level. Independent of manure application, *Pseudomonas* might be the most abundant genus in soils, occurring at a proportion of 15.6%–91.5%. Previous studies have revealed high prevalences of *intI1* in *Pseudomonas* from manured soil, pigsties, and sediments (Agersø and Sandvang 2005; Rosewarne et al., 2010; Byrne-Bailey et al., 2011). It has been reported that *intI1* is broadly distributed among more than 70 species and is often carried by some pathogenic species, including *Pseudomonas aeruginosa* (Zhang et al., 2018a). Further studies need to be carried out to explore the pathogenic host species of *intI1* and the associated risks. Moreover, a considerable amount of *Fimbriimonas* (0.9%–32.2%) were observed in DG, NH and WQ soils, but were almost undetectable in other soils. Other dominant host genera with relative abundances over 1% were only detected in certain samples, such as *Mesorhizobium*, *Desulfurispirillum*, and *Pichia*.

As for *ermF*, the potential host bacteria composition at the genus level is given in Fig. 5. *Bacteroides* might be the most abundant genus with a proportion of 50.3%–99.1% across all samples. The *ermF* gene was first reported in an anaerobic colony of *Bacteroides* spp. and is often linked with conjugative transposons located on the chromosome (Chung et al., 1999). In a study of swine manure anaerobic digestion, genera *Bacteroides* were claimed to be the possible hosts of *ermF* that is a well-known macrolide resistance gene (Zhang et al., 2018b). Macrolide resistance genes are usually reported present in *Bacteroides* (Johnsen et al., 2017). Additionally, other dominant members, such as *Mesorhizobium* (3.6% in JH) and *Barnesiella* (1.1% in BD), were found in manured soils. Interestingly, many host genera (0.1%–0.4%) were detected only in CK, such as *Azospirillum*, *Sphingomonas*, *Staphylococcus*, *Cronobac-*

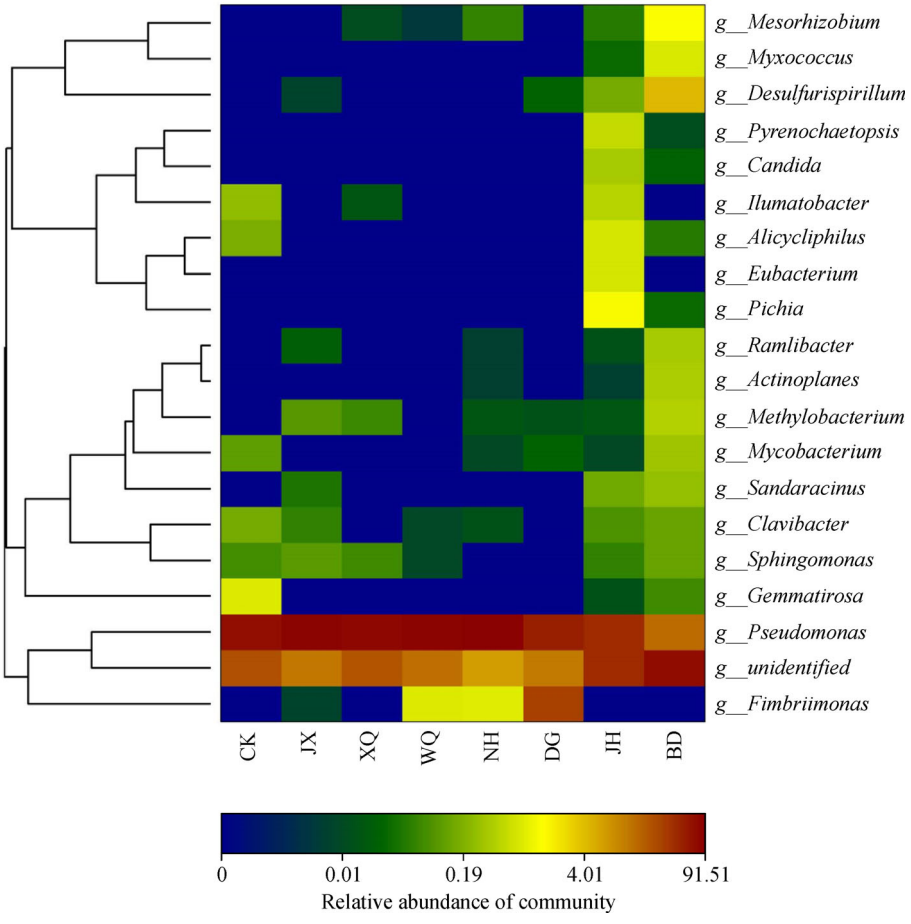

Fig. 4 The host bacteria composition for *intI1* at the genus level (top 20 genera).

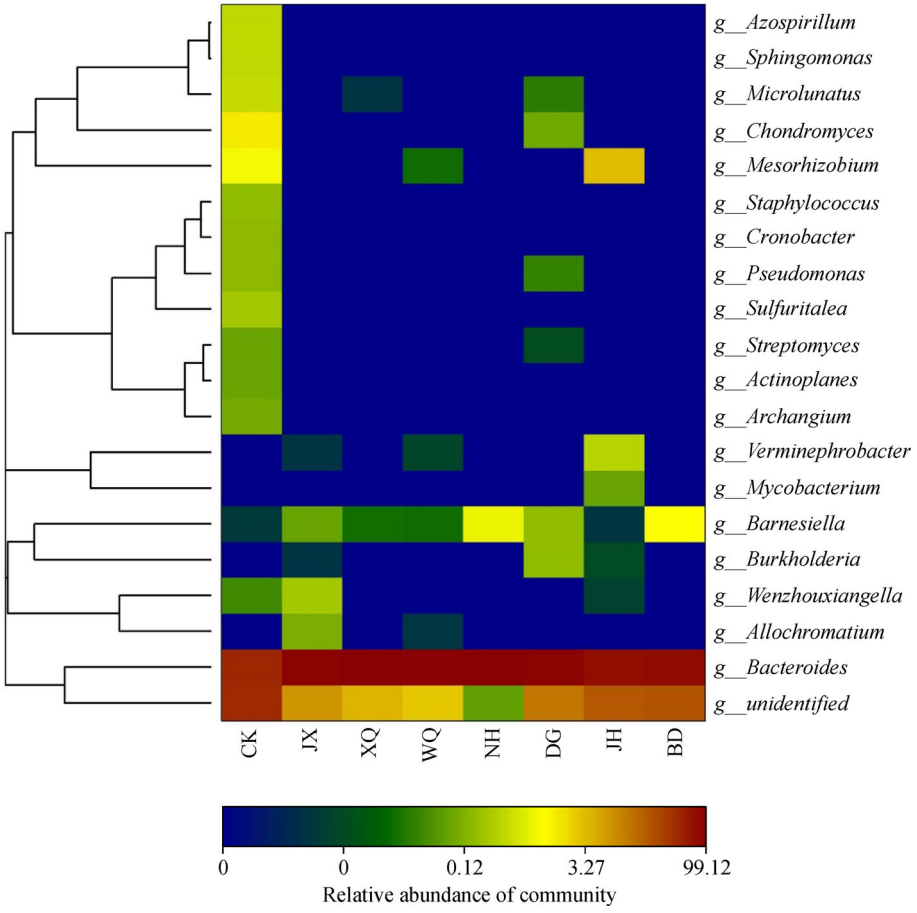

Fig. 5 The host bacteria composition for *ermF* at the genus level (top 20 genera).

Table 5 Correlation analysis among ARGs, MGEs, soil properties and heavy metals

Genes	pH	TC	OM	TN	C/N	16S rRNA	Cr	Ni	Cu	Zn	As	Pb	*intI1*	*Tn*916/1545	MGEs
tetA	−0.619	0.809*	0.673	0.798*	0.103	0.712*	−0.277	−0.292	0.876**	0.927**	0.275	0.737*	0.706	0.730*	0.711*
tetW	−0.541	0.557	0.662	0.416	0.367	0.402	0.257	−0.110	0.464	0.503	−0.242	0.175	0.476	0.436	0.469
tetX	−0.305	0.773*	0.654	0.840**	0.009	0.913**	−0.469	−0.671	0.634	0.657	−0.253	0.678	0.778*	0.746*	0.772*
sulI	−0.591	0.775*	0.824*	0.683	0.152	0.946**	−0.143	−0.521	0.927**	0.899**	−0.244	0.799*	0.998**	0.990**	0.997**
sulII	−0.588	0.775*	0.822*	0.685	0.149	0.949**	−0.148	−0.525	0.925**	0.897**	−0.245	0.802*	0.998**	0.990**	0.998**
ermF	−0.579	0.791*	0.820*	0.716*	0.135	0.968**	−0.182	−0.541	0.920**	0.897**	−0.240	0.814*	0.999**	0.989**	0.998**
aac(6')-Ib-cr	−0.404	0.844**	0.767*	0.867**	0.134	0.845**	−0.317	−0.619	0.621	0.671	−0.324	0.525	0.745*	0.696	0.736*
bla_{TEM}	−0.101	−0.097	0.057	−0.188	0.245	−0.241	0.503	0.235	−0.336	−0.298	−0.451	−0.544	−0.206	−0.263	−0.217
ARGs	−0.583	0.809*	0.835**	0.735*	0.144	0.970**	−0.185	−0.552	0.920**	0.901**	−0.248	0.804*	0.998**	0.986**	0.997**

Notes: * indicates statistical significance at $P < 0.05$; ** indicates statistical significance at $P < 0.01$. ARGs denotes the sum of antibiotic resistance genes; MGEs denotes the sum of *intI1* and *Tn916/1545*.

ter, *Sulfuritalea*, *Actinoplanes*, and *Archangium*. This finding implies that the application of organic manure possibly reduced the diversity of the host range of *ermF* in soils but accelerated the enrichment of certain bacteria, i.e., *Bacteroidetes* whose proportion increased from 50.3% in CK to over 75% in manured soils. This result might be attributed to the better survival of *Bacteroidetes* in the eutrophic environment resulting from manure application (Wang et al., 2018).

3.4 Relationships among ARGs, MGEs, and physicochemical properties

Correlation analysis among the absolute abundances of

ARGs and MGEs, heavy metal contents and soil physicochemical parameters is shown in Table 5. Total number of ARGs gene copies had significantly positive correlations with TC ($P < 0.05$), organic matter ($P < 0.01$), TN ($P < 0.05$) and 16S rRNA ($P < 0.01$) in soil. On the contrary, soil pH ($r = -0.583$, $P>0.05$) and C/N ($r = 0.144$, $P>0.05$) exhibited no significant links with ARGs in the present study. Studies of the relationships among pH, C/N and ARGs remain inconclusive (Wu et al., 2010; Wang et al., 2014; Chen et al., 2016; Guo et al., 2018), possibly reflecting the different types of soils and ARGs investigated among different studies. The results of the present study demonstrate that various soil physicochemical properties, such as TC, TN, and OM, could influence the abundances of ARGs.

Significant positive links were observed between ARGs and certain heavy metals including Cu ($P < 0.01$), Zn ($P < 0.01$) and Pb ($P < 0.05$). Similar correlation patterns between ARGs and heavy metals have been found in other manured soils (Ji et al., 2012; Peng et al., 2017; Guo et al., 2018). This phenomenon is not surprising, since heavy metals and antibiotics are concurrently used as feed supplements, suggesting the potential for co-selection and cross-selection of resistance traits (Zhu et al., 2013). Metal feed additives, such as Cu and Zn were claimed to be at above-background concentrations in Chinese feedlots (Shi et al., 2011). Pb had significant correlation with some ARGs, as well as Cu and Zn. However, other metals (Cr, Ni, and As) did not exhibit strong correlations with ARGs in this study, possibly due to their relatively low concentrations in target samples; for example, Cd and As occurred at low levels. With respect to Cr, the weak relationship between ARGs and Cr could be associated with the fact that the genotoxicity of Cr is primarily related to the dissolved fraction but not the particle fraction in soils (Ji et al., 2012). Since antibiotic and heavy metal resistance are commonly located in the same genetic elements, elevated mobilization under heavy metal selection pressure could contribute to the spread of ARGs in the environment.

Furthermore, total ARGs exhibited impressively strong correlations with both of the MGEs, i.e., *intI1* and *Tn916/1545* ($P < 0.01$). Individual ARGs other than *tetW* and bla_{TEM} were also significantly and positively associated with *intI1* or *Tn916/1545*. The remarkable positive correlations between ARGs and MGEs have been extensively reported (Zhu et al., 2013; Chen et al., 2016; Peng et al., 2017). ARGs are frequently located on MGEs, including integrons, transposons and plasmids which are the vehicles for ARGs spread via HGT (Zhao et al., 2017). It has been recognized that MGEs, particularly integrons, can be used by bacteria to stockpile and express various exogenous resistance genes and play critical roles in ARGs propagation (Chen et al., 2016). The affinity of MGEs and ARGs indicates the potential for the HGT of ARGs in manure-amended agricultural soils. In the present study, the total abundances of MGEs were much higher in the manured soils than in the control soil (Fig. 2); consequently, the ARGs in manured soils might have greater HGT potential than do those in non-manure-amended soil.

In this study, ARGs exhibited strong correlations with certain soil physicochemical properties, heavy metals and MGEs, indicating that the profile and propagation of ARGs might be influenced by the combined impacts of multiple factors, as reported by other studies (Chen et al., 2016; Zhao et al., 2017). The *r*-values (Table 5) describing the extent of ARGs correlations to various factors, indicated that the MGEs (e.g., *intI1* and *Tn916/1545*), Cu and Zn might be the major factors affecting the profile and spread of ARGs, whereas TC, OM, TN and Pb were likely the minor factors.

4 Conclusions

This study comprehensively demonstrated the effects of long-term manure application on the enrichment of ARGs and MGEs in agricultural soils. All the analyzed eight ARGs and two MGEs were detected in both the manured and control soils. The total relative abundances of ARGs in manured soils were 5.0–18.1 fold greater than that those in CK, except for the relative abundance in BD, which was comparable to that of CK. The manured soils had much higher prevalences of *Tn916/1545* (2.5–69.1 times) than did CK. However, the relative abundances of *intI1* in manured soils were not always greater (0.6–3.7 fold) than the abundance in CK. High-throughput sequencing analysis indicated that the bacteria carrying *intI1* and *ermF* were likely mainly affiliated with *Proteobacteria* and *Bacteroides*, respectively, at the phylum level. At the genus level, *Pseudomonas* and *Bacteroidetes* might be the dominant host bacteria for *intI1* and *ermF* in soils, independent of manure application. Correlation analysis revealed that ARGs had strong associations with soil physicochemical properties (e.g., TC, TN, and OM), heavy metals (Cu, Zn and Pb) and MGEs, indicating that the profile and spread of ARGs might be associated with multiple factors.

Acknowledgements This study was supported by the National Natural Science Foundation of China (Grant No. 21607114) and Science and Technology Correspondent Project of Tianjin (No. 18JCTPJC55100). We also appreciated the help from Professors Xiaomei Wang and Xiaodong Xie during sample analysis. Thanks to Chunjie Li and Fahui Liang for sample collection.

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11783-019-1180-x and is accessible for authorized users.

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