

Coexistence between antibiotic resistance genes and metal resistance genes in manure-fertilized soils

Na Zhang^{a,b}, Philippe Juneau^c, Ruilin Huang^{a,b}, Zhili He^{d,e}, Bo Sun^a, Jizhong Zhou^e, Yuting Liang^{a,*}

^a State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, No. 71 East Beijing Road, Nanjing 210008, China

^b University of Chinese Academy of Sciences, No. 19A Yuquan Road, Beijing 100049, China

^c Department of Biological Sciences, GRIL-TOXEN, Ecotoxicology of Aquatic Microorganisms Laboratory, Université du Québec à Montréal, C.P. 8888, Succ. Centre-Ville, H3C 3P8 Montréal, Québec, Canada

^d Environmental Microbiomics Research Center, School of Environmental Science and Engineering, Sun Yat-Sen University, Guangzhou 510006, China

^e Institute for Environmental Genomics, Department of Microbiology & Plant Biology, and School of Civil Engineering and Environmental Sciences, University of Oklahoma, Norman, OK 73019, USA

ARTICLE INFO

Handling Editor: Naoise Nunan

Keywords:

Multidrug resistance genes
Metal resistance genes
Coexistence
Manure fertilization
Potential hosts

ABSTRACT

Antibiotics and metals introduced during manure application are believed to be the major drivers of the bloom and spread of antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) in agricultural environments. However, the coexistence of multidrug resistance (MDR) and metal resistance and the effects of manure application on the coexistence are less studied. Here, we profiled the coexistence patterns of 9919 MDR genes (MDRGs) and 25,312 MRGs in paddy fields with manure or inorganic fertilization. The abundances of both MDRGs and MRGs were higher under manure fertilization than inorganic fertilization. Network analysis determined that coexistence between MDRGs and MRGs was much more frequent in manure-fertilized soils than in inorganic-fertilized soils. Manure application drove this coexistence by not only directly introducing resistance genes but also increasing co-selection through antibiotics and metals, and stimulating their potential common hosts, including species of *Actinobacterium* WWH12, *Bacillus*, *Geobacter*, *Solirubrobacter*, Acidobacteriales, Bacillales, Chloroflexi, Methylogiellaceae and Xanthobacteraceae. Soil antibiotic resistance was significantly predicted by the coexistence of major multidrug efflux genes (*mfs*, *mex*, *abc*, *mate* and *smr*) and MRGs. The coexistence between MDRGs and MRGs explained 18% of the increase in soil antibiotic resistance. Such coexistence is nonnegligible and permits the promotion of soil antibiotic resistance, thus posing a potential threat to both agroecosystems and humans.

1. Introduction

The increasing prevalence of antibiotic resistance genes (ARGs) in the environment has become a global threat to human health (Arias and Murray, 2009; Forsberg et al., 2012). Annual global antibiotic consumption in agriculture has reached 63,000–240,000 tons (Kuppusamy et al., 2018). Generally, antibiotics are poorly absorbed by animals, and 30–90% of the parent compounds is excreted (Kemper, 2008; Qiao et al., 2018). The amount of 36 major antibiotics excreted was estimated to be 53,800 tons in 2013, of which 84% was produced by animals (Zhang et al., 2015). Multidrug resistance (MDR), which refers to acquired nonsusceptibility to antibiotic agents of different classes, is seriously

threatening, as few antibiotic agents are effective and limited new antibiotic agents have been developed (Magiorakos et al., 2012; WHO, 2014). With the growing use of antibiotics in animal farming, it is of critical importance to understand the existence pattern of MDR genes (MDRGs) and its driving factors in agroecosystems (Gullberg et al., 2014; Blanco et al., 2016).

In addition to antibiotics, metals (e.g., Cu, Zn, or As) have also been commonly used in feed additives for growth promotion and disease control in livestock production, leading to their release and accumulation in soils following agricultural applications of animal manure (Perez et al., 2011; Zhu et al., 2013; Dębski, 2016). Bacterial antibiotic and metal resistance systems share some common structural and functional

* Corresponding author.

E-mail address: ytliang@issas.ac.cn (Y. Liang).

<https://doi.org/10.1016/j.geoderma.2020.114760>

Received 27 February 2020; Received in revised form 25 September 2020; Accepted 27 September 2020

Available online 16 October 2020

0016-7061/© 2020 Elsevier B.V. All rights reserved.

characteristics (Baker-Austin et al., 2006). Cross-resistance (the same genes conferring resistance to multiple types of antibiotics and metals) and co-resistance (multiple genes encoding antibiotic and metal resistance located in the same mobile genetic elements [MGEs]) are known mechanisms underlying the metal-driven selection of antibiotic resistance (Silveira et al., 2014; Zhu et al., 2017). The best-known examples of genes conferring cross-resistance are MDR efflux pumps (Blanco et al., 2016). Bacterial multidrug efflux pumps are antibiotic resistance determinants present in all microorganisms (Martinez et al., 2009). Prokaryotes harbor five major families of broad-spectrum efflux pumps, including the adenosine triphosphate-binding cassette superfamily (*abc*), the multidrug and toxic compound extrusion family (*mate*), the major facilitator superfamily (*mfs*), the resistance-nodulation-division family (*rnd*) and the small multidrug resistance family (*smr*) (Blanco et al., 2016). These efflux systems can actively remove and extrude both antibiotics and metals from the bacterial cell into the external environment before they reach their intracellular targets (Amaral et al., 2014). The co-occurrence of ARGs and metal resistance genes (MRGs) can often be found on MGEs such as plasmids and the class I integron (Di Cesare et al., 2016). However, previous studies on the coexistence of ARGs and MRGs are mostly confined to analyses of several specific ARGs and MRGs, such as resistance genes for tetracycline (TC), sulfonamide (SA), macrolide, Zn, Cu and As, in environments such as wastewater treatment plants and biogas reactors (Di Cesare et al., 2016; Li et al., 2017; Luo et al., 2017). The coexistence relationship between MDRGs and various MRGs in soils remains unclear.

In agricultural production, land applications of manure to meet crop nutrient requirements and maintain soil fertility have a long agricultural history and have been promoted due to an increasing preoccupation for sustainable soil productivity, ecological stability, and disposal of unwanted wastes (Peng et al., 2015). To our knowledge, the effects of manure applications on the coexistence of MDRGs and MRGs in agricultural soils have not been widely explored. In order to ensure agroecosystem function and human health, gaining a better understanding of the coexistence patterns of the both kind of resistances after agricultural manure application is necessary. To gain more insight into the coexistence of MDRGs and MRGs under increasingly serious contamination by antibiotics and metals, we collected 18 pig manure samples, 39 manure-fertilized soils and 39 inorganic-fertilized soils from three typical rice planting regions representing classic soil types and climates in eastern China. A high-throughput functional gene array, GeoChip 5.0 (Shi et al., 2019), which contained 14,977 probes covering five multidrug efflux system gene families and four β -lactamase resistance gene families and 42,627 probes covering 105 MRG families, was employed to analyze MDRG and MRG coexistence patterns. In this study, we hypothesize that (i) MDRGs are widely present in agricultural soils and enriched by manure application; (ii) the coexistence of MDRGs and MRGs is frequent in agroecosystems and increases under the direct and indirect effects of manure application; and (iii) the elevated coexistence of MDRGs and MRGs contributes to soil antibiotic resistance in agroecosystems.

2. Materials and methods

2.1. Site description and sampling

Details for all methods are provided in [Supplementary Information \(SI\) Section S1](#). Briefly, a total of 39 manure-fertilized paddy soils and 39 inorganic-fertilized paddy soils were collected from three agricultural regions in eastern China (26.00 °N to 31.60 °N, 115.00 °E to 119.48 °E) in October 2013 ([Table S1](#)). The pig manure used as fertilizer was from nearby livestock farms, and manure-fertilized soil (0–20 cm) samples were taken after the harvest of single rice. Pigs in these farms were treated with similar anthropogenic antibiotic inputs. Soil (0–20 cm) samples with inorganic fertilization were collected from adjacent paddy fields without records of manure fertilization. In our data analysis and results interpretation, we considered manure-fertilized and inorganic-

fertilized soils collected from “adjacent sites” in the same agricultural area as paired samples. A total of 18 pig manure samples were collected from nearby livestock farms that were applied to manure-fertilized paddy fields. Standard methods were used to characterize soil chemical properties ([Table S2](#)), metals and antibiotics ([SI Section S1](#)).

2.2. GeoChip hybridization to detect MDRGs and MRGs

MDRG and MRG diversity was studied using GeoChip 5.0, a high-throughput functional gene array containing 14,977 probes covering 33,758 coding sequences from MDRGs and 42,627 probes covering 94,470 coding sequences from MRGs (Shi et al., 2019). Details on GeoChip hybridization, imaging and data preprocessing are provided in [SI Section S1](#).

2.3. Illumina sequencing analysis of 16S rRNA gene amplicons

Soils were studied by 16S rRNA bacterial amplicon sequencing using the Illumina MiSeq 2 × 250 bp sequencing platform (Illumina, San Diego, CA, USA). A total of 2,724,333 filtered sequences and 21,919 ASVs were obtained. The primer set including 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') was selected, targeting the bacterial V4 region in 16S rRNA genes (Caporaso et al., 2012). Details on sample preparation and sequencing are also provided in [SI Section S1](#).

2.4. Quantification of typical ARGs

Considering the antibiotics used in pig production, five TC resistance genes (*tetW*, *tetM*, *tetO*, *tetQ* and *tetH*), two SA resistance genes (*sul1* and *sul2*), two quinolone (FQ) resistance genes (*gyrA* and *qnrA*), and one macrolide resistance gene (*ermF*) were detected by qPCR to reveal soil antibiotic resistance. The copy numbers of ARGs and the 16S rRNA gene were determined by qPCR carried out in triplicate using a CFX96 Real-time PCR System (Bio-Rad, USA). The specific primer sets for 10 target ARGs and the 16S rRNA gene are listed in [Table S3](#). Details on the qPCR system and protocol and data analysis are provided in [SI Section S1](#).

2.5. Data analysis

Univariate patterns of association were tested using Pearson correlation coefficients. The response ratios of MDRG and MRG abundance between manure-fertilized and inorganic-fertilized soils were calculated (Luo et al., 2006). Partial redundancy analysis was used to delineate the effects of explanatory variables on soil MDRG diversity. Canonical correspondence analysis (CCA) was used to identify the effects of variables on soil MDRG and MRG structure. Random forest classification and regression analysis were used to predict soil antibiotic resistance. Network analysis was performed to investigate the coexistence of MDRGs with bacterial taxa, MDRGs with MRGs, and MDRGs and MRGs with bacterial taxa in soils. Permutational multivariate analysis of variance (PERMANOVA) and Pearson correlation analysis were conducted to determine the effect of the soil type and soil properties on the positive links between soil MDRGs and MRGs in the network. Structural equation models (SEMs) were constructed to evaluate the direct and indirect effects of climate, soil pH, total organic carbon (TOC), antibiotics, metals, soil bacterial diversity, and manure MDRG and MRG diversity on the positive links between soil MDRGs and MRGs in the network. The *priori* model was constructed first based on theoretical relationships ([Fig. S1](#)). Details are provided in [SI Section S1](#).

3. Results

3.1. Comparison of antibiotic and metal concentrations and MDRG and MRG diversity in soils with different fertilizers

TCs showed the highest concentrations among the target antibiotics in the manures (6.7–20.1 mg/kg on average, $p < 0.05$) (Fig. S2a). High concentrations of oxytetracycline, sulfamethyldiazine and enrofloxacin were detected in the manure-fertilized soils. None of the target antibiotics were detected in inorganic-fertilized paddy fields. It should be noted that this result might be limited by the range of analyte types and the detection limit of the method used to quantify antibiotics. The concentrations of Zn and Cu, which were used as feed additives, were highest in manures (475 to 2183 and 349 to 771 mg/kg on average, respectively) and were similar in manure-fertilized and inorganic-fertilized soils (Fig. S2b).

The diversities of MDRGs and MRGs in soils and manures were examined by a GeoChip 5.0 assay. In total, 9919 unique probes related to nine gene families of MDRGs were hybridized, including five multidrug efflux system gene families for various classes of antibiotic resistance (*abc*, *mate*, *mex*, *mfs*, and *smr*) and four β -lactamase (Classes A-D) resistance gene families. An average of 5652 probes were in inorganic-fertilized soils, and 5970 probes were in manure-fertilized soils (Fig. S3, Table S4). The diversity of MDRGs was significantly related to bacterial diversity ($r = -0.241$, $p = 0.037$; Fig. S4a). Multidrug efflux genes related to various classes of antibiotic resistance were dominant, with 9,434 probes (Fig. S3). This result may be partially due to the different probe numbers designed on the GeoChip. Significant positive response ratios ranged from 0.21 to 0.29 ($p < 0.01$) between manure-fertilized and inorganic-fertilized soils ($p < 0.01$), indicating higher abundances of MDRGs in response to manure fertilization (Fig. S5a). For example, the abundances of the β -lactamase resistance genes *blaC* and *blaA* (with the highest response ratios of 0.29 and 0.24, respectively) in manure-fertilized soils were significantly higher than those in inorganic-fertilized soils. A total of 25,312 unique probes related to 101 MRG families were detected, with averages of 11,659 and 12,521 probes in inorganic-fertilized and manure-fertilized soils, respectively (Table S5). Similarly, MRG diversity showed significant correlations with bacterial diversity ($r = -0.241$, $p = 0.037$; Fig. S4b). Manure fertilization substantially increased MRG abundance, with an average response ratio of 0.26 ($p < 0.01$) (Fig. S5b).

Greater than 60% of the variation in soil MDRG diversity was explained by environmental variables, manure fertilization, the soil

bacterial community and MRG diversity in the partial redundancy analysis (Fig. S6). Environmental variables and manure fertilization were the dominant contributors, accounting for 59.6% of the total variance, alone or combined with other factors. Similar effects of these abiotic and biotic factors on soil MDRG and MRG diversity were detected by CCA (Fig. S7). Mean annual temperature (MAT), soil pH, TOC, total potassium (TK), total phosphorus (TP), Zn, Cu, Pb, Ni, Cr, SAs and FQs, and manure-derived MDRG and MRG diversity were significant factors influencing both soil MDRG and MRG structure ($p < 0.01$).

3.2. Coexistence of MDRGs and MRGs and potential bacterial hosts in soils increased under manure fertilization

Analysis of the network between resistance genes and bacterial taxa was suggested to provide potential host information if the resistance genes and the coexisting bacterial taxa possessed significantly similar abundance trends among samples (Li et al., 2015). The similar abundance trends might be explained by the specific bacterial taxa host-specific resistance genes (Forsberg et al., 2014; Feng et al., 2018). Networks among MDRGs, MRGs and bacterial taxa were explored to reveal the co-selection potential of MDRGs and MRGs and to identify possible hosts for coexisting resistance (Fig. 1, S8; Tables S6, S7). Multidrug efflux genes were dominant and generally occurred together with resistance genes for As, Cd, Cr, Cu, Hg, Ni and Te, especially for MRG-Ni (Fig. 1, S8). The numbers of linkages (edge numbers) between bacteria and MDRGs were slightly higher in manure-fertilized soils than in inorganic-fertilized soils at 262 and 223, respectively (Table S6). Specifically, the linkages between bacteria and MRGs were much more common in manure-fertilized soils (130) than in inorganic-fertilized soils (63). Proteobacteria, Actinobacteria and Firmicutes predominantly co-occurred with MDRGs and MRGs. Resistant bacteria, including species of *Actinobacterium WWH12*, *Bacillus*, *Geobacter*, *Solirubrobacter*, Acidobacteriales, Bacillales, Chloroflexi, Methyloligellaceae and Xanthobacteraceae, were identified as potential common hosts for both MDRGs and MRGs (Table S7). Compared with inorganic fertilization, manure fertilization increased not only the number of potential hosts for coexisting resistance genes but also their abundances (Fig. S9). Furthermore, these potential common hosts had more connections with diverse MDRGs and MRGs, such as *mfs*, *mex*, *abc*, *blaD*, *nika*, *arsB*, *czcD*, *copA*, *cutA*, *znuC*, *terD*, *mer* and *psaA_5f0_Mn*, under manure application than in soils with inorganic fertilizer (Table S7). These results indicated that manure application increased potential host bacteria and enhanced their coexistence with MDRGs and MRGs.

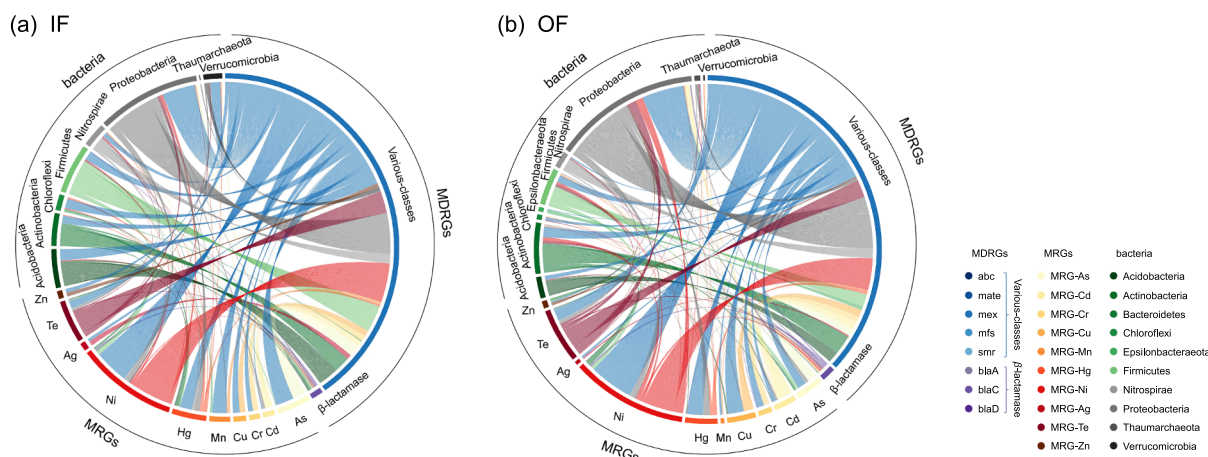


Fig. 1. Coexistence of multidrug resistance genes (MDRGs) and metal resistance genes (MRGs) with bacterial taxa. Circular layouts cluster the associations (i.e., edges) among MDRGs, MRGs and bacteria in networks of (a) inorganic-fertilized (IF) soils and (b) manure-fertilized (OF) soils, respectively. The sector size in the outer circle indicates the total relative proportion of respective associations with others, while the flows through the center of the circle illustrate the relative importance of associations between individuals.

3.3. Drivers of the coexistence of MDRGs and MRGs in soils

PERMANOVA showed that 77.3% of the variance in soil MDRG and MRG coexistence could be explained by fertilization (49.1%) and soil type (28.2%; Table S8). Soil pH, TOC, TP, available phosphorus (AP) and TK were significantly positively correlated with the coexistence of soil MDRGs and MRGs ($p < 0.001$; Table S9). SEMs were constructed to investigate the direct and indirect effects of climate (combined with MAT and mean annual precipitation [MAP]), soil attributes (pH and TOC), selective pressures (antibiotics and metals), soil bacterial diversity, and manure-derived MDRG and MRG diversity on the coexistence of soil MDRGs and MRGs (Fig. 2). For inorganic-fertilized fields, metals were the most important driver that directly increased the coexistence of MDRGs and MRGs ($r = 0.317$, $p < 0.001$; standardized total effect = 0.309). The coexistence was shown to be directly impacted by soil pH ($r = 0.214$, $p < 0.001$) and indirectly impacted by pH effects on metal speciation ($r = -0.237$, $p < 0.001$) and soil bacterial diversity ($r = 0.239$, $p = 0.008$). For manure-fertilized fields, in addition to the significant effects of metals ($r = 0.452$, $p = 0.022$; standardized total effect = 0.452), manure-derived MDRGs ($r = 0.355$, $p = 0.032$) and MRGs ($r = 0.338$, $p = 0.035$) had a significant positive influence on the coexistence of MDRGs and MRGs in soils. Antibiotics also directly enhanced coexistence ($r = 0.397$, $p < 0.001$; standardized total effect = 0.380). Compared with inorganic fertilization, manure fertilization increased the contributions of soil bacterial community diversity ($r = 0.188$, $p = 0.022$). TOC was an important factor (standardized total effect = 0.337) that affected the bioavailable concentrations of antibiotics ($r = 0.402$, $p < 0.001$) and metals ($r = 0.278$, $p < 0.001$).

Furthermore, the influence of metals and antibiotics on the coexistence between specific MDRGs and MRGs was estimated. The positive associations between the predominant MDRGs (multidrug efflux genes, including *abc*, *mate*, *mex*, *mfs*, and *smr*) and MRGs in the co-occurrence networks were selected to characterize their synergistic increase effects. The differences in coexistence between manure- and inorganic-fertilized soils were compared (Fig. 3a). Coexistences between *mfs*, *mex* and all MRGs were increased under manure fertilization. In addition, there were more positive associations between *abc* and As and Ni resistance genes in manure-fertilized soils than in inorganic-fertilized soils. Random forest models showed that the coexistence of a specific MDRG and all MRGs was well explained by selective pressures from metals and antibiotics (p

= 0.01, $R^2 > 0.80$). The concentrations of Pb, Ni, Cr, Cd and FQs were major predictors of the coexistence of *mfs*, *mex*, *abc* and MRGs (increase in mean square error (MSE) % >15, $p < 0.05$) (Fig. 3b).

3.4. Prediction of soil antibiotic resistance by MDRG and MRG coexistence

Soil antibiotic resistance, including resistance to TCs (*tetW*, *tetM*, *tetO*, *tetQ* and *tetH*), SAs (*sul1* and *sul2*), FQs (*gyrA* and *qnrA*), and macrolide (*ermF*), was further estimated by qPCR (Fig. S10). The random forest model indicated that 60% of the total variation in soil antibiotic resistance could be explained by the coexistence of predominant MDRGs and MRGs at the 0.01 level with 1000 trees (Fig. 4). The coexistence of *mfs*, *mex*, *mate*, and *abc* with MRGs was the most important predictor, with increases in the MSE (%) of 18.6, 17.4, 16.6, and 16.4, respectively, followed by the coexistence of *smr* and MRGs. Based on these results, the coexistence of MDRGs and MRGs predicted antibiotic resistance in soils.

4. Discussion

4.1. Manure application increased the abundance of MDRGs in soils

Numerous studies have demonstrated that agricultural applications of manure boost ARGs in soils (Xie et al., 2018; Blau et al., 2019; Zhou et al., 2019). In this study, manure fertilization significantly increased soil MDRG abundance. Among the general mechanisms of antibiotic resistance, multidrug efflux pumps excluding drugs serve as critical one in MDR (Hernando-Amado et al., 2016). We found a large number and wide distribution of broad-spectrum efflux pumps such as *mfs*, *mex*, *abc*, *smr* and *mate* in soils, suggesting that they play a generalized role in protecting the cell from multiple antibiotics encountered in the agricultural environment. Consistent with previous findings, among the five efflux pumps, *mfs* was the most prevalent and clinically relevant efflux pump superfamily in bacteria (Spengler et al., 2017). In addition, the β -lactamase resistance genes *blaC* and *blaA* were also increased under manure applications. β -lactams are one of the top antibiotics used in livestock production to prevent and treat diseases (Zhang et al., 2015). Udikovic-Kolic et al. (2014) found that soils with manure contained a higher abundance of β -lactam-resistance genes (*bla*_{CEP-04}) than those

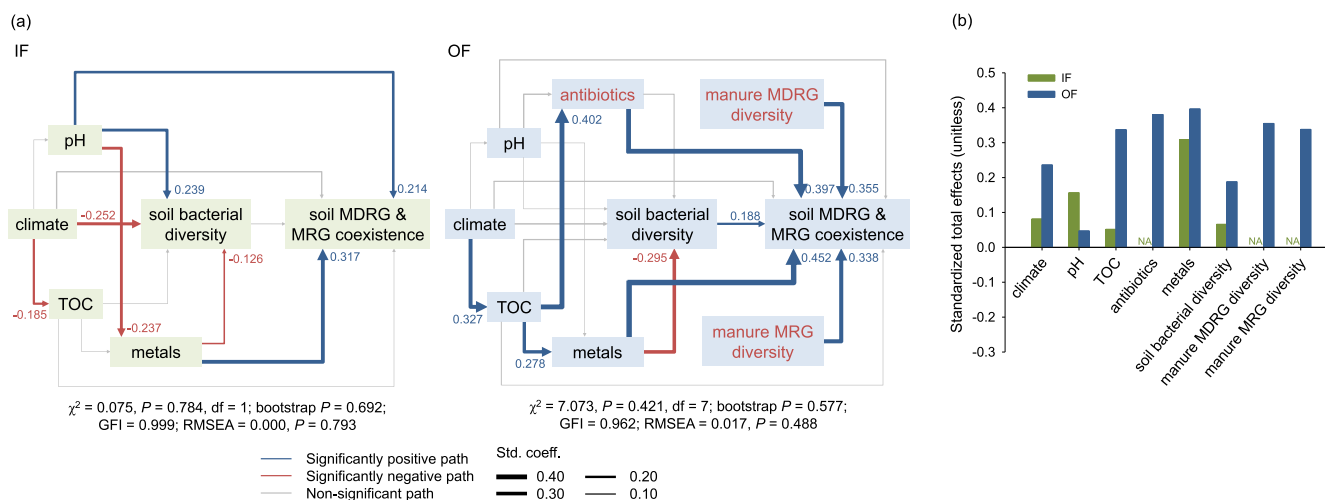


Fig. 2. Structural equation models (SEMs) investigating drivers of coexistence of multidrug resistance genes (MDRGs) and metal resistance genes (MRGs). SEMs based on the effects of variables on soil MDRG and MRG coexistence in inorganic-fertilized (green) and manure-fertilized (blue) soils. Blue, red and gray arrows represent significant positive, significant negative (both $p < 0.05$) and nonsignificant paths ($p > 0.05$), respectively. The path coefficients are indicated as standardized effect sizes of the relationships. (b) The standardized total effects (direct plus indirect effects) derived from the SEM used in (a). TOC: total organic carbon. IF: inorganic-fertilized soils; OF: manure-fertilized soils. Std. coeff.: standardized coefficient. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

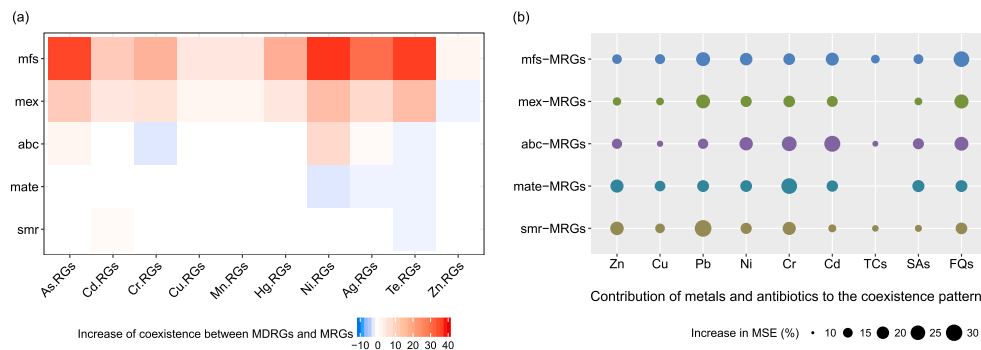


Fig. 3. Influence of metals and antibiotics on the coexistence of specific multidrug resistance genes (MDRGs) and metal resistance genes (MRGs). (a) Heatmap showing variation in coexistence between specific MDRGs and MRGs in manure-fertilized soils compared with inorganic-fertilized soils. Colors represent differences in MDRG and MRG coexistence (positive associations) between manure-fertilized (OF) and inorganic-fertilized (IF) soils. (b) The bubble plot shows a random forest model predicting the importance (% increase in mean square error [MSE]) of selective pressures from metals and antibiotics on the coexistence of specific MDRGs and all MRGs. The circle size represents the significant importance in the prediction ($p < 0.05$). TCs: tetracyclines; SAs: sulfonamides; FQs: quinolones.

Importance of coexistence between MDRGs and MRGs for predicting soil antibiotic resistance

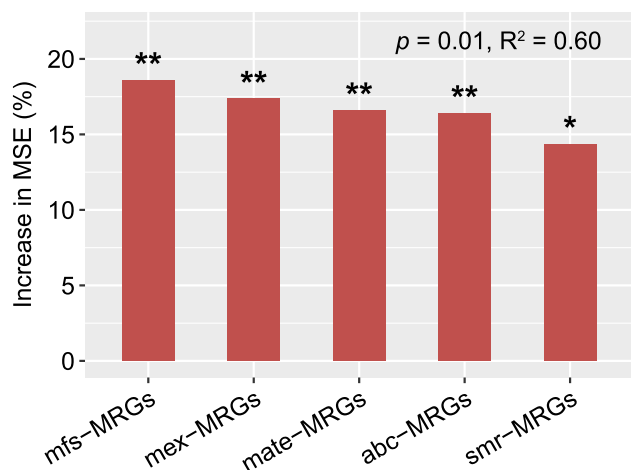


Fig. 4. Prediction of soil antibiotic resistance by the coexistence of multidrug resistance genes (MDRGs) and metal resistance genes (MRGs). Mean predictor importance (% increase in mean square error [MSE]) of coexistence between MDRGs and MRGs for soil antibiotic resistance based on random forest analyses. The significance levels of each predictor are as follows: * $p < 0.05$ and ** $p < 0.01$.

treated with inorganic fertilizer, and this result was attributed to the enrichment of resident soil bacteria that harbor β -lactamases (*Pseudomonas* sp., *Janthinobacterium* sp. and *Psychrobacter pulmonis*) by manure amendment. GeoChip has been used as a quick and high-throughput tool to assess ARG and MRG profiles in various environments (Low et al., 2016; Gao et al., 2020). However, GeoChip is a “closed format” detection technology (Zhou et al., 2015), meaning that it depends on the known sequence information and probe design. Our results may be limited by gene design in GeoChip, and continuous GeoChip updates are needed. In addition, other “open format” technologies, such as metagenomic analysis (Su et al., 2017a, 2017b), are suggested to study more resistance genes.

4.2. Manure application enhanced coexistence of MDRGs and MRGs in soils and co-selection by antibiotics and metals

In addition to the enrichment of MDRGs, our network analysis revealed that manure fertilization also substantially enhanced the coexistence of MDRGs and MRGs. The coexistence of typical ARGs and

MRGs in microbial communities during manure composting has been reported previously (Yin et al., 2017). Genes encoding resistance to metals such as Cu, Ag, As, Sb, Co, Ni, Cd, Fe, Zn and Hg frequently coexist with resistance genes for many classes of antibiotics, e.g., SAs, β -lactams, amphenicols, TCs and aminoglycosides (Pal et al., 2015, 2017). Cross-resistance and co-resistance are known to be the main mechanisms of co-selection for antibiotic and metal resistance (Singer et al., 2016). Our results showed that the coexistence of MRG-Ni and MDRGs was dominant in the networks. This dominance might be attributed to their shared resistance mechanism, the use of efflux pumps, which can confer cross-resistance to metals and antibiotics (Pal et al., 2017). For instance, the *czcCBA-* (*czcC*, *czcB*, *czcA* encoding an outer membrane factor, a fusion protein, and a resistance-nodulation-cell division protein, respectively), *cznCBA-*, *cnrCBA-*, and *nccCBA-* mediated efflux systems play important roles in both Ni resistance and multidrug detoxification (Baker-Austin et al., 2006; Stähler et al., 2006; Hu et al., 2017). The difference in coexistence patterns between β -lactamase resistance genes and specific MRGs may be partly due to their different functional preferences. Genes with closely related functions, such as functional interactions to produce resistance to specific antibiotics and metals, are often co-occurring as the closest genetic neighbors. Genome analysis of numerous complete genomes found that genes resistant to β -lactam, kasugamycin and bacitracin are the ARG types most likely to co-occur with common MRG types (excluding multidrug and unclassified ARGs) (Li et al., 2017).

Antibiotics and metals are widely used in animal feed additives to promote growth and prevent diseases and are then excreted with manures (Kuppusamy et al., 2018; Imran et al., 2019). Under combined contamination by antibiotics and metals following manure application, the coexistence of MDRGs and MRGs was promoted in agroecosystems. Owing to shared structural and functional resistance systems, antibiotics and metals could contribute to coexisting selective pressures and act as complementary drivers of coexistence (Hu et al., 2016; Guo et al., 2018). Our SEM results indicated that metal content was a major driver influencing the coexistence of MDRGs and MRGs in both inorganic-fertilized and manure-fertilized soils, and antibiotics were an important factor in manure-fertilized soils. Zhou et al. (2016) found significant correlations among ARGs, MRGs and metals in feces ($p < 0.01$) and suggested that metals promoted the emergence of metal resistance and participated in co-selection for ARGs. Spectral alterations post exposure to As(V) and TC by surface-enhanced Raman scattering indicated that As(V)-induced bacterial resistance could simultaneously block TC action, confirming the contribution of cross-resistance to As(V)-enhanced antibiotic resistance to TC (Cui et al., 2016). Cu resistance genes (*trcB*) and ARGs (*ermB* and *tetM*) are commonly found together in plasmids, and Cu may co-select resistance to multiple antibiotics, especially for the macrolide resistance gene (Amachawadi et al., 2013).

4.3. Potential host-resistant bacteria contributed to the coexistence of MDRGs and MRGs in soils

The most common co-selection scenario is likely the presence of chromosomal MRGs and plasmid-borne ARGs in the same strain (Pal et al., 2015). Certain bacterial taxa comprising many pathogens are particularly prone to carrying both MRGs and ARGs. Although direct information on genomes (plasmids and chromosomes) carrying MDRGs and MRGs was not available in this study, network analysis of MDRGs, MRGs and bacterial taxa revealed that it was common for microbes (such as species of *Actinobacterium WWH12*, *Bacillus*, *Geobacter*, *Solirubrobacter*, Acidobacteriales, Bacillales, Chloroflexi, Methyloligellaceae and Xanthobacteraceae) to have both MDRGs and MRGs. A study of 4582 plasmids (mainly from Proteobacteria, Firmicutes and Spirochaetes) suggested that plasmids hosted by *Escherichia*, *Staphylococcus*, *Salmonella* and *Klebsiella* tended to carry both ARGs and MRGs (Pal et al., 2015). The coexistence of ARGs and MRGs was found to be positively correlated with the class I integron-integrase gene, which resulted in co-resistance (Liu et al., 2019). Our results showed that the abundance of potential host bacteria in manure-fertilized soils was higher than that in inorganic-fertilized soils. The enrichment of potential hosts may provide more carriers for MDRGs and MRGs. SEM analysis suggested that the increased contributions of bacterial diversity to the coexistence of MDRGs and MRGs in soils supplemented with manure fertilization might therefore result from co-selection. Notably, although network analysis is considered to be a useful tool to provide insights into the coexistence of resistance genes and potential hosts in complex environmental samples (Li et al., 2015; Luo et al., 2017), it could not distinguish the contributions of manure-derived and soil-borne bacteria or decipher the location

of resistance genes in this study. In future studies, increasing the time series sampling before and after fertilization is suggested to explore the contribution of manure-derived and soil-borne bacteria and explain the dissemination of resistant bacteria and resistant genes. Additionally, other approaches, such as functional metagenomics and single-cell genomic technologies (Dos Santos et al., 2017; Feng et al., 2018; Su et al., 2017a, 2017b), are needed to actually test the potential host-resistant bacteria predicted by network analysis, investigate the location of genes and the coexistence patterns of MDRGs and MRGs at the genome/plasmid level, and elucidate their mechanisms of co-resistance and cross-resistance and contributions to coexistence.

4.4. Effects of MDRG and MRG coexistence on soil antibiotic resistance in agroecosystems

Horizontal gene transfer of resistance genes located in MGEs is often assumed to be a major mechanism for resistance disseminated to other strains (Amachawadi et al., 2013; Soucy et al., 2015). Recipient microorganisms acquiring ARGs and/or MRGs can then quickly adapt to habitats with selective pressures from antibiotics and/or metals (Pal et al., 2015). Our results suggested that the coexistence of MDRGs and MRGs was the most important predictor of soil antibiotic resistance under manure application (Fig. 5, increase in MSE (%) = 18, $p < 0.01$). In manure-fertilized soils, the enhanced coexistence of MDRGs and MRGs may increase soil antibiotic resistance in agroecosystems by promoting the horizontal transfer of resistance genes. This result was supported by previous studies demonstrating that plasmids with both ARGs and MRGs are conjugated significantly more often than those not carrying both types of resistance genes, which indicates a high level of

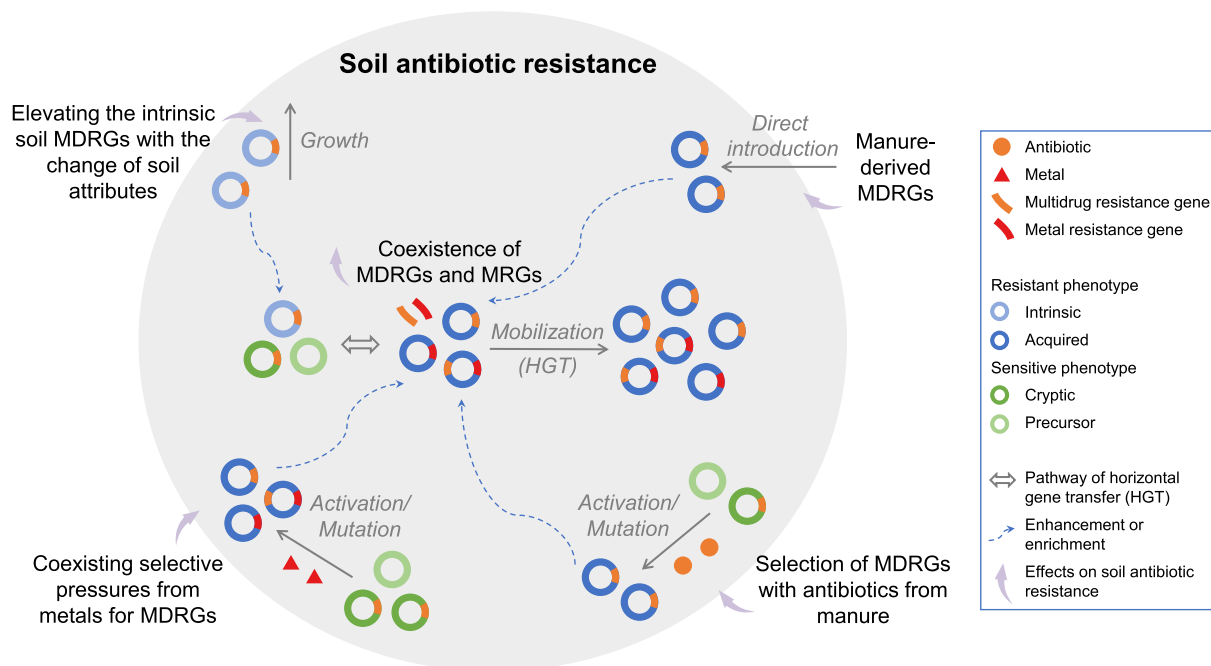


Fig. 5. A schematic representation of possible mechanisms via which manure fertilization influences soil antibiotic resistance. Soil antibiotic resistance is defined as all genes encoding antibiotic resistance-related proteins or similar proteins likely to evolve towards potent antibiotic resistance, encompassing both intrinsic and acquired resistance genes as well as cryptic resistance genes (which are not necessarily expressed) and precursor genes (which encode proteins with modest antibiotic resistance activity or affinity to antibiotics that might evolve into effective resistance genes) (Wright, 2007). Manure fertilization could increase soil antibiotic resistance in the following ways: (a) by directly introducing manure-derived multidrug resistance genes (MDRGs), (b) by imposing selection for MDRGs by antibiotics from manure, (c) by exerting coexisting selective pressures from metals for MDRGs, (d) by elevating intrinsic soil MDRGs, and (e) by enhancing the coexistence of MDRGs and MRGs with manure fertilization, which might contribute to soil antibiotic resistance via horizontal gene transfer (conjugation, transduction or transformation). The solid gray arrows indicate the changes in soil antibiotic resistance under manure fertilization. The dotted blue arrows indicate the enhancement of MDRG and MRG coexistence or the enrichment of intrinsic MDRGs. The random forest model revealed that 54% of the variation in the soil antibiotic resistance level was explained by the direct introduction of manure-derived MDRGs, selective pressures from antibiotics and metals, elevation of intrinsic soil MDRGs with a change in soil attributes, and coexistence of MDRGs and MRGs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

gene transfer between strains and resistance to a wide range of antibiotics (Pal et al., 2015, 2017). Genetically coupled resistance in MGEs with high mobility potential could theoretically be directly connected to large proportions of the soil bacterial gene pool, thus increasing the probability of spreading resistance to specific species or even across phylum boundaries (Klümper et al., 2015).

Naturally, ARGs are selected by antibiotics and provide a competitive advantage against antibiotic producers, allowing host bacteria to survive at high concentrations of antibiotics (Nesme and Simonet, 2015). Resistance genes are usually associated with some fitness costs to provide resistance for bacteria. Mobilization of resistance genes provides a new mechanism for gene replication and subsequent functionalization in bacteria, which may increase the expression levels of resistance genes or contribute to the fine-tuning of their expression. Both methods are beneficial under the selection of antibiotics (Martinez, 2014; Bengtsson-Palme et al., 2017). In other words, the mobilization of resistance genes may provide a fitness cost benefit mechanism for bacteria to resist various selection pressures. Therefore, when MDRGs and MRGs coexist, their high migration potential may provide a mechanism for increasing soil antibiotic resistance at a lower adaptation cost.

Based on the above empirical studies and our findings together, we proposed a schematic representation to illustrate the possible ways in which manure fertilization influences soil antibiotic resistance in agroecosystems (Fig. 5). MDRGs from manure could be introduced directly into soils and survive with hosts. Bacteria resident in soils containing intrinsic MDRGs could be enriched by manure. Intrinsic resistance genes and cryptic resistance genes (which are not necessarily expressed), as well as precursor genes (which encode proteins with modest antibiotic resistance activity or affinity to antibiotics), could acquire resistance by activation or adaptive mutations with the selection of antibiotics and metals (Wright, 2007). These mechanisms also enhanced the coexistence of MDRGs and MRGs or enriched resident bacteria containing intrinsic MDRGs, which further contributed to soil antibiotic resistance via horizontal gene transfer. The random forest model showed that 54% of the variation in soil antibiotic resistance was explained by the direct introduction of manure-derived MDRGs and selective pressures from antibiotics and metals, elevating the intrinsic soil MDRGs and coexistence of MDRGs and MRGs ($p = 0.01$). It should be noted that this result might be overstated by our sample data on a single time point and a limited number of farms. This concept model needs to be verified by more extensive research.

In conclusion, we focused on the increased abundances and coexistence of MDRGs and MRGs due to manure application in agricultural soils and the danger they pose on increasing soil antibiotic resistance in agroecosystems. The results indicated that agricultural application of manure increased the abundance and coexistence of MDRGs and MRGs in agroecosystems. This might be attributed to the directly introduced resistance genes, increased co-selective pressures from antibiotics and metals, and stimulation of hosts that acquire and transfer resistance genes under manure fertilization. Such coexistence could further increase soil antibiotic resistance, posing a threat to agroecosystem function and human health. Our findings highlight the necessity of considering both antibiotic and metal concentrations and coexisting MDRGs and MRGs when designing integrated strategies to control antibiotic resistance under intensive agricultural applications of manure. Future studies should focus on identifying different MGEs and their relationship with the coexistence of MDRGs and MRGs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was supported by the National Natural Scientific Foundation of China (Nos. 41877060, 41430856, and 41622104), Top-Notch Young Talents Program of China (W03070089), Youth Innovation Promotion Association of Chinese Academy of Sciences (2016284), and Natural Sciences and Engineering Research Council of Canada (RGPIN-2017-06210).

Authors' contributions

All authors contributed intellectual input and assistance to this study and manuscript preparation. Y.L. developed the original framework. Y. L., N.Z., R.H. contributed sampling, reagents and data analysis. N.Z. and Y.L. wrote the manuscript with help from P.J., Z.H., B.S., and J.Z.

All authors declare no competing financial interests.

Availability of data

The GeoChip data are available in the repository Figshare, <https://doi.org/10.6084/m9.figshare.9691319>. Raw sequence data for 16S rRNA gene amplicons were deposited under NCBI BioProject Accession No. PRJNA589177 (<https://www.ncbi.nlm.nih.gov/sra/PRJNA589177>). Bacterial ASV data are available in the repository Figshare <https://doi.org/10.6084/m9.figshare.12673709>.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.geoderma.2020.114760>.

References

- Amachawadi, R.G., Scott, H.M., Alvarado, C.A., Mainini, T.R., Vinasco, J., Drouillard, J. S., Nagaraja, T.G., 2013. Occurrence of the transferable copper resistance gene *ctrB* among Fecal Enterococci of U.S. Feedlot Cattle Fed Copper-Supplemented Diets. *Appl. Environ. Microbiol.* 79 (14), 4369–4375.
- Amaral, L., Martins, A., Spengler, G., Molnar, J., 2014. Efflux pumps of gram-negative bacteria: what they do, how they do it, with what and how to deal with them. *Front Pharmacol* 4.
- Arias, C.A., Murray, B.E., 2009. Antibiotic-resistant bugs in the 21st Century – A clinical super-challenge. *N Engl. J. Med.* 360 (5), 439–443.
- Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of antibiotic and metal resistance. *Trends Microbiol.* 14 (4), 176–182.
- Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2017. Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiol. Rev.* 42, 68–80.
- Blanco, P., Hernando-Amado, S., Reales-Calderon, J., Corona, F., Lira, F., Alcalde-Rico, M., Bernardini, A., Sanchez, M., Martinez, J., 2016. Bacterial multidrug efflux pumps: much more than antibiotic resistance determinants. *Microorganisms* 4, 14.
- Blau, K., Jacquiod, S., Sørensen, S.J., Su, J., Zhu, Y., Smalla, K., Jechalke, S., 2019. Manure and doxycycline affect the bacterial community and its resistome in lettuce rhizosphere and bulk soil. *Front Microbiol* 10.
- Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., Gormley, N., Gilbert, J.A., Smith, G., Knight, R., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6 (8), 1621–1624.
- Cui, L.I., Zhang, Y.-J., Huang, W.E., Zhang, B.-F., Martin, F.L., Li, J.-Y., Zhang, K.-S., Zhu, Y.-G., 2016. Surface-enhanced raman spectroscopy for identification of heavy metal arsenic(V)-mediated enhancing effect on antibiotic resistance. *Anal. Chem.* 88 (6), 3164–3170.
- Dębski, B., 2016. Supplementation of pigs diet with zinc and copper as alternative to conventional antimicrobials. *Pol J Vet Sci* 19, 917–924.
- Di Cesare, A., Eckert, E.M., D'Urso, S., Bertoni, R., Gillan, D.C., Wattiez, R., Como, G., 2016. Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res.* 94, 208–214.
- Dos Santos, D.F.K., Istvan, P., Quirino, B.F., Kruger, R.H., 2017. Functional metagenomics as a tool for identification of new antibiotic resistance genes from natural environments. *Microb. Ecol.* 73 (2), 479–491.
- Feng, J., Li, B., Jiang, X., Yang, Y., Wells, G.F., Zhang, T., Li, X., 2018. Antibiotic resistome in a large-scale healthy human gut microbiota deciphered by metagenomic and network analyses: Antibiotic resistome. *Environ. Microbiol.* 20 (1), 355–368.
- Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., Dantas, G., 2014. Bacterial phylogeny structures soil resistomes across habitats. *Nature* 509 (7502), 612–616.

- Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., Dantas, G., 2012. The shared antibiotic resistome of soil bacteria and human pathogens. *Science* 337 (6098), 1107–1111.
- Gao, Q., Dong, Q., Wu, L., Yang, Y., Hale, L., Qin, Z., Xie, C., Zhang, Q., Van Nostrand, J. D., Zhou, J., 2020. Environmental antibiotics drives the genetic functions of resistome dynamics. *Environ. Int.* 135, 105398. <https://doi.org/10.1016/j.envint.2019.105398>.
- Gullberg, E., Albrecht, L.M., Karlsson, C., Sandegren, L., Andersson, D.I., Baquero, F., 2014. Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *mBio* 5 (5). <https://doi.org/10.1128/mBio.01918-14>.
- Guo, T., Lou, C., Zhai, W., Tang, X., Hashmi, M.Z., Murtaza, R., Li, Y., Liu, X., Xu, J., 2018. Increased occurrence of heavy metals, antibiotics and resistance genes in surface soil after long-term application of manure. *Sci. Total Environ.* 635, 995–1003.
- Hernando-Amado, S., Blanco, P., Alcalde-Rico, M., Corona, F., Reales-Calderón, J.A., Sánchez, M.B., Martínez, J.L., 2016. Multidrug efflux pumps as main players in intrinsic and acquired resistance to antimicrobials. *Drug Resist. Updates* 28, 13–27.
- Hu, H.-W., Wang, J.-T., Li, J., Li, J.-J., Ma, Y.-B., Chen, D., He, J.-Z., 2016. Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils: Copper induced changes in antibiotic resistance. *Environ. Microbiol.* 18 (11), 3896–3909.
- Hu, H.-W., Wang, J.-T., Li, J., Shi, X.-Z., Ma, Y.-B., Chen, D., He, J.-Z., 2017. Long-term nickel contamination increases the occurrence of antibiotic resistance genes in agricultural soils. *Environ. Sci. Technol.* 51 (2), 790–800.
- Imran, M.d., Das, K.R., Naik, M.M., 2019. Co-selection of multi-antibiotic resistance in bacterial pathogens in metal and microplastic contaminated environments: An emerging health threat. *Chemosphere* 215, 846–857.
- Kemper, N., 2008. Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol. Ind.* 8 (1), 1–13.
- Klümper, U., Riber, L., Dechesne, A., Sannazzaro, A., Hansen, L.H., Sørensen, S.J., Smets, B.F., 2015. Broad host range plasmids can invade an unexpectedly diverse fraction of a soil bacterial community. *ISME J* 9 (4), 934–945.
- Kuppasamy, S., Kakarla, D., Venkateswarlu, K., Megharaj, M., Yoon, Y.-E., Lee, Y.B., 2018. Veterinary antibiotics (VAs) contamination as a global agro-ecological issue: A critical view. *Agric. Ecosyst. Environ.* 257, 47–59.
- Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J.M., Zhang, T., 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J* 9 (11), 2490–2502.
- Li, L.-G., Xia, Y.u., Zhang, T., 2017. Co-occurrence of antibiotic and metal resistance genes revealed in complete genome collection. *ISME J* 11 (3), 651–662.
- Liu, K., Sun, M., Ye, M., Chao, H., Zhao, Y., Xia, B., Jiao, W., Feng, Y., Zheng, X., Liu, M., Jiao, J., Hu, F., 2019. Coexistence and association between heavy metals, tetracycline and corresponding resistance genes in vermicomposts originating from different substrates. *Environ. Pollut.* 244, 28–37.
- Low, A., Ng, C., He, J., 2016. Identification of antibiotic resistant bacteria community and a GeoChip based study of resistome in urban watersheds. *Water Res.* 106, 330–338.
- Luo, G., Li, B., Li, L.-G., Zhang, T., Angelidaki, I., 2017. Antibiotic resistance genes and correlations with microbial community and metal resistance genes in full-scale biogas reactors as revealed by metagenomic analysis. *Environ. Sci. Technol.* 51 (7), 4069–4080.
- Luo, Y.Q., Hui, D.F., Zhang, D.Q., 2006. Elevated CO2 stimulates net accumulations of carbon and nitrogen in land ecosystems: a meta-analysis. *Ecology* 87, 53–63.
- Magiorakos, A.-P., Srinivasan, A., Carey, R.B., Carmeli, Y., Falagas, M.E., Giske, C.G., Harbarth, S., Hindler, J.F., Kahlmeter, G., Olsson-Liljequist, B., Paterson, D.L., Rice, L.B., Stelling, J., Struelens, M.J., Vatopoulos, A., Weber, J.T., Monnet, D.L., 2012. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18 (3), 268–281.
- Martínez, J.L., 2014. General principles of antibiotic resistance in bacteria. *Drug Discov. Today: Technol.* 11, 33–39.
- Martínez, J.L., Sánchez, M.B., Martínez-Solano, L., Hernandez, A., Garmendia, L., Fajardo, A., Alvarez-Ortega, C., 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. *FEMS Microbiol. Rev.* 33 (2), 430–449.
- Nesme, J., Simonet, P., 2015. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria: The soil resistome. *Environ. Microbiol.* 17 (4), 913–930.
- Pal, C., Asiani, K., Arya, S., Rensing, C., Stekel, D.J., Larsson, D.G.J., Hobman, J.L., 2017. Metal resistance and its association with antibiotic resistance. In: Poole, R.K. (Ed.), *Advances in Microbial Physiology*, pp. 261–313.
- Pal, C., Bengtsson-Palme, J., Kristiansson, E., Larsson, D.G.J., 2015. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genom.* 16 (1) <https://doi.org/10.1186/s12864-015-2153-5>.
- Peng, S., Wang, Y., Zhou, B., Lin, X., 2015. Long-term application of fresh and composted manure increase tetracycline resistance in the arable soil of eastern China. *Sci. Total Environ.* 506–507, 279–286.
- Perez, V.G., Waguespack, A.M., Bidner, T.D., Southern, L.L., Fakler, T.M., Ward, T.L., Steidinger, M., Pettigrew, J.E., 2011. Additivity of effects from dietary copper and zinc on growth performance and fecal microbiota of pigs after weaning. *J. Anim. Sci.* 89, 414–425.
- Qiao, M., Ying, G.-G., Singer, A.C., Zhu, Y.-G., 2018. Review of antibiotic resistance in China and its environment. *Environ. Int.* 110, 160–172.
- Shi, Z., Yin, H., Van Nostrand, J.D., Voordeckers, J.W., Tu, Q., Deng, Y., Yuan, M., Zhou, A., Zhang, P., Xiao, N., Ning, D., He, Z., Wu, L., Zhou, J., 2019. Functional gene array-based ultrasensitive and quantitative detection of microbial populations in complex communities. *mSystems* 4.
- Silveira, E., Freitas, A.R., Antunes, P., Barros, M., Campos, J., Coque, T.M., Peixe, L., Novais, C., 2014. Co-transfer of resistance to high concentrations of copper and first-line antibiotics among enterococcus from different origins (humans, animals, the environment and foods) and clonal lineages. *J Antimicrob Chemoth* 69, 899–906.
- Singer, A.C., Shaw, H., Rhodes, V., Hart, A., 2016. Review of antimicrobial resistance in the environment and its relevance to environmental regulators. *Front. Microbiol.* 7, 1728.
- Soucy, S.M., Huang, J., Gogarten, J.P., 2015. Horizontal gene transfer: Building the web of life. *Nat. Rev. Genet.* 16 (8), 472–482.
- Spengler, G., Kincses, A., Gajdacs, M., Amaral, L., 2017. New roads leading to old destinations: efflux pumps as targets to reverse multidrug resistance in bacteria. *Molecules* 22, 468.
- Stähler, F.N., Odenbreit, S., Haas, R., Wilrich, J., Vliet, A.H.M.V., Kusters, J.G., Kist, M., Bereswill, S., 2006. The novel helicobacter pylori CznABC metal efflux pump is required for cadmium, zinc, and nickel resistance, urease modulation, and gastric colonization. *IAI* 74 (7), 3845–3852.
- Su, J., An, X., Li, B., Chen, Q., Gillings, M.R., Chen, H., Zhang, T., Zhu, Y., 2017a. Metagenomics of urban sewage identifies an extensively shared antibiotic resistome in China. *Microbiome* 5 (1). <https://doi.org/10.1186/s40168-017-0298-y>.
- Su, J., Cui, L., Chen, Q., An, X., Zhu, Y., 2017b. Application of genomic technologies to measure and monitor antibiotic resistance in animals. *Ann. Ny. Acad. Sci.* 1388, 121–135.
- Udikovic-Kolic, N., Wichmann, F., Broderick, N.A., Handelsman, J.o., 2014. Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization. *Proc. Natl. Acad. Sci. USA* 111 (42), 15202–15207.
- WHO, 2014. Antimicrobial resistance: global report on surveillance. World Health Organization, Geneva, Switzerland.
- Wright, G.D., 2007. The antibiotic resistome: The nexus of chemical and genetic diversity. *Nat. Rev. Microbiol.* 5 (3), 175–186.
- Xie, W.-Y., Yuan, S.-T., Xu, M.-G., Yang, X.-P., Shen, Q.-R., Zhang, W.-W., Su, J.-Q., Zhao, F.-J., 2018. Long-term effects of manure and chemical fertilizers on soil antibiotic resistome. *Soil Biol. Biochem.* 122, 111–119.
- Yin, Y., Gu, J., Wang, X., Song, W., Zhang, K., Sun, W., Zhang, X., Zhang, Y., Li, H., 2017. Effects of copper addition on copper resistance, antibiotic resistance genes, and intl1 during swine manure composting. *Front. Microbiol.* 8, 344.
- Zhang, Q.-Q., Ying, G.-G., Pan, C.-G., Liu, Y.-S., Zhao, J.-L., 2015. Comprehensive evaluation of antibiotics emission and fate in the river basins of China: Source analysis, multimedia modeling, and linkage to bacterial resistance. *Environ. Sci. Technol.* 49 (11), 6772–6782.
- Zhou, B., Wang, C., Zhao, Q., Wang, Y., Huo, M., Wang, J., Wang, S., 2016. Prevalence and dissemination of antibiotic resistance genes and coselection of heavy metals in Chinese dairy farms. *J. Hazard. Mater.* 320, 10–17.
- Zhou, J., He, Z., Yang, Y., Deng, Y.e., Tringe, S.G., Alvarez-Cohen, L., 2015. High-throughput metagenomic technologies for complex microbial community analysis: open and closed formats. *mBio* 6 (1). <https://doi.org/10.1128/mBio.02288-14>.
- Zhou, X., Zhou, X., Qiao, M., Qiao, M., Su, J., Su, J., Zhu, Y., Zhu, Y., 2019. High-throughput characterization of antibiotic resistome in soil amended with commercial organic fertilizers. Springer, Berlin Heidelberg, Berlin/Heidelberg, pp. 641–651.
- Zhu, Y.-G., Johnson, T.A., Su, J.-Q., Qiao, M., Guo, G.-X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M., 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. USA* 110 (9), 3435–3440.
- Zhu, Y.-G., Zhao, Y.i., Li, B., Huang, C.-L., Zhang, S.-Y., Yu, S., Chen, Y.-S., Zhang, T., Gillings, M.R., Su, J.-Q., 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. *Nat. Microbiol.* 2 (4) <https://doi.org/10.1038/nmicrobiol.2016.270>.