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RESEARCH ARTICLE

Antibiotic resistance genes in manure-amended paddy soils across eastern China: Occurrence and influencing factors

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HIGHLIGHTS

- Manure fertilization resulted in antibiotic residues and increased metal contents.
- The *tet* and *sul* genes were significantly enhanced with manure fertilization.
- Soil physicochemical properties contributed to 12% of the variations in ARGs.
- Soil metals and antibiotics co-select for ARGs.

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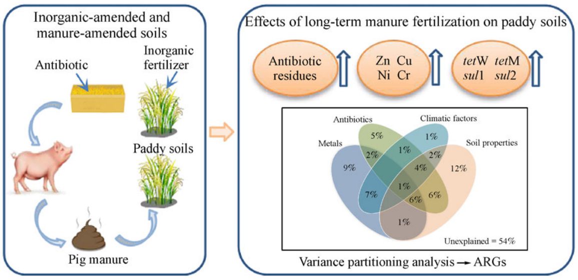
GRAPHIC ABSTRACT



ABSTRACT

Pig manure, rich in antibiotics and metals, is widely applied in paddy fields as a soil conditioner, triggering the proliferation of antibiotic resistance genes (ARGs) in soil. However, comprehensive studies on the effects of manure fertilization on the abundance of ARGs and their influencing factors are still insufficient. Here, pig manure and manure-amended and inorganic-amended soils were collected from 11 rice-cropping regions in eastern China, and the accumulation of antibiotics, metals, and ARGs was assessed simultaneously. The results showed that manure fertilization led to antibiotic residues and increased the metal content (i.e., Zn, Cu, Ni, and Cr). Tetracycline and sulfonamide resistance genes (*tet*M, *tet*O, *sul*1, and *sul*2) were also significantly enhanced with manure fertilization. According to variance partitioning analysis, the most important factors that individually influenced ARGs were soil physicochemical properties, accounting for 12% of the variation. Significant correlations between soil nutrients and ARGs indicated that manure application enhanced the growth of resistant microorganisms by supplying more nutrients. Metals and antibiotics contributed 9% and 5% to the variations in ARGs, respectively. Their co-occurrence also increased the enrichment of ARGs, as their interactions accounted for 2% of the variation in ARGs. Interestingly, Cu was significantly related to most ARGs in the soil ($r = 0.26–0.52$, $p < 0.05$). Sulfapyridine was significantly related to *sul*2, and tetracycline resistance genes were positively related to doxycycline. This study highlighted the risks of antibiotic and ARG accumulation with manure fertilization and shed light on the essential influencing factors of ARGs in paddy soils.

1 Introduction

To promote animal growth and prevent disease, antibiotics are added to animal feed, and most antibiotics can not be absorbed and metabolized by animals (Rahman et al., 2018). In fact, international health organizations disadvise the use of antibiotics for animal growth promotion, and several countries, such as the European Union (EU), have recognized this and taken steps to reduce the use of antibiotics animal production (Heuer et al., 2011a). However, antibiotics are still widely used in the livestock industry in some Asian countries to increase production

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and economic benefits. Zhang et al. (2013) found that tetracyclines, sulfonamides, fluoroquinolones, and macrolides are the most commonly used antibiotics and are often detected in high concentrations in the environment. There are many ways for antibiotics to enter the environment, one of which is the application of animal manure in agriculture as organic fertilizers (Tang et al., 2015). Tetracyclines and sulfonamides are commonly used antibiotics for animals, so they have been frequently detected in agricultural fields and manure (Guo et al., 2018). Quinolones and macrolides are also used to treat a variety of pathogenic infections in hospital and out-of-hospital settings. Four-quinolones are the third-most-prescribed drugs, followed by macrolides. As two important classes of antibiotics, they are widely used to prevent and treat animal diseases. High concentrations of quinolones and macrolides can be found in livestock manure and manure-amended soils (Wang et al., 2019).

A major problem caused by antibiotic contamination in the environment is the rapid increase in the abundance of resistance genes (ARGs) in bacteria (Ji et al., 2012). ARGs spread in the environment, causing significant risk to humans. As organic fertilizers are reservoirs of antibiotics, the abundance of ARGs increases with the increase in antibiotics through organic fertilizers (Tang et al., 2015). Heuer et al. (2011b) found that the abundance of sulfonamide resistance genes (*sul*1 and *sul*2) was significantly increased in soils treated with three successive fertilizers containing antibiotics compared to soils treated with antibiotic-free fertilizer. In addition, Van den Meersche et al. (2020) found that fertilization with animal manure directly added ARGs to the soils, and this direct mechanism may be more important than the selective pressure exerted by antibiotics. However, some studies showed that the release and spread of ARGs had nothing to do with their corresponding antibiotics (Ji et al., 2012), as the antibiotics will be degraded and absorbed in soils. Thus, the effect of antibiotic contamination on ARGs in soils is still controversial.

In addition to antibiotics, metals such as copper (Cu), zinc (Zn), chromium (Cr), and manganese (Mn) are also widely used as animal feed additives due to their antibacterial and growth-promoting effects (Ji et al., 2012). A previous study showed that 55%, 69%, and 51% of cadmium (Cd), Cu, and Zn in agricultural soils were derived from livestock manure, respectively (Luo et al., 2009). Unlike antibiotics, which are subject to easy degradation in soils, metals can exhibit long-term selection pressure on microorganisms (Guo et al., 2018). An increasing number of studies have shown that metals are directly related to ARGs (Knapp et al., 2011; Guo et al., 2018). Knapp et al. (2011) reported that ARGs (*sul*1 and *sul*2) have a strong correlation with some metals (Cu, Zn, and Hg) in soils. These metals could be further combined with antibiotics to exhibit synergistic selection pressure (Baker-Austin et al., 2006). Metal resistance genes (MRGs) and ARGs have been coselected by a variety of mechanisms in the environment (Ji et al., 2012), as metals and antibiotic resistance are frequently combined in the same mobile gene elements, such as plasmids. Hence, the abundance of ARGs might be enhanced by long-standing coselection pressure imposed by metals (Guo et al., 2018). However, most of the abovementioned studies on the changes in metals and antibiotics after manure application and their effects on ARGs are not comprehensive. Long-term field studies with both manure and inorganic fertilization are necessary to understand the effects of manure application on soil antibiotics, metals, and ARGs.

Once ARGs are formed and disseminated under the selection pressure of antibiotics and metals, they can persist even then the selection pressure has disappeared (Kim et al., 2016). In addition to antibiotics and metals, many factors can affect the enrichment and transmission of ARGs in soils. For example, light, high temperature, anaerobic treatment, and biological manure treatment were reported to accelerate the degradation of ARGs in the environment (Pei et al., 2007; Van den Meersche et al., 2019). Soil properties also affect the abundance and distribution of ARGs, as they can change the bacterial community and further change antibiotic resistance, such as the soil pH, total nitrogen (TN), total organic nitrogen (TON), total phosphorus (TP), and organic matter (OM) (Cui et al., 2018). Wang et al. (2020) concluded that TN and OM were the most important soil properties affecting ARGs in greenhouse soils. Although the variations in ARGs were associated with environmental factors, their relative contributions and cross-impacts were rarely estimated.

Here, a total of 11 rice planting regions in eastern China were selected to analyze the abundance of four classes of ARGs, including tetracycline, sulfonamide, quinolone, and macrolide resistance genes, in pig manure, manure-amended soils (>10 years), and inorganic-amended soils. We aimed to explore 1) how long-term manure fertilization affects the accumulation of antibiotics and metals in paddy soils; 2) how manure fertilization affects the occurrence of ARGs; and 3) to what extent antibiotic residues, metals, and other environmental factors contribute to ARG abundance.

2 Materials and methods

2.1 Sample collection

Fresh pig manure and manure-amended and inorganic-amended soils were collected from 11 rice planting regions in eastern China between October and December 2013, including Taizhou (TZ), Mingguang (MG), Danyang (DY), Changzhou (CZ), Lu'an (LA), Shanghai (SH), Jiaxing (JX), Yiwu (YW), Shanggao (SG), Zhangshu (ZS), and Fuzhou (FZ). The locations, climate type, mean annual temperature, mean annual precipitation, and soil type of the sampling regions are summarized in Table S1.

In each region, an area of paddy fields greater than 5 ha with a single rice planting and similar fertilization regimes that had continued for more than 10 years was selected. The soil sampling sites were cultivated and irrigated consistently. Pig manure was collected from nearby farms, and manure-amended soil samples were collected immediately after a single rice sample was harvested. Inorganic-amended soil samples were collected from nearby paddy fields where only chemical fertilizers were applied. We established a 50 m × 50 m rectangular plot at each sampling site. Thirteen subplots were placed apart along the transect, resembling an "L", with distances between the adjacent subplots of 0, 1, 1, 3, 6, 14, and 25 m (Fig. S1). Three soil cores (5 cm diameter) from surface soil (0–20 cm) were sampled randomly in a circle with a 0.5 m diameter from each subplot and mixed. Every pool of samples from each subplot was analyzed separately. Pig manure samples were collected on a farm near each rice planting region. A total of 6 fresh pig manure samples were collected from the manure collection pool at each farm. All samples were transported immediately to the laboratory on ice. In the laboratory, visible residues were manually removed from the samples and subdivided into two subsamples. One subsample was stored at 4°C for geochemical measurements, and the other was stored at −80°C for molecular analysis.

2.2 Soil physicochemical analysis and metal quantitation

The physicochemical properties of all soil samples were analyzed according to the recommended soil-testing procedures (Lu, 1999), including pH, moisture content, sulfate (SO_4^{2-}), total organic carbon (TOC), total nitrogen (TN), nitrate nitrogen (NO_3^--N), ammonium nitrogen (NH_4^+-N), total organic nitrogen (TON), total phosphorus (TP), available phosphorus (AP), and total potassium (TK).

Six kinds of metals, including Zn, Cu, Pb, Ni, Cr, and Cd, which are closely related to human activities or often used as feed additives, were determined for both pig manure and soil samples. A total of 0.5 g soil sample or 0.1 g pig manure sample was placed into a digestion tank (Topwave, Analytik Jena, Germany), and the sample was dissolved in the HF/HCl/HNO_3 (GR, Sinopharm Chemical Reagent Co., LTD, China) mixture (1:2:6 mL). When the temperature dropped below 60°C, the solution was diluted with water containing 0.2% nitric acid to 50 mL. The concentration of metals in the samples was detected by flame atomic absorption spectrometry (novAA300, Analytik Jena, Germany) after the digestion solution was diluted to a certain multiple (González-Alcaraz and van Gestel, 2015).

2.3 Detection of antibiotics in soil and pig manure

According to the antibiotic treatment history of pigs, the selected antibiotics included four tetracyclines (TCs: tetracycline, TC; chlortetracycline, CTC; oxytetracycline, OTC; and doxycycline, DC), five sulfonamides (SAs: sulfamethyldiazine, SM1; sulfamethazine, SM2; sulfadiazine, SDZ; sulfapyridine, SPY; and sulfamethoxazole, SMX), four quinolones (FQs: norfloxacin, NFC; enrofloxacin, ENR; ciprofloxacin, CIP; and lomefloxacin, LOM), and two macrolides (MALs: tylosin, TYL; roxithromycin, RTM).

The standard substances of the target antibiotics were purchased. Then, 0.5 g of each standard antibiotic was accurately weighed and dissolved in methanol (LC, TEDIA, USA) in a 10 mL brown volumetric flask to prepare 50 mg/mL standard mother liquor, then gradient diluted into standard solutions of different concentrations. Nine different concentrations (1, 5, 10, 20, 50, 100, 200, 500, 1000 μg/L) of a standard solution were selected as the standard curve. The standard recovery of the standard curve was between 58.7% and 97.6%. For the four classes of antibiotics, the detection limit (LOD) ranged from 0.0023 to 0.0858 mg/L, and the quantitative limit (LOQ) ranged from 0.0069 to 0.2600 mg/L. In addition, the method detection limit (MQL) was 0.20–2.70 μg/kg in soils and 0.01–0.15 mg/kg in manure (Table S2).

The target antibiotics were extracted from soils and pig manure samples by ultrasonic extraction and solid-phase extraction, as previously reported (Martínez-Carballo et al., 2007). The antibiotics in the samples were detected by LC/MS/MS liquid chromatography triple quadrupole mass spectrometry (Agilent 6460, Agilent, USA) in multi-reaction monitoring mode. The proper peak assignment and peak purity were determined based on retention time and characteristic ions. Prior to the filtration of all sample solutions, the internal standard (simeton) was added as a standard substitute to compensate for matrix effects during analysis (Zhang et al., 2013).

2.4 Total genome extraction from soil and quantification of resistance genes

Microbial genomic DNA was extracted from the samples using the method developed by Zhou et al. (1996) and quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, USA). Considering the antibiotic treatment history of pigs, the selected ARGs included tetracycline resistance genes (*tet*W, *tet*M, *tet*O, *tet*Q, and *tet*H), sulfonamide resistance genes (*sul*1 and *sul*2), quinolone resistance genes (*gyr*A and *qnr*A), and macrolide resistance genes (*erm*F).

According to Liang et al. (2017), all qualitative PCR tests were performed in a 50 μL reaction system. The specific components of the reaction system are shown in Table S3. The specific primers designed are presented in Table S4. The CFX96 real-time PCR System (Bio-Rad, USA) was used for quantitative real-time PCR analysis of target ARGs and 16S rRNA in a 20 μL system. The real-time PCR primers for ARGs and the 16S rRNA gene were the same as those used in the qualitative PCR (Table S4). Details for the procedures of qualitative PCR and quantitative real-time PCR are provided in Supplementary Information.

In addition, colistin and β-lactam antibiotics are widely used in the food-producing animal industry in China. Geochip 5.0 was used to determine the abundances of ARGs in soils from three representative sampling sites (Changzhou, Shanggao, and Fuzhou of China). Details for the Geochip are provided in Supplementary Information.

2.5 Data analysis

The significance of the differences in environmental variables, metals, antibiotic concentrations, and ARGs was tested using Duncan's multiple-range test at the $p < 0.05$ level after one-way analysis of variance by SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Linear regressions were conducted between antibiotic concentrations in manure-amended paddy soils and pig manure in R 3.6.3 with the 'lm' function. To avoid overfitting, we further performed cross-validation using the 'bootstrap' package. Pearson's correlation analysis between ARGs and antibiotics, metals, and other environmental variables was used to determine their correlations in R 3.6.3. Canonical correspondence analysis with the 'vegan' package in R was used to evaluate the contributions of antibiotics, metals, climate factors, and soil physicochemical properties to ARGS variation by means of variance partition analysis (VPA). Heatmaps and boxplots were visualized with the 'pheatmap' and 'ggplot2' packages in R.

3 Results

3.1 Soil physicochemical properties with manure application

The soil physicochemical properties of both inorganic-amended and manure-amended paddy fields are presented in Tables 1 and S5. The manure application in paddy fields significantly increased the soil pH and moisture while decreasing the soil TOC ($p < 0.01$). The manure application also greatly elevated the soil nutrients, including NO_3^--N, NH_4^+-N, TON, TP, and AP ($p < 0.05$). However, there was no significant difference in the contents of SO_4^{2-}, TN, and TK between the inorganic-amended and manure-amended paddy soils ($p > 0.05$). In general, the application of manure had a significant effect on soil physicochemical properties.

3.2 Investigation of antibiotics in pig manure and soils

To investigate the antibiotic distributions in manure-amended paddy soils in eastern China, four tetracyclines (TC, OTC, CTC, and DC), five sulfonamides (SM1, SM2, SDZ, SPY, and SMX), four quinolones (NFC, ENF, CIP, and LOM), and two macrolides (TYL and RTM) were analyzed by LC-MS/MS in pig manure and paddy soils sampled from 11 regions. High concentrations of tetracyclines were detected in all pig manure samples, ranging from 6.70 to 140.55 mg/kg (Fig. 1(a)). Pig manure from JX contained high levels of TC (70.02±5.42 mg/kg) and OTC (57.75±2.64 mg/kg), and manure from YW was rich in OTC (60.48±86.11 mg/kg). Sulfonamides were detected in most pig manure samples, except for TZ and ZS, which ranged from n.d. to 6.59 mg/kg. Quinolones were also detected in all pig manure samples (0.75–14.11 mg/kg), while macrolides were only detected in MG (0.86 mg/kg) and SH (0.55 mg/kg) at low concentrations.

Similar to the patterns observed in pig manure, manure-amended paddy soils were also rich in tetracyclines,

Table 1 Soil physicochemical properties in inorganic-amended and manure-amended paddy soils

Soil physicochemical properties	Inorganic-amended soils (n = 143)	Manure-amended soils (n = 143)	p
pH	4.3–7.3	5.4–7.3	< 0.001
Soil moisture (%)	5.9–35.8	8.4–35	0.008
SO_4^{2-} (mg/kg)	34.7–138.8	18.1–286.9	0.632
TOC (mg/kg)	56.4–196.6	44.6–163.6	0.002
TN (g/kg)	0.6–2	0.6–2.3	0.192
NO_3^--N (mg/kg)	2.5–36.6	3.3–120.8	< 0.001
NH_4^+-N (mg/kg)	15.9–71.4	20.9–83.3	0.028
TON (mg/kg)	6.1–67.7	7.8–154.3	< 0.001
TP (g/kg)	0.4–1.5	0.4–2.5	< 0.001
AP (mg/kg)	0.3–3.3	0.4–11.7	< 0.001
TK (g/kg)	8–44.5	11.8–52.9	0.142

Notes: TOC: total organic carbon; TN: total nitrogen; TON: total organic nitrogen; TP: total phosphorus; AP: available phosphorus; TK: total potassium.

ranging from 144.22 to 3838.51 μg/kg (Fig. 1(a)). Soils in TZ contained the highest levels of OTC (3491.06±1905.14 μg/kg), and soil in JX was rich in TC (3343.97±840.56 μg/kg). Sulfonamides were detected in soils from MG, CZ, LA, SH, JX, and SG, ranging from 10.31 to 219.59 μg/kg. In all soil samples, high concentrations of quinolones were detected (49.11–611.19 μg/kg), while macrolides were only detected in MG (14.05 μg/kg). By correlating the antibiotic content in soils with the antibiotics in pig manure, we found significant positive relationships between the antibiotics in soils and in pig manure for TC (raw_r^2 = 0.97, cv_r^2 = 0.89, $p < 0.001$), SM1 (raw_r^2 = 0.95, cv_r^2 = 0.94, $p < 0.001$), SDZ (raw_r^2 = 0.60, cv_r^2 = 0.39, $p = 0.005$), and LOM (raw_r^2 = 0.38, cv_r^2 = 0.13, $p = 0.043$) (Fig. 1(b)). We also tried to detect 15 antibiotics in inorganic-amended paddy soils, while all target antibiotics were below the LOD.

3.3 Investigation of metals in pig manure and soils

Metals in pig manures and inorganic-amended and manure-amended paddy soils sampled from 11 regions were also determined in our study, including Zn, Cu, Pb, Ni, Cr, and Cd. The highest total concentrations of all metals were found in pig manure in each region, ranging from 894.41 to 3614.39 mg/kg (Fig. 2(a)). The total concentrations of metals in pig manure were the highest in ZS and the lowest in FZ. Inorganic-amended paddy soils contained the most metal in SH (430.60 mg/kg) and the least metal in MG (196.63 mg/kg). The total concentrations of metals in manure-amended paddy soils were the highest in MG (608.35 mg/kg) and the lowest in TZ (237.77 mg/kg). Six metals were also detected in both inorganic-amended and manure-amended paddy soils. The accumulation of Zn was the highest in inorganic-amended paddy soils, ranging from 57.64 to 172.47 mg/kg, and the accumulation of Cd was the lowest, ranging from 1.70 to 6.30 mg/kg (Fig. 2(b)). Similarly, the concentrations of Zn in manure-amended paddy soil were the highest (61.68–281.08 mg/kg), and the concentrations of Cd were the lowest (0.57–7.52 mg/kg). In manure-amended paddy soils, Zn, Cu, Ni, and Cr were significantly higher in concentration than in inorganic-amended soils ($p < 0.05$). We also tried to correlate the metals in soils with those in pig manure. However, there were no significant correlations for any of the six metals ($p > 0.05$).

3.4 The abundance of antibiotic resistance genes

All 10 ARGs were detected using qPCR in all 11 paddy fields, including both inorganic-amended and manure-

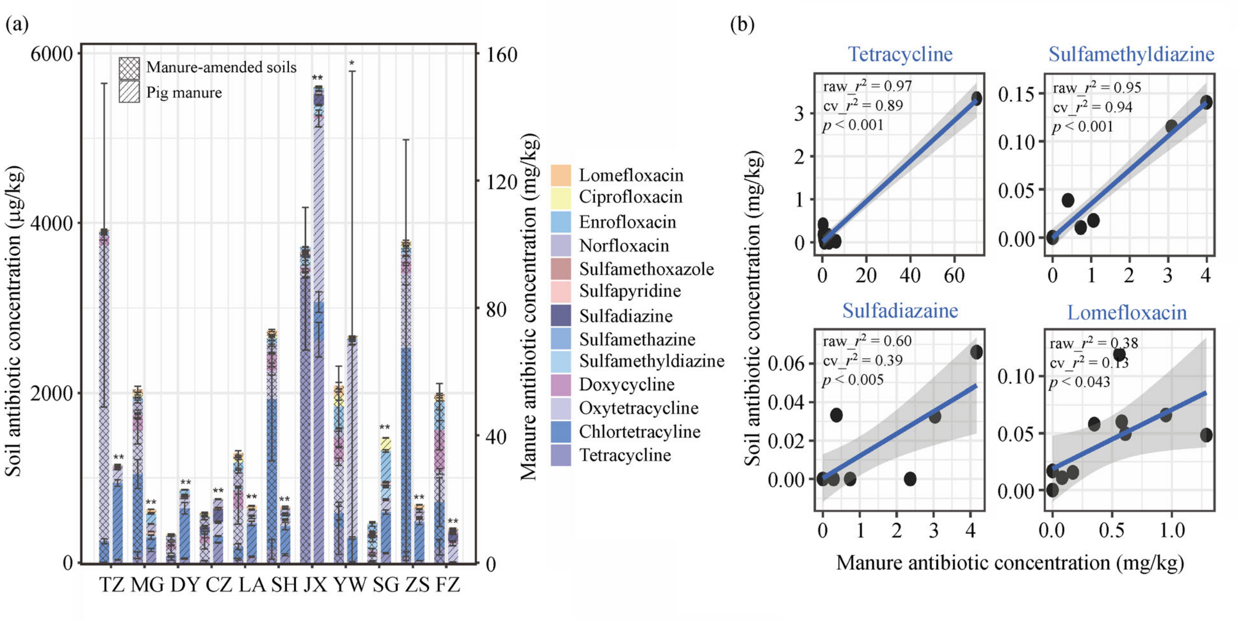

Fig. 1 (a) Antibiotic concentrations in manure-amended paddy soils and pig manure used as fertilizer in 11 rice planting regions in eastern China; (b) Correlating the antibiotics in manure-amended paddy soils with those in pig manure. Four classes of antibiotics include tetracyclines (TCs: tetracycline; chlortetracycline; oxytetracycline; doxycycline), sulfonamides (SAs: sulfamethyldiazine; sulfamethazine; sulfadiazine; sulfapyridine; sulfamethoxazole), quinolones (FQs: norfloxacin; enrofloxacin; ciprofloxacin; lomefloxacin), and macrolides (MALs: tylosin; roxithromycin). TZ: Taizhou; MG: Mingguang; DY: Danyang; CZ: Changzhou; LA: Lu'an; SH: Shanghai; JX: Jiaxing; YW: Yiwu; SG: Shanggao; ZS: Zhangshu; FZ: Fuzhou. The left *y*-axis of Fig. 1 (a) represents antibiotic concentrations in manure-amended paddy soils, and the right *y*-axis represents antibiotic concentrations in pig manure. raw_r^2 and cv_r^2 indicate the r^2 value of linear regression with and without cross-validation, respectively. * and ** above the bars indicate significant differences in antibiotic concentrations between pig manure and manure-amended paddy soils at $p < 0.05$ and $p < 0.01$, respectively.

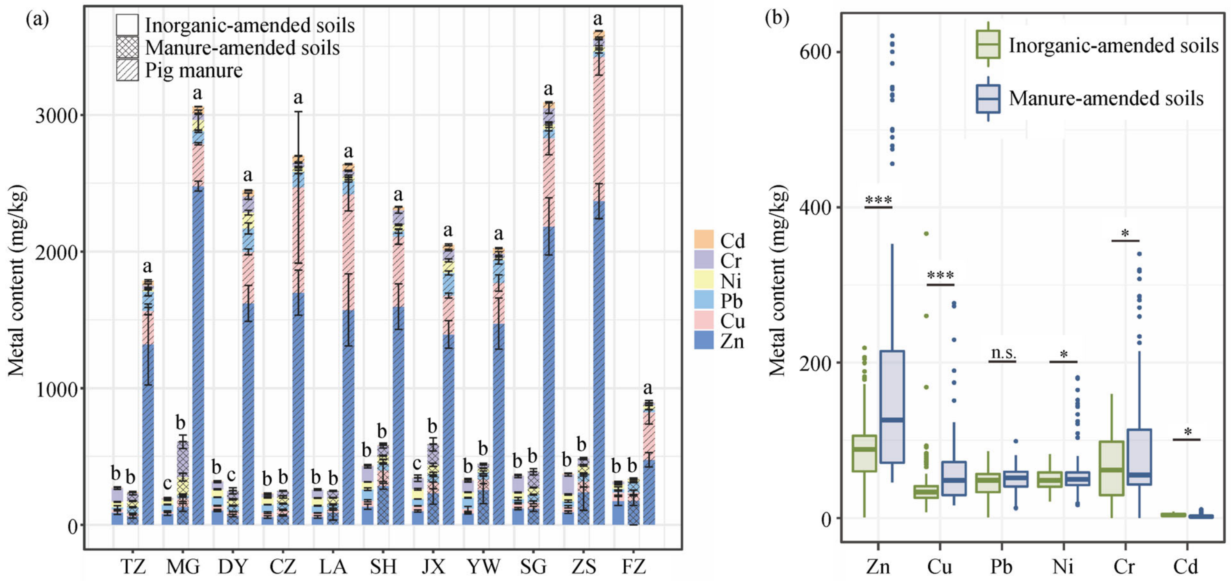

Fig. 2 (a) Metals in inorganic-amended and manure-amended paddy soils and pig manure used as fertilizer in 11 rice planting regions in eastern China; (b) Comparison of the metals in inorganic-amended and manure-amended paddy soils. TZ: Taizhou; MG: Mingguang; DY: Danyang; CZ: Changzhou; LA: Lu'an; SH: Shanghai; JX: Jiaxing; YW: Yiwu; SG: Shanggao; ZS: Zhangshu; FZ: Fuzhou. Different letters above the bars indicate differences in metal concentrations between inorganic-amended and manure-amended paddy soils and pig manure at $p < 0.05$. * and *** indicate significant differences in metals in inorganic-amended and manure-amended paddy soils at $p < 0.05$ and $p < 0.001$, respectively. n.s.: not significant.

amended fields. Except for YW and FZ, the abundance of ARGs in manure-amended fields was higher than in inorganic-amended soils at other sampling points. Among all the sampling sites, SH contained the highest amounts of resistance genes, followed by JX in manure-amended soils. The abundance of sulfonamide resistance genes was significantly higher than those of other resistance genes in manure-amended fields except in LA, and the abundance of quinolone resistance genes was the lowest of the four classes of ARGs at all sites.

The relative abundance (gene copies/16S rDNA copies) of tetracycline resistance genes in paddy soils ranged from 0.0003±0.0001 to 0.35±0.62 (Fig. 3). The relative abundance of sulfonamide resistance genes were highest among all the ARGs, varying from 0.0009±0.0006 to 0.82±0.44. The relative abundance of quinolone and macrolide resistance genes in paddy soils varied from 6.11E-06 to 1.7E-02 and 4.33E-05 to 8.7E-01, respectively. Among all these genes, the relative abundance of *tet*M, *tet*O, *sul*1, and *sul*2 were significantly higher in manure-amended soils than in inorganic-amended soils ($p < 0.05$). The relative abundance of *tet*W, *tet*Q, and *erm*F were higher in manure-amended soils than in inorganic-amended soils ($p > 0.05$). The relative abundance of *tet*H, *gyr*A, and *qnr*A were small, and they did not differ much in inorganic-amended and manure-amended soils. As the β-lactam antibiotics are widely used in the food-producing animal industry in China, we also detected the β-lactam resistance genes by Geochip 5.0. The results showed that the abundance of β-lactam resistance genes in manure-amended soils was significantly higher than that in inorganic-amended soils in CZ and SG (Fig. S2).

3.5 Relationships between antibiotic resistance genes and antibiotics, metals, and environmental variables

To link the ARGs in paddy soils with the antibiotics, metals, and environmental variables, Pearson's correlations were conducted for both the inorganic-amended (Fig. S3) and manure-amended (Fig. 4) paddy soils. In inorganic-amended soils, the content of Cu in paddy soils were positively and significantly related to the relative abundances of ARGs ($r = 0.37$–0.59, $p < 0.05$), except for *tet*M and *qnr*A. Significant positive correlations ($p < 0.01$) were found between SO_4^{2-} in soil and *tet*M, and TN in soil was significantly correlated with *qnr*A ($p < 0.05$). For manure-amended soils, antibiotics, metals, and environmental variables were all potentially related to ARGs. Tetracycline resistance genes were positively related to DC ($r = 0.31$–0.34, $p < 0.05$). The sulfonamide resistance gene (*sul*2) was positively related to SM1 ($r = 0.36$, $p < 0.05$) and SPY ($r = 0.41$, $p < 0.05$). The quinolone resistance gene *gyr*A was negatively correlated with TC ($r = -0.27$, $p < 0.05$) and positively correlated with TYL ($r = 0.28$, $p < 0.05$), and *qnr*A was positively correlated with TC ($r = 0.43$, $p < 0.05$) and negatively correlated with LOM ($r = -0.25$, $p < 0.05$). The macrolide resistance gene *erm*F showed positive correlations with DC ($r = 0.26$, $p < 0.05$).

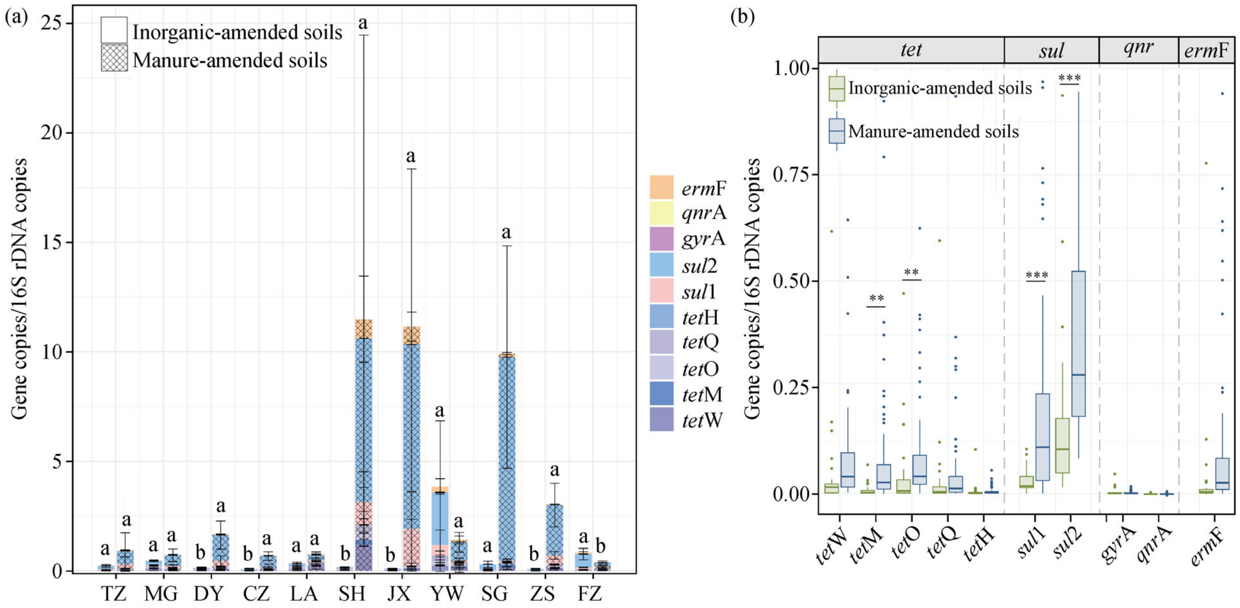

Fig. 3 (a) The relative abundance of antibiotic resistance genes in inorganic-amended and manure-amended paddy soils in 11 rice planting regions in eastern China; (b) Comparison of the relative abundance of antibiotic resistance genes in inorganic-amended and manure-amended paddy soils. *tet*, *sul*, *qnr*, and *ermF* indicate tetracycline, sulfonamide, quinolone, and macrolide resistance genes, respectively. TZ: Taizhou; MG: Mingguang; DY: Danyang; CZ: Changzhou; LA: Lu'an; SH: Shanghai; JX: Jiaxing; YW: Yiwu; SG: Shanggao; ZS: Zhangshu; FZ: Fuzhou. Different letters above the bars indicate the difference in the sum relative abundance of antibiotic resistance genes between inorganic-amended and manure-amended paddy soils at $p < 0.05$. ** and *** indicate significant differences in antibiotic resistance genes in inorganic-amended and manure-amended paddy soils at $p < 0.01$ and $p < 0.001$, respectively. n.s.: not significant.

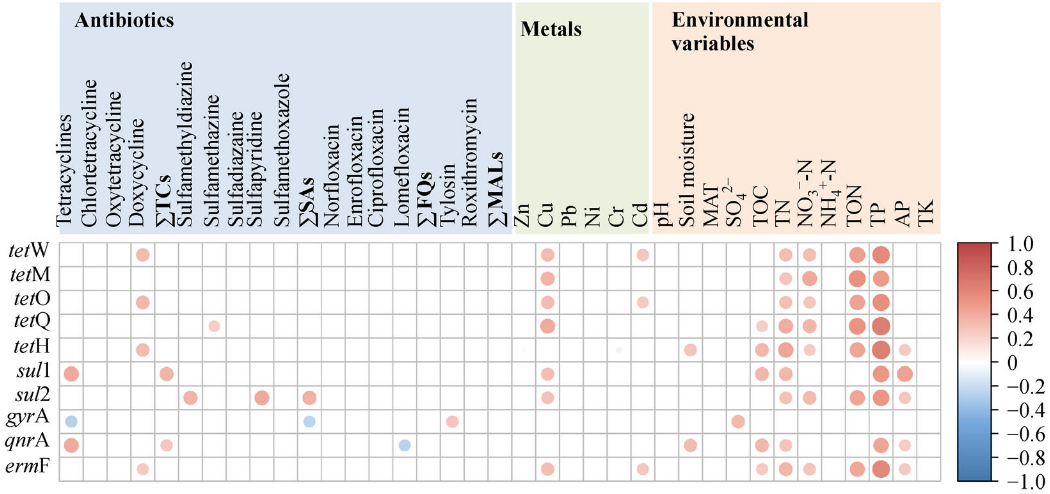

Fig. 4 Pearson's correlations between the antibiotic resistance genes and the antibiotics, metals, and other environmental variables in manure-amended paddy soils. The color points indicate a significant correlation coefficient ($p < 0.05$; bluer indicates a stronger negative correlation, and redder indicates a stronger positive correlation). TCs: tetracyclines; SAs: sulfonamides; FQs: quinolones; MALs: macrolides; MAT: mean annual temperature; TOC: total organic carbon; TN: total nitrogen; TON: total organic nitrogen; TP: total phosphorus; AP: available phosphorus; TK: total potassium.

Soil metals, including Cu and Cd, showed positive and significant correlations with ARGs (r = 0.26–0.52, $p < 0.05$). Environmental variables were also important to the ARGs, presenting positive relationships between soil physicochemical properties (soil moisture, SO_4^{2-}, TOC, TN, NO_3^--N, TON, TP, and AP) and ARGs ($r = 0.23$–0.65, $p < 0.05$).

The contributions of antibiotics, metals, climatic factors, and soil physicochemical properties to the ARG variation in manure-amended paddy soils were quantified by variance partitioning analysis (Fig. 5). A total of 46% of the variation was explained by these four components. Antibiotics, metals, climatic factors, and soil physicochemical properties independently explained 5%, 9%, 1%, and 12% of the total variations observed, respectively. Interactions among the four components also influenced the ARG variation, in which the interactions between metals and climatic factors explained 7%; the interactions between antibiotics and soil physicochemical properties explained 6%, and the interactions among antibiotics, metals, and soil physicochemical properties explained 6%. More than 50% of the ARG variations could not be explained by these four components.

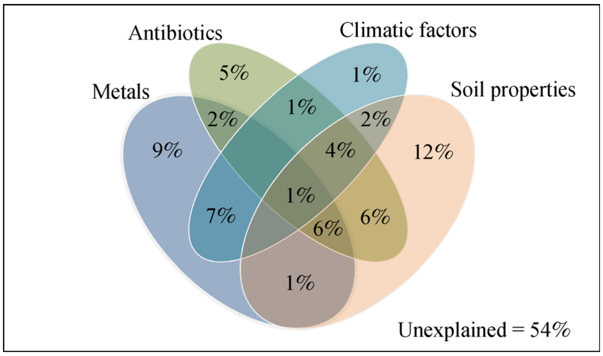

Fig. 5 Variation partitioning analysis of the antibiotic resistance genes explained by antibiotics, metals, climatic factors, and soil properties. Metals included Zn, Cu, Pb, Ni, Cr, and Cd. Antibiotics included tetracyclines, sulfonamides, quinolones, and macrolides. Climatic factors included mean annual temperature and precipitation. Soil properties included soil moisture, SO_4^{2-}, total organic carbon, total nitrogen, NO_3^--N, total organic nitrogen, total phosphorus, and available phosphorus.

4 Discussion

4.1 Manure fertilization increased the contents of antibiotics and metals in paddy soils

By measuring the antibiotics in inorganic-amended and manure-amended paddy soils, we found that manure fertilization can significantly aggravate antibiotic pollution in soils. In this study, there were high concentrations of antibiotics in manure-amended soils, but no antibiotics were detected in inorganic-amended soils. These findings suggested that large proportions of antibiotics in soils were derived from manure fertilization. Van den Meersche et al. (2020) suggested that antibiotic-resistant bacteria were excreted in feces together with antibiotic residues and might enter the environment upon fertilization where the fecal bacteria were disseminated. In our study, we also found that most antibiotics in manure-amended soils could be detected in pig manure, and they were significantly correlated. Consistent with our results, many studies have shown that antibiotic residues can be repeatedly detected in manure and soils modified with manure (Pan et al., 2011; An et al., 2015;Van den Meersche et al., 2020). As antibiotics fed to poultry are difficult to fully absorb, most of them are discharged into the soils with manure, increasing residue concentrations of antibiotics in soils (An et al., 2015).

Among all the antibiotics measured, tetracyclines were present in the highest quantities in pig manure and manure-amended soils in our study. By analyzing tetracyclines, sulfonamides, fluoroquinolones, and tylosin remaining in pig, chicken, and cow manure from large-scale farms in north-east China, Li et al. (2013) also showed that tetracyclines were most abundant, with a concentration of 56.81 mg/kg. Tetracyclines can inhibit the synthesis of bacterial proteins to achieve antibacterial effects, and they are widely used in livestock and poultry breeding as veterinary drugs and growth promoters (Chopra and Roberts, 2001). Some tetracyclines in animals can not be completely metabolized or absorbed because they are bioactive substances, acting highly effectively at low doses and excreted as parent compounds or metabolites after a short time of residence (Kemper, 2008). Previous studies indicated that 70%–90% of tetracyclines could be excreted through animal urine or feces (Mahmoud and Abdel-Mohsein, 2019). Moreover, compared to other antibiotics (e.g., sulfamethazine, norfloxacin, erythromycin, and chloramphenicol), tetracyclines have the longest half-life and the strongest adsorption (Pan and Chu, 2016).

Manure fertilization also increased the concentration of metals in paddy soils, including Zn, Cu, Ni, and Cr, in our study. By measuring the metals in pig manure sampled from Qingyang, China, Liu et al. (2020) concluded that the content of Zn and Cu in the manure was higher than those of other heavy metals through meta-analysis. The high residue of Zn and Cu in pig manure was associated with the addition of large amounts of Zn and Cu to the feed. Zn is used in the European Union to inhibit/cure postweaning diarrhea in piglets, and Zn and Cu antibacterial activity was able to reduce fermentation in the intestinal tract of animals and inhibit intestinal pathogens (Ji et al., 2012). Meanwhile, we found that the difference in the concentrations of Pb between inorganic-amended and manure-amended soils was not significant, indicating that the short-term and rational use of manure has little effect on the accumulation risk of Pb in soils (Deng et al., 2020). In addition, there were no significant correlations for all six metals between metals in pig manure and soils. In fact, there are many sources of metals in soils. Yang et al. (2020) found that the largest contribution to soil heavy metals in Jiangsu, China, was from natural sources and traffic sources, and the most considerable contribution to soil heavy metals in Zhejiang Province (China) was from agricultural and industrial sources.

4.2 Effects of manure fertilization on antibiotic resistance genes in paddy soils

Tetracycline and sulfonamide resistance genes (*tet*M, *tet*O, *sul*1, and *sul*2) were significantly enhanced with manure fertilization in paddy soils in this study. Feces were a natural gene pool of ARGs. However, ARGs in feces are unlikely to persist, and they can inevitably enter other environmental media, such as soil, through a series of transmission pathways, resulting in the wider distribution of ARGs in the environment (Kolz et al., 2005; Heuer et al., 2011a). Agricultural activities such as fecal accumulation and fertilization bring high concentrations of ARGs and resistant microbial communities into the soils and lead to the spread of ARGs (Heuer et al., 2011a).

This study showed that sulfonamide resistance genes (*sul*1 and *sul*2) had higher relative abundance than other ARGs for both inorganic-amended and manure-amended soils. In addition, high levels of tetracycline resistance genes were also detected in the manure-amended soils. Consistent with our results, Guo et al. (2018) also reported a high relative abundance of sulfonamide and tetracycline resistance genes in surface soils after long-term application of manure in a field experiment. A previous study reported that sulfonamides and tetracyclines were used as feed additives as antibiotics in the breeding industry (Pan et al., 2011). Sulfonamides are synthetic antibacterial drugs that can not be synthesized by microorganisms. Therefore, the sulfonamide resistance obtained by soil microorganisms is mainly due to the selection pressure of sulfonamide on microbes.

The abundance of quinolone resistance genes in inorganic-amended and manure-amended soil samples was much lower than that of the sulfonamide and tetracycline resistance genes in this study. Manure fertilization had little effect on quinolone resistance genes. Guo et al. (2018) found that the percentage of fluoroquinolone resistance genes decreased with the increase in the amount of pig manure. In addition, the abundance of macrolide resistance genes was found to be higher in manure-amended soils than in inorganic-amended soils in our study. These findings were consistent with the results of Wang et al. (2019). The abundance of macrolide resistance genes in soils was significantly higher than that of quinolone resistance genes. It has been suggested that quinolone resistance genes act as a reverse proton efflux pump system, which can discharge quinolones from the inside of cells into the extracellular space, further reducing the selection pressure (Wang et al., 2019). Therefore, the abundance of quinolone resistance genes was lower than that of macrolide resistance genes.

4.3 Potentially important environmental factors contributing to ARGs in paddy soils

According to the results of Pearson's correlation analysis and VPA, the most important factors that individually influenced ARGs were soil physicochemical properties, accounting for 12% of the variation in ARGs. In particular, soil nutrients (i.e., TN, NO_3^--N, TON, TP, and AP) showed significant and positive contributions to the ARGs. The results indicated that the long-term application of pig manure could provide more nutrients and greatly promote the growth of resistant microorganisms in paddy soil. Previous research in aquatics also indicated the positive role of nutrients in affecting the abundance of ARGs (Zhang et al., 2018). TP had a great influence on *tet*W, *tet*Q, *tet*H, and *erm*F. Recent studies have shown that pH, TN, and TP are important environmental factors for the formation of bacterial communities, which might further lead to changes in ARGs in soils (Cui et al., 2018; Wang et al., 2020).

Soil metals also contributed to the increase in antibiotic resistance genes, accounting for 9% of the variation in ARGs. Consistent with our results, Zhou et al. (2016) also found that the abundance of ARGs showed a significant positive correlation with the total concentration of metals. In this study, Cu was significantly correlated with most ARGs. This was consistent with the previous study conducted by Guo et al. (2018). Berg et al. (2005) concluded that Cu exposure was a specific selection for Cu-resistant microbes and further coselection for antibiotic resistance in agricultural soils. These results indicated the significant risks of Cu contamination on the persistence and spread of ARGs.

Antibiotics exert direct selection pressure on the accumulation of ARGs, which contributed to 5% of the variations individually. In this study, SPY was the most important factor affecting *sul*2, and tetracycline resistance genes were positively related to DC. Some studies have indeed shown that the abundance of ARGs is significantly associated with the corresponding homologous antibiotics (Zhang et al., 2018). Antibiotics exert selection pressure on microorganisms, resulting in the emergence and high concentrations of resistance genes in feces, and this selection pressure determines the types and levels of resistant bacteria (Heuer et al., 2011a). However, quinolone and macrolide resistance genes were not significantly correlated with the content of quinolones and macrolides, respectively. This might be related to the low concentrations of quinolones and macrolides detected in the soils, as the selection pressure of antibiotics is dependent on their concentration. ARGs in manure can directly enter the soil through fertilization, and this direct mechanism does not involve the participation of antibiotics (Van den Meersche et al., 2020). Indeed, a previous study demonstrated that ARGs could exist in the absence of antibiotics (Johnsen et al., 2009). They found that the antimicrobial-resistance determinants, following a significant reduction in the selection pressure, depend on factors other than drug consumption alone. These results indicated that ARGs were not simply determined by antibiotics; other environmental factors also matter.

Variance partition analysis showed that climatic factors scarcely influenced the ARGs in paddy soils individually, but they influenced the ARGs by interacting with soil metals. We speculated that climatic factors might affect metals and thus ARGs in soils, as a previous study found that drier conditions and higher temperatures increased metal bioaccumulation (González-Alcaraz and van Gestel, 2015). In addition, we found that the interaction between antibiotics and metals increased the enrichment of ARGs. Previous studies have shown that resistance occurs not only due to the selection pressure of antibiotics but also due to the promotion of the transmission of genetic elements by contaminants through cross-resistance and coresistance processes (Baker-Austin et al., 2006). That study suggested that metal contamination represented long, widespread, and persistent selection pressure that may contribute to the maintenance and transmission of antibiotic resistance factors. In fact, 54% of the ARG variations could not be explained by antibiotics, metals, soil physicochemical properties, and climatic factors. This indicated that many other factors affect ARGs in soils. For example, Wang et al. (2020) found that soil type was also an important factor influencing ARGs. In addition, local differences in planting methods, irrigation methods, and water sources could also affect the abundance of ARGs in soils (Rahman et al., 2018).

5 Conclusions

Our study demonstrated the effect of manure application on soil physicochemical properties and the levels of antibiotics, metals, and ARGs in soils. The results showed that the long-term application of pig manure significantly improved soil pH, TON, NO_3^--N, NH_4^+-N, TP, and AP. Additionally, manure application resulted in antibiotic residues and increased the ARGs in paddy soils. Both antibiotics and metals exhibited collaborative selection pressure on ARGs and increased the abundance of ARGs. Soil nutrients also greatly influenced on ARGs, among which TP was the most critical factor affecting *tet*W, *tet*Q, *tet*H, and *erm*F. Climatic factors scarcely influenced the ARGs in paddy soils individually, but they influenced the ARGs by interacting with soil metals. This study discussed the occurrence and influencing factors of ARGs in paddy soils after manure fertilization, which may help develop effective strategies to reduce ARG pollution in agricultural soils.

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