Long-Term Nickel Contamination Increases the Occurrence of Antibiotic Resistance Genes in Agricultural Soils

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Supporting Information

ABSTRACT: Heavy metal contamination is assumed to be a selection pressure on antibiotic resistance, but to our knowledge, evidence of the heavy metal-induced changes of antibiotic resistance is lacking on a long-term basis. Using quantitative PCR array and Illumina sequencing, we investigated the changes of a wide spectrum of soil antibiotic resistance genes (ARGs) following 4–5 year nickel exposure (0–800 mg kg^{−1}) in two long-term experimental sites. A total of 149 unique ARGs were detected, with multidrug and β -lactam resistance as the most prevailing ARG types. The frequencies and abundance of ARGs tended to increase along the gradient of increasing nickel concentrations, with the highest values recorded in the treatments amended with 400 mg nickel kg^{−1} soil. The abundance of mobile genetic elements (MGEs) was significantly associated with ARGs, suggesting that nickel exposure might enhance the potential for horizontal transfer of ARGs. Network analysis demonstrated significant associations between ARGs and MGEs, with the integrase *intI1* gene having the most frequent interactions with other co-occurring ARGs. The changes of ARGs were mainly driven by nickel bioavailability and MGEs as revealed by structural equation models. Taken together, long-term nickel exposure significantly increased the diversity, abundance, and horizontal transfer potential of soil ARGs.

INTRODUCTION

The increasing emergence and propagation of antibiotic resistance genes (ARGs) in clinical and environmental settings is becoming a major threat to human health in the 21st century.¹ In the face of almost no new antibiotics in development, antibiotic resistance would threaten the effects of modern medicines, and cause rising difficulties in the treatment of infections.^{2,3} Compelling evidence suggests that the environment is a vast reservoir of antibiotic resistance and that there is evidence for recent exchange of ARGs between environmental bacteria and human pathogens, emphasizing the potential clinical importance of environmental antibiotic resistance.⁴ ARGs are often located on mobile genetic elements (MGEs), and are assumed to be disseminated to other microorganisms and pathogens via the pathway of horizontal

gene transfer (HGT), and to be migrated into the food chain after land application of animal manure.⁵ Globally, the proliferation of antibiotic resistance in natural and clinical environments has been mainly attributed to the intensive use of antibiotics in agriculture and medicine.^{3,6} However, apart from the selection pressure imposed by antibiotic use, other components and mechanisms might also contribute to the selection and dissemination of antibiotic resistance.^{6,7} Despite the rising burdens of antibiotic resistance, the potential contribution of environmental ARGs to the clinical reservoirs

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of resistance remains largely unknown,⁸ and it is essential to identify the sources and movement of ARGs for design of appropriate strategies to minimize their spread.

Heavy metals, which are common and persist in environmental settings, may be one such factor that indirectly selects for antibiotic resistance.⁹ As alternatives to antibiotics, heavy metals are widely used as feed supplements in the livestock production for growth promotion and disease control,¹⁰ and are subject to release following land application of fertilizers, pesticides, sewage sludge, and animal manure.^{11,12} Most antibiotics are readily sequestered and degraded via photolytic and other pathways, and therefore the selection pressure imposed by antibiotic residues rapidly declines over time in the environment.^{13,14} In contrast, heavy metal can accumulate to several orders of magnitude higher than antibiotics and is predicted to persist in agricultural soils with long half-lives.^{15,16} Growing evidence suggested that metal-induced change of antibiotic resistance is an important phenomenon and has a strong selection pressure on bacterial assemblages in various environmental settings.^{12,15–18} Therefore, it is hypothesized that the ubiquitous and rapidly increasing heavy metal contamination in agricultural soils may impose a strong long-term selection pressure for environmental antibiotic resistance.^{7,11}

The possibility of heavy metal-induced coselection for antibiotic resistance has been highlighted by several mechanisms, particularly if multiple genes encoding for antibiotic and metal resistance are located on the same MGEs (coresistance) or the same genes confer resistance to multiple types of antibiotics and metals (cross-resistance).^{9,11} Other potential coselection mechanisms used by prokaryotes include indirect but shared regulatory responses to the exposure of antibiotics and metals such as biofilm induction.⁹ To date, only a few studies have investigated the metal-induced selection of antibiotic resistance in environmental settings, including soils amended with copper (Cu),^{15,19} freshwater microcosms applied with nickel (Ni) and cadmium,¹⁷ activated sludge bioreactor,²⁰ and municipal solid waste leachates.^{18,21} The majority of these studies, however, relied only on culture-dependent approaches and correlative evidence, and focused on a limited number of antibiotics and ARGs.^{15,22,23} Aside from these cases, experimental studies which directly manipulate metal exposure levels to test for metal-driven changes of antibiotic resistance are rare, particularly in the complex soil environment where a direct assessment of metal-induced effect is often hindered by the presence of multiple contaminants.^{6,24} Therefore, it is imperative to understand the role of heavy metal as a selection agent in maintaining environmental antibiotic resistance.

This study was designed to investigate the impacts of long-term Ni exposure on the diversity, abundance, and horizontal transfer potential of ARGs in two experimental sites with contrasting soil properties. We selected Ni as the model metal contaminant because it is abundant and widely detected in agro-ecosystems,^{25–28} and is also an essential micronutrient for various cellular functions and components.⁹ The major sources of Ni in agricultural soils are atmospheric deposition, fertilizers and agrochemical, livestock manures, sewage irrigation and sewage sludge.²⁹ A quantitative PCR (qPCR) array containing 296 primer sets was used to survey a wide spectrum of ARGs and MGEs in parallel.²⁴ We hypothesized that (i) Long-term Ni exposure can modify the profiles of multiple antibiotic resistance, with increasing Ni concentrations selecting for enhanced resistance levels; (ii) Changes in ARG patterns are

closely associated with MGEs, owing to the genetic linkage of ARGs in MGEs; and (iii) changes in the bacterial community structure, as an important determinant of ARGs,³⁰ might be also associated with changes in ARGs in Ni-contaminated soils.

MATERIALS AND METHODS

Site Description and Soil Sampling. Two long-term experimental stations of the Chinese Academy of Agricultural Sciences, located in Dezhou (37.33°N, 116.63°E), Shandong, and Qiyang (26.75°N, 111.88°E), Hunan, China, were selected to provide contrasting soil properties. The soil in Dezhou is classified as a fluvo-aquic soil with a pH of 7.9, whereas the soil in Qiyang is classified as a red soil with a pH of 4.3. The detailed information about the climatic conditions in the two experimental sites was provided in Hu et al. (2016).¹⁹ The field trials were established in July 2007 with six application rates of Ni (as NiCl₂) (0, 50, 100, 200, 400, and 800 mg Ni kg⁻¹ soil) in a randomized block design with four replicate plots (2 × 3 m for each plot) in both sites. The top 20 cm soils (approximately 1200 kg for each plot) taken from the field were thoroughly mixed with NiCl₂ powder and returned to the plots. The selected Ni concentrations represented the levels (<1.0–800 mg Ni kg⁻¹ dry soil) found in a wide range of environmental compartments impacted by agriculture and industry.^{25–28} However, it should be noted that the spikes of 400 and 800 mg Ni kg⁻¹ soil in this study are not particularly common and only environmental relevant in some heavily contaminated sites. Wheat in rotation with maize was grown at both sites under conventional agricultural management practices. Inorganic fertilizers (urea, calcium superphosphate, and potassium sulfate) containing no heavy metals were applied to the plots according to local farming practices. The two sites had not been treated with organic manures or biosolids in the previous years. In July 2011 and July 2012, five soil cores (0–10 cm) taken from each plot were composited into a single sample, and each treatment included four plots. Soil samples were transported to the laboratory on ice, passed through a 2.0 mm mesh, and archived at 4 °C before physicochemical analyses and at –80 °C prior to molecular analysis.

Soil Physicochemical Analysis and DNA Extraction. Soil physicochemical properties including soil pH, gravimetric water content, soil organic carbon (SOC), total nitrogen (TN), soil microbial biomass carbon (SMBC), extractable Ni concentrations were measured using conventional methods as described previously.¹⁹ The detailed information about the soil physicochemical properties is shown in [Supporting Information \(SI\) Table S1](#). The MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) was used to extract genomic DNA from 0.25 g of soil as per the manufacturer's instructions, and the quality of DNA was assessed by NanoDrop ND-2000c UV–vis spectrophotometer (NanoDrop Technologies, Wilmington, DE).

qPCR Analysis of ARGs and MGEs. A qPCR array was performed to determine the diversity and abundance of ARGs using the same primer sets as described previously.^{24,31} The qPCR array contained a total of 296 primer sets, including 285 primer sets which can target all the major classes of antibiotic resistance (including aminoglycosides, β -lactam, FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol), MLSB (macrolide-lincosamide-streptogramin B), sulfonamides, tetracyclines, vancomycin, multidrug, and others), 10 primer sets targeting MGEs (including eight transposase genes and two class 1 integrase genes), and one

targeting 16S rRNA gene.¹⁹ The 5 μ L reaction containing 2.5 μ L SensiMix SYBR No-ROX reagent (Bioline, London, UK), 1 μ L 10-fold diluted DNA, and 0.4 μ L each primer (10 μ M) was conducted on a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, Hercules, CA).¹⁹ Three technical replicates were included for each soil sample and run in three independent 384-well plates, with each including 296 primer sets, calibrator (as 16S rRNA gene for the same DNA sample for all the plates), and negative control. Amplification conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. The results were screened with the following criteria: (i) a threshold cycle value (C_T) of 31 was used as the detection limit;¹⁹ (ii) samples with more than two replicates above the detection limit were regarded as positive quantification; and (iii) amplicons with multiple melting curves were removed from the analysis. The $2^{-\Delta C_T}$ method where $\Delta C_T = (C_{T \text{ detected ARGs}} - C_{T \text{ 16S rRNA gene}})$ was used to calculate the relative abundances of ARGs and MGEs compared to the 16S rRNA gene in each soil sample according to a comparative C_T method.³²

High-Throughput Illumina Sequencing. The hyper-variable V4–V5 regions of the bacterial 16S rRNA gene were amplified using the primers 515F/806R which has few taxonomic biases.³³ The primers were modified by adding adaptor A to the forward primer, and adaptor B with 12 nucleotide barcodes to the reverse primer. The details for the PCR reaction mixture, amplification conditions and PCR product purification, and the paired-end sequencing on the Miseq platform have been described in our previous study.¹⁹ The obtained raw sequences were merged using fast length adjustment of short reads (FLASH),³⁴ quality filtered and assigned to individual samples using Quantitative Insights Into Microbial Ecology (QIIME) following the standard operation procedure.³⁵ A chimera filtering approach, UPPARSE, was used to cluster the quality-filtered reads into operational taxonomic units (OTUs) at a 97% identity level on the Bio-Linux platform.³⁶ A set of representative sequences retrieved from each OTU were aligned by using PyNAST³⁷ against the Greengenes reference set.³⁸ The Ribosome Database Project Classifier with a confidence threshold of 0.80 was used to conduct the taxonomic classification.³⁹ The same number of sequences (47,011) was randomly selected for each sample to compensate for variability in sequencing depth before the downstream analysis. The Illumina sequencing reads of the bacterial 16S rRNA gene have been deposited in the European Nucleotide Archive under the accession number ERP017104.

Co-Occurrence Analysis and Network Generation. Network analysis has been widely used to explore the underlying interaction/association among microbial taxa in complex microbial communities.⁴⁰ A correlation matrix was constructed by calculating the pairwise correlation coefficients among the different taxonomic levels of bacteria (including the phylum, family and genus levels) and ARG subtypes which occurred in $\geq 50\%$ of all the samples in both sites to minimize the artificial association bias in Cytoscape (version 2.8.3)⁴¹ plug-in CoNet (version 1.1)⁴² as previously described.¹⁹ Briefly, the pairwise interactions/associations were calculated using two correlation methods (Pearson and Spearman) and two dissimilarity methods (Kullback–Leibler and Bray–Curtis). We reduced the potential compositionality biases and false-positive correlations by running the ReBoot procedure with 100 permutations, and refined the resultant distribution with 1000 bootstraps. We combined the P values from the four

methods using the Brown method, and only included significant correlations with a P value < 0.05 in the downstream analysis. We performed the Benjamini–Hochberg (BH) multiple test as a correction after the merging of P values. The BH method adjusts the false discovery rate (FDR), which controls the expected number of false positives among all significant relationships. The chance of falsely rejecting the null hypothesis is 0.05. We imported the resultant pairwise correlations into Cytoscape plug-in Network Analyzer⁴³ for visualization, and explored network topology using the Circular (for the ARGs–MGEs networks) and Frucherman Reingold (for the ARGs–bacteria networks) algorithms in the Gephi platform.⁴⁴ The final networks only displayed statistically robust correlations with a correlation coefficient (ρ -value) > 0.8 and a significance level (P -value) < 0.05 .¹⁹

Statistical Analysis. One-way analysis of variance was performed to test the difference in the diversity and relative abundances of ARGs and MGEs and the relative abundance of bacterial phyla across different treatments in SPSS 20 (IBM, Armonk, NY). The abundance of ARGs, MGEs, and bacterial taxa was log-transformed before statistical analysis to meet the normality assumptions. The relationships between the relative abundances of ARGs and MGEs were analyzed using Spearman's rank correlation test. The log-transformed relative abundances of ARG subtypes were displayed in heat maps using the *ggplot2* package⁴⁵ in the R platform.⁴⁶ The impacts of long-term Ni exposure on the ARG compositions were visualized by the nonmetric multidimensional scaling (NMDS) ordinations based on the Bray–Curtis dissimilarity distances, and statistically evaluated by permutational multivariate analysis of variance (PerMANOVA) using the *vegan* package⁴⁷ with 999 permutations in R. Statistically significant differences were accepted at $P < 0.05$.

Structural equation models (SEMs) were constructed to evaluate the direct or indirect effects of Ni contamination, soil properties (Table S1), the bacterial abundance and community composition, and MGEs on the ARG patterns. SEM is an *a priori* approach offering the capacity to visualize the casual relationships between variables by fitting data to the models representing causal hypotheses.⁴⁸ The Mantel test was performed to examine the pairwise correlations among these variables using the *Ecodist* package in R,⁴⁹ and the matrix of resultant R values was imported into AMOS (SPSS Inc., Chicago, IL) for SEMs construction using the maximum-likelihood estimation method. The data used for the Mantel test included the extractable Ni concentrations, soil properties (including soil pH, gravimetric water content, SOC, TN, and SMBC), the bacterial 16S rRNA gene abundance, the bacterial community composition at the 97% OTU level, and the relative abundance of MGEs and ARGs. The theoretical model assumptions were as follows (SI Figure S1): (i) Ni exposure might have direct influences on MGEs and the ARG patterns; (ii) Ni exposure could indirectly influence the patterns of MGEs and ARGs by changing the bacterial abundance and community compositions; and (iii) Basic soil properties might have been changed by the long-term Ni exposure, with potential consequences on the bacterial communities, MGEs and ARGs. We used high goodness-of-fit index (> 0.90), low Akaike information criteria (AIC), nonsignificant chi-square test ($P > 0.05$), and low root-mean-square errors of approximation (RMSEA < 0.05) to indicate the overall goodness of fit for SEMs. Furthermore, we calculated the standardized total effects of distance from Ni contamination,

soil properties, bacterial abundance, bacterial community composition, and MGEs on ARGs. The net effect of one factor upon another is calculated by summing all direct and indirect pathways between the two factors.

RESULTS AND DISCUSSION

Long-Term Ni Exposure Selected for Increasing Diversity and Abundance of ARGs. The qPCR array containing 285 primer sets targeting ARGs and 10 sets targeting MGEs was used to evaluate the changes in the diversity and abundance of ARGs after 4–5 year Ni exposure in the two agricultural soils. A total of 126 and 140 unique ARGs were detected in the fluvo-aquic and red soils, respectively (Figure 1). In the fluvo-aquic soil, the numbers of ARGs

Figure 1. Changes in the numbers of detected ARGs and MGEs along the gradient of Ni exposure in the fluvo-aquic soil (a) and the red soil (b). Error bars indicate standard errors ($n = 4$). Different letters above the bars indicate a significant difference in ARGs.

significantly increased from 61 to 72 (in the control treatment) to 114–121 (in the Ni400 treatment) along the gradient of Ni concentrations ($P < 0.05$), but the highest values were recorded in the Ni400 treatments in both years (Figure 1a). Likewise in the red soil, the numbers of ARGs detected in the control treatments (45–64) showed an increasing tendency toward high Ni concentrations, with the highest value (116–126) observed in Ni400 as well (Figure 1b). The numbers of ARGs detected in Ni400 were not significantly different from those in Ni800 in both soils ($P > 0.05$). The detected ARG types were mainly dominated by the multidrug and β -lactam resistance, which comprised 26.2% and 21.4% of the total ARGs in the fluvo-aquic soil, and 23.6% and 20.7% in the red soil, respectively (Figure 1). Other abundant ARGs could potentially encode resistance to MLSB (15.1% and 16.4%), aminoglycoside (12.7% and 12.9%), tetracycline (8.7% and 10.0%), and vancomycin (7.9% and 7.8%) in the fluvo-aquic

and red soils, respectively. The frequencies of these individual types of ARGs tended to increase along the gradient of increasing Ni concentrations, and generally exhibited similar patterns as that observed for total ARGs (Figure 1). We further calculated the relative abundances of ARGs (SI Figure S2) by normalizing to the bacterial 16S rRNA gene, which mimicked the patterns observed for the diversity of ARGs.

Our findings are in line with previous observations that exposure of bacterial communities to heavy metals coselected for increased bacterial resistance to multiple antibiotics.^{7,17,19,50} The two contrasting agricultural soils exhibited similar ARG patterns in response to 4–5 year Ni exposure. The treatments were applied into the field in 2007 with the Ni concentrations ranging from 0 to 800 mg kg⁻¹ soil, but the extractable Ni concentrations decreased to 2.2 ± 0.3 – 76.3 ± 1.1 mg kg⁻¹ (58.9 ± 1.6 in Ni400) in the fluvo-aquic soil and 0.8 ± 0.03 – 93.7 ± 1.6 mg kg⁻¹ (48.7 ± 3.4 in Ni400) in the red soil (SI Table S1) after 4–5 years of land application. The decrease of Ni extractability and availability can be mainly attributed to the aging processes, such as adsorption and diffusion Ni into soil components, and the precipitates of Ni on the surface of clay minerals and Al oxides.⁵¹ It is hypothesized that increasing levels of Ni exposure would exert increasing selection pressure on antibiotic resistance, and thus the abundance of Ni-induced ARGs would increase along the gradient of Ni concentrations. At the same time, however, ARGs are carried by soil bacteria, and the bacterial abundance was observed to continuously decrease along the increasing Ni concentration gradient (SI Figure S3) due to the toxicity effect of Ni on soil bacteria.^{6,11} Therefore, two forces (the increasing selection pressure and the decreasing bacterial abundance with increasing Ni levels) are driving the changes of ARGs toward opposite directions along the Ni gradient, and the maximum level of ARGs would appear if the two forces reached a balance. In this study, the extractable Ni concentrations around 48.7–58.9 mg kg⁻¹ in Ni400 seemed to be the balance point, where the maximum selection effect on antibiotic resistance was observed, but the Ni800 treatments (with extractable Ni of 76.3–93.7 mg kg⁻¹) are assumed to have excessive Ni bioavailability resulting in a significant decrease in soil bacterial abundance (SI Figure S3). Therefore, soil bacteria could develop increasing antibiotic resistance before reaching the optimum Ni concentrations for selection of antibiotic resistance in Ni400, but a decreased level of ARGs was observed in Ni800 of both soils. Our findings caution that if Ni accumulates to critical concentrations (as the extractable Ni concentrations found in Ni400) in the environment, it can trigger strong indirect selection for antibiotic resistance. However, it is noteworthy that the microbial toxicity of Ni is highly dependent on the environmental conditions (e.g., soil pH, organic matter contents, the redox potential, vegetation and climatic factors),^{11,52,53} which can affect the valence of the metal ions and therefore the bioavailability and selection pressure of Ni in soils. Therefore, it is desirable to investigate how environmental ARGs respond to the Ni concentrations by including diverse soil types and various climatic conditions in future studies.

Long-Term Ni Exposure Selected for Increasing Levels of MGEs. The HGT of ARGs among environmental microbial assemblages is an important pathway for the spread of antibiotic resistance in agricultural soils.^{4,54} Most of ARG cassettes are found in co-occurrence with heavy metal resistance genes in MGEs such as integrons, transposons and plasmids, which are frequently detected in agricultural

systems⁵⁵ and increase in their abundance and diversity under the pressure of environmental pollution.^{56–58} In this study, the qPCR array detected six MGEs (including two integrase genes and four transposase genes) in both soils (Figure 1). The numbers and relative abundances of detected MGEs exhibited an increasing tendency along the gradient of Ni concentrations (Figure 1 and SI Figure S2), mimicking the patterns of total ARGs in both soils. Spearman's correlation analysis revealed that the relative abundance of total MGEs had significantly positive relationships with those of total ARGs and several major types of ARGs (e.g., aminoglycoside, β -lactam, multidrug, tetracycline, and vancomycin resistance genes in the fluvo-aquic soil, and aminoglycoside, β -lactam, MLSB, multidrug, tetracycline, and vancomycin resistance genes in the red soil) (Table 1), suggesting that MGEs might play an important role in the

Table 1. Correlations between the Relative Abundance of ARGs and MGEs in the Fluvo-Aquic Soil and the Red Soil As Revealed by the Spearman's Rank Correlation Test^a

	fluvo-aquic soil	red soil
	MGEs	MGEs
total ARGs	0.815 (<0.001)	0.855 (<0.001)
FCA	0.092 (0.542)	0.128 (0.526)
aminoglycoside	0.687 (<0.001)	0.450 (<0.001)
beta lactamase	0.723 (0.025)	0.711 (<0.001)
MLSB	0.173 (0.428)	0.489 (0.031)
sulfonamide	0.153 (0.569)	0.253 (0.158)
tetracycline	0.615 (0.038)	0.529 (<0.001)
vancomycin	0.726 (<0.001)	0.571 (<0.001)
multidrug	0.776 (<0.001)	0.658 (<0.001)
other	0.152 (0.324)	0.187 (0.214)

^aValues represent the correlation coefficient *R*-values derived from the Spearman's rank correlation test, and values in the brackets represent the *P*-values. Significant correlations (*P* < 0.05) are in bold numbers.

spread of ARGs in Ni polluted soils, in line with previous findings.^{19,56,57} However, the frequencies of HGT for soil ARGs are influenced by many abiotic factors (e.g., temperature, soil pH, nutrients, and soil types) and biotic factors,⁵⁹ which together with MGEs regulate the actual rates of HGT in the complex soil environment.

Different ARG Patterns in Response to Long-Term Ni Exposure. We further explored the changes in the relative abundances of individual ARG subtypes along the gradient of Ni exposure using the qPCR array results in heat maps (Figure 2). The resistance profiles of samples (columns in heat maps) were clearly clustered into three categories corresponding to the different Ni concentrations: low (dominated by samples from the control and Ni50 treatments), medium (dominated by samples from Ni100 and Ni200), and high (dominated by samples from Ni400 and Ni800) Ni exposure levels. This finding was further supported by the gradual shifts of ARGs along the gradient of increasing Ni concentrations along the first axis of NMDS ordinations based on the Bray–Curtis dissimilarity matrices (Figure 3), which revealed that Ni exposure had significant effects on the ARG patterns (corroborated by the PerMANOVA test, *P* < 0.05 for both soils).

The ARG profiles (rows in heat maps) could be categorized into three patterns (Figure 2): Cluster A had very low abundance or was almost absent at low Ni levels, but markedly increased in their abundances in medium and high Ni levels.

Cluster B represented a minor group of ARGs in Ni-contaminated soils, persisted in a majority of samples with low abundance, and was almost absent in control and low Ni concentrations. However, this cluster showed apparent enrichment in medium and high Ni levels in the fluvo-aquic soil, which might be attributed to the selection of less abundant soil indigenous ARGs or evolution of new ARGs under the selection pressure posed by Ni amendment.⁹ Cluster C, as the most dominant pattern, was prevalent with high abundance across all the samples, suggesting that these ARGs might be carried by soil indigenous microbes which are tolerant of Ni pressure and might have developed antibiotic resistance before the Ni amendment.^{37,60} All these three clusters tended to increase in their relative abundances with increasing Ni concentrations in both soils (PerMANOVA, *P* < 0.05 for all the three clusters). The similar patterns of ARGs were also observed in a recent study of the impacts of copper contamination on ARGs in the same experimental stations,¹⁹ suggesting that similar strategies might be used by soil microorganisms to cope with heavy metal pressure. The clear enrichment of ARGs might be attributed to the increasing selection pressure imposed by the gradient of increasing Ni concentrations. Alternatively, although Ni exposure might not necessarily cause increased antibiotic resistance, it might influence the ARG patterns possibly by selecting for Ni-tolerant bacterial species carrying ARGs. Therefore, Ni represents an important metal element which could exert significant influences on the patterns of soil antibiotic resistance and select for increasing resistance levels.

Co-Occurrence Patterns Among ARGs and MGEs. The nonrandom co-occurrence patterns among ARG subtypes and MGEs were explored using the network analysis, and only strong ($\rho > 0.8$) and significant (*P* < 0.05) correlations were displayed. The ARGs-MGEs network included 69 nodes (each node represented a subtype of ARGs or MGEs) and 282 edges, and the entire network was clearly separated into four modules (Figure 4). Compared with random associations, ARGs within the same module may interact more frequently among themselves than with ARGs in other modules, and are supposed to be carried by some specific microbial taxa or MGEs.⁶¹ Module I was the largest module comprising 29 nodes, followed by Modules II, III and IV including 21, 13, and 6 nodes, respectively. Each module contained a variety of ARG types, and no single module harbored only a single type of ARGs. In each module the most frequently connected node was defined as the “hub”. The hubs and the co-occurring ARGs in the same module might be harbored in the same multidrug-resistant bacterial taxa or MGEs,^{19,61,62} and thus these hubs were proposed as proxies to predict the occurrence and abundance of the corresponding co-occurring ARGs.⁶¹ Of particular interest, the “*intI1*”, an important integrase gene, was the hub for Module I comprising 28 co-occurring ARGs, suggesting that the co-occurring ARGs might be harbored in class 1 integrons subject to a synchronous transmission during HGT.⁵⁶

Our findings strongly supported previous studies that suggested using the *intI1* gene (an integrase gene of class 1 integrons) as a potential proxy for anthropogenic pollution due to its widespread distribution, co-occurrence with other ARGs, and sensitive responses to metal exposure in environmental samples.^{56–58} Therefore, the remarkable diversity of ARGs and MGEs and their significant co-occurrence patterns in the Ni-contaminated agricultural soils may together offer a high

Co-Occurrence Patterns Among ARGs, MGEs, and Bacterial Community. ARG profiles have been reported to vary as a function of the taxonomic composition of bacterial communities, suggesting that the host range of ARGs is a major determinant of ARGs.^{31,63,64} In this study, the soil bacterial community compositions were determined by targeting the bacterial 16S rRNA gene on the Miseq Illumina sequencing platform, which yielded a total of 15 421 630 high-quality bacterial sequences, with an average of 160 642 sequences per sample. The bacterial community in both soils was dominated

To identify the bacterial taxa hypothetically contributing to the prevalence of ARGs, the network analysis was performed to explore the co-occurrence patterns between the ARG types and bacterial taxa (at the levels of phylum, family and genus) (SI Figures S5, S6, and S7). The nonrandom co-occurrence event between ARGs and bacterial taxa might represent an appropriate scenario to track the potential ARG hosts in environmental settings with complex microbial communities.^{61,64} In the network between ARGs and bacterial phyla (SI Figure S5), Cyanobacteria, Actinobacteria, Gemmatimonadetes, Crenarchaeota, and MVP-21 were among the most

Figure 3. NMDS ordinations derived from the Bray–Curtis dissimilarity matrices showing the changes in the relative abundance of ARGs and MGEs along the gradient of Ni exposure in the fluvo-aquic soil (a) and red soil (b). The 2D stress values for both plots are lower than 0.10 indicated that these data are well-represented by the two-dimensional ordinations.

prevalent predicted taxa (as possible ARG hosts), with each phyla containing ARGs encoding resistance to multiple types of antibiotics. For instance, Actinobacteria and Crenarchaeota were had more connections with diverse multidrug resistance genes, and simultaneously they might also harbor genes potentially encoding resistance to β -lactam. Cyanobacteria were predicted to encompass vancomycin, tetracycline, multidrug, aminoglycoside, and β -lactam resistance genes. Our results are consistent with previous findings that Actinobacteria

and Cyanobacteria are well-known groups of bacteria producing antibiotics, and are frequently detected with multiple drug resistance.^{31,64,65} Meanwhile, multidrug, β -lactam, MLSB, and aminoglycoside resistance genes showed the strongest relationships with the bacterial communities, and they were predicted to be shared among different bacterial phyla. This finding might explain the observations of widespread β -lactam resistance in soils,^{60,64} because β -lactamases are carried by multiple bacterial phyla which can extend the prevalence of β -lactam resistance to various environmental settings. Therefore, the same ARG types can be distributed in different ARG hosts, and the same bacterial phyla are predicted to carry various types of ARGs, which was further supported by the networks between ARGs and bacterial taxa at the family and genus levels (Figures S6 and S7). These findings indicated that the phylogenetic structure of the bacterial community might be not an important determinant of ARGs in Ni-contaminated soils as those observed in other studies.^{19,31,60,64} This phenomenon might be attributed to the elevated HGT of ARGs mediated by MGEs in Ni-contaminated soils, which can dis-couple ARGs content and bacterial phylogeny and allows for swift acquisition of resistance.⁶⁶

Factors Influencing the Patterns of ARGs in Ni-Contaminated soils. The abundant and diverse ARGs and their increasing tendency along the gradient of Ni concentrations prompted us to identify the main factors shaping soil ARG patterns. SEM is an a priori approach allowing for an intuitive graphical representation of complex networks of relationships found in ecosystems.⁴⁸ We constructed SEMs to test the casual relationships between Ni exposure, basic soil properties, the bacterial community abundance and compositions, MGEs, and the ARG patterns in both soils (Figure 5). The results showed that 75% and 58% of the total variance of the ARG patterns in the fluvo-aquic and red soils, respectively, could be explained by our SEMs. As for the fluvo-aquic soil (Figure 5a), Ni exposure had significantly positive influences on soil properties ($\lambda = 0.61$, $P < 0.001$), MGEs ($\lambda = 0.47$, $P < 0.01$), and ARGs ($\lambda = 0.64$, $P < 0.001$). Strongly positive effects of MGEs ($\lambda = 0.40$, $P < 0.001$) on ARGs was observed as well. As for the red soil (Figure 5b), long-term Ni exposure resulted in significant changes in soil properties ($\lambda = 0.53$, $P < 0.001$). Soil properties were observed to have significant effects on the

Figure 4. Network showing the co-occurrence patterns among the detected ARG subtypes and MGEs across all the treatments in both soils. The nodes coded with different colors represent different ARGs and MGEs, and the edges correspond to strong and significant correlations between nodes. The size of each node is proportional to the number of significant correlations between nodes. The thickness of the edges is proportional to the correlation coefficient.

Figure 5. Structural equation models showing the direct and indirect effects of Ni exposure, soil properties, bacterial abundance, bacterial community composition, and MGEs on the ARG patterns in the fluvo-aquic (a,c) and red soils (b,d). Red and black arrows indicate positive and negative relationships, respectively. Continuous and dashed arrows indicate significant and nonsignificant relationships, respectively. Numbers adjacent to arrows are path coefficients, and width of the arrows is proportional to the strength of path coefficients. R^2 values denote the proportion of variance explained for each variable. Significance levels are indicated: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. (c,d) Standardized total effects (direct plus indirect effects) derived from the structural equation models. The hypothetical models fit our data well, as suggested by $\chi^2 = 0.6$, $P = 0.91$, GFI = 0.99, AIC = 36.56, and RMSEA = 0.00 for the fluvo-aquic soil (a) and $\chi^2 = 0.3$, $P = 0.85$, GFI = 0.99, AIC = 38.33, and RMSEA = 0.00 for the red soil (b).

bacterial abundance ($\lambda = 0.37$, $P < 0.01$) and ARGs ($\lambda = 0.59$, $P < 0.001$). MGEs were also an important factor ($\lambda = 0.29$, $P < 0.01$) in affecting the ARG patterns in the red soil. As indicated by the standardized total effects from SEMs, the patterns of ARGs in both soils were directly influenced by Ni exposure and MGEs, followed by soil properties as an indirect driver, while the contributions of the bacterial community composition to the ARG patterns were trivial and even negligible (Figure 5c and d). Taken together, our findings by incorporating all the data into SEMs challenged the previous perceptions of low HGT potential for ARGs in natural settings,⁶⁴ and suggested that the HGT mediated by MGEs, rather than changes in bacterial community composition, were the most dominant factor shaping the ARG profiles in long-term Ni-contaminated sites. Beyond the critical role of MGEs in the adaptation of bacterial communities against heavy metal perturbations, the heavy metal-induced coselection effects might have also contributed to the changes in ARGs which requires in-depth investigations of the expression levels of ARGs and Ni resistance genes in future studies.

The results of this study supported the findings obtained from another recent study,¹⁹ in which a wide spectrum of ARGs and MGEs were found to be enriched along a gradient of increasing Cu concentrations in the same experimental sites. These findings reinforce our understanding of the significant selection pressure on antibiotic resistance imposed by individual types of heavy metals (e.g., Ni and Cu), and future studies are required to elucidate how the different types of heavy metals interact to shape the patterns of environmental ARGs. Despite the significant effects of Ni and Cu on ARGs in

the two studies, Ni seemed to have stronger selection pressure on antibiotic resistance compared with Cu, as revealed by the more marked increases of ARGs along the Ni gradient (Figure 1). The experimental designs of these two studies did not allow a clear decipher of the mechanisms for the different effects of Ni and Cu. It is supposed that, however, most bacteria in soils might have evolved more tolerance to Cu than to Ni, because Cu is generally more abundant than Ni in various environments,¹¹ and the spikes of 400 and 800 mg Ni kg⁻¹ soil in this study are not particularly common. We also found that the bacterial community composition was the most important determinant of ARGs in the Cu-contaminated soils,¹⁹ but had very minor roles in the Ni-induced changes in ARGs (Figure 5), which might be attributed to the higher HGT potential for ARGs in Ni-contaminated soils than in Cu-contaminated soils. However, previous studies suggested that both Cu and Ni resistance operons can be either chromosomally encoded or plasmid encoded,^{67,68} which cannot explain why there were less bacterial composition-ARGs relationships in the Ni-contaminated soils. Therefore, it is highly desirable to unravel the microbial mechanisms for Ni-induced antibiotic resistance and the link between antibiotic resistance operons and Ni resistance operons in future studies.

In conclusion, we provide strong field-based evidence that Ni exposure significantly enhanced the diversity, abundance and HGT potential of a wide spectrum of ARGs in two long-term Ni-contaminated fields. Our results suggest that Ni contamination could allow for indirect selection of bacteria conferring multiresistance, and thereby contribute to the emergence and dissemination of antibiotic resistance. These findings are

important for the future evaluation of public health risks associated with the heavy metal-induced selection of antibiotic resistance, and highlight the necessity to take heavy metals into consideration when designing appropriate strategies to reduce the continuous spread of ARGs.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b03383.

Table S1 Basic properties of the fluvo-aquic and red soil samples collected from Dezhou (DZ) and Qiyang (QY), respectively; **Figure S1** A priori structural equation model showing the direct and indirect effects of Ni contamination, soil properties, bacterial abundance, bacterial community composition, MGEs on ARG patterns; **Figure S2** Changes in the relative abundances of ARGs and MGEs normalized to the bacterial 16S rRNA gene along the gradient of Ni exposure in the fluvo-aquic soil (a) and red soil (b); **Figure S3** Changes in the bacterial 16S rRNA gene abundance across different Ni-contaminated treatments and the fluvo-aquic soil (a) in the red soil (b); **Figure S5** The network showing the co-occurrence patterns between ARG types and bacterial taxa at the phylum level across all the treatments; **Figure S6** The network showing the co-occurrence patterns between ARG types and bacterial taxa at the family level across all the treatments. **Figure S7** The network showing the co-occurrence patterns between ARG types and bacterial taxa at the genus level across all the treatments (PDF)

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Notes

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