University Library



A gateway to Melbourne's research publications

Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
Hu, HW;Wang, JT;Li, J;Li, JJ;Ma, YB;Chen, D;He, JZ

Title:
Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils

Date:
2016-11-01

Citation:
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. Environmental Microbiology, 18 (11), pp.3896-3909. https://doi.org/10.1111/1462-2920.13370.

Persistent Link:
https://hdl.handle.net/11343/124172

Title page

Field-based evidence for copper contamination induced changes of antibiotic resistance in agricultural soils

Hang-Wei Hu[1,2], Jun-Tao Wang[1], Jing Li[1], Jun-Jian Li[2], Yi-Bing Ma[3], Deli Chen[2], Ji-Zheng He[1,2]*

[1]State Key Laboratory of Urban and Regional Ecology, Research Centre for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

[2]Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia

[3]National Soil Fertility and Fertilizer Effects Long-term Monitoring Network, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing 100081, China

*Author for correspondence: Ji-Zheng He, E-mail: jizheng.he@unimelb.edu.au, Tel: (+61)-3-90358890

Running title: Copper induced changes in antibiotic resistance

Originality-Significance Statement

The emerging spread of antibiotic resistance genes (ARGs) and their subsequent acquisition by clinically relevant microorganisms is a serious threat to human health. The widespread heavy metal contamination is assumed to contribute to the proliferation of ARGs due to their shared mechanisms of resistance, however, our knowledge of the heavy metal-induced changes in antibiotic resistance is lacking in a long-term basis. This study provided comprehensive field-based evidence that 4-5 year copper contamination significantly changed the diversity, abundance, and mobility potential of a broad spectrum of ARGs in two contrasting agricultural soils. Our findings are important for prediction of ARG behaviours in the contaminated soil environment and development of appropriate strategies to minimize their dissemination.

Summary

Bacterial resistance to antibiotics and heavy metals are frequently linked, suggesting that exposure to heavy metals might select for bacterial assemblages conferring resistance to antibiotics. However, there is a lack of clear evidence for the heavy metal-induced changes of antibiotic resistance in a long-term basis. Here, we used high-capacity quantitative PCR array to investigate the responses of a broad spectrum of antibiotic resistance genes (ARGs) to 4-5 year copper contamination (0-800 mg kg^{-1}) in two contrasting agricultural soils. In total, 157 and 149 unique ARGs were detected in the red and fluvo-aquic soil, respectively, with multidrug and β-lactam as the most dominant ARG types. The highest diversity and abundance of ARGs were observed in medium copper concentrations (100-200 mg kg^{-1}) of the red soil and in high copper concentrations (400-800 mg kg^{-1}) of the fluvo-aquic soil. The abundances of total ARGs and several ARG types had significantly positive correlations with

mobile genetic elements (MGEs), suggesting mobility potential of ARGs in copper-contaminated soils. Network analysis revealed significant co-occurrence patterns between ARGs and microbial taxa, indicating strong associations between ARGs and bacterial communities. Structural equation models showed that the significant impacts of copper contamination on ARG patterns were mainly driven by changes in bacterial community compositions and MGEs. Our results provide field-based evidence that long-term Cu contamination significantly changed the diversity, abundance, and mobility potential of environmental antibiotic resistance, and caution the un-perceived risk of the ARG dissemination in heavy metal polluted environments.

Keywords

Antibiotic resistance, horizontal gene transfer, heavy metals, agriculture, co-selection

Introduction

Antibiotics are widely used in the world to effectively treat infections in humans and animals and contribute to our agriculture and food production industries (Marshall and Levy, 2011). The emerging prevalence and spread of antibiotic resistance genes (ARGs, collectively known as 'resistome') among bacteria and pathogens has, however, put antibiotic use at risk, and represents a serious threat to public health in the 21st century (Berendonk *et al.*, 2015). The recently released global report from the World Health Organization (WHO) highlighted that the magnitude of antibiotic resistance has reached alarming levels in many parts of the world (WHO, 2014), and a growing recognition of an environmental dimension to antibiotic resistance development has prompted calls for investigation into the importance of antibiotic resistance in environmental settings (Ashbolt *et al.*, 2013). Along with the rising burdens of ARGs, it is assumed that environmental ARGs can multiply in their hosts, be horizontally transferred to other bacterial assemblages, and be in high risk of transmission into the food chain (Marti *et al.*, 2013; Berendonk *et al.*, 2015). Environmental ARGs are also subject to dispersal via leaching to waters, runoff into rivers and sediments, transport of air dust, and delivery of ARGs-carrying products (Pruden *et al.*, 2012; Han *et al.*, 2016). Therefore, ARGs have been recognized as an emerging environmental contaminant posing a major worldwide human health risk (Pruden *et al.*, 2006).

Agricultural soils are considered as an important environmental reservoir for ARGs and pathogens (Nesme *et al.*, 2014), due to land application of animal manures containing residual antibiotics and ARGs (Hu *et al.*, 2016). However, it was found that the concentrations of most antibiotics in manure rapidly declined within 30 days after application to soils (Kopmann *et al.*, 2013), and manure-derived ARGs cannot thrive in the soil environment and gradually decreased over time after manure treatment (Heuer *et al.*, 2008; Hu *et al.*, 2016). Therefore, the selection pressure imposed by most antibiotic residues is transient in environmental

81 settings (Kolpin *et al.*, 2002), suggesting that components and mechanisms, other than
82 antibiotics, might exist to govern the evolution and dissemination of ARGs (Alonso *et al.*,
83 2001; Berg *et al.*, 2010). In fact, owing to the structural and functional characteristics
84 commonly shared by many prokaryotic antibiotic- and metal-resistance systems (Baker-
85 Austin *et al.*, 2006), there is growing concern that heavy metals such as copper (Cu), zinc,
86 mercury, and arsenic may indirectly enhance the spread of ARGs (Alonso *et al.*, 2001; Ji *et al.*,
87 2012). Heavy metals are commonly added to animal feeds for growth promotion and disease
88 control and can accumulate in soils after land application of metal-containing fertilizers,
89 manure, and sewage sludge (Bolan *et al.*, 2004; Wright *et al.*, 2006; Seiler and Berendonk,
90 2012). In contrast to antibiotic residues which are prone to degradation and sequestration
91 (Chee-Sanford *et al.*, 2009), heavy metals (notably Cu) can be persistent in agricultural soils
92 with estimated half-life of hundreds to thousands of years (Berg *et al.*, 2005), and thus may
93 provide a long-standing selection pressure for antibiotic resistance.

94 The types and levels of metal contamination have been often associated with specific patterns
95 of antibiotic resistance suggesting that several mechanisms underlie the metal-driven co-
96 selection process (Baker-Austin *et al.*, 2006). It has been known that genes encoding
97 resistance to antibiotics and metals are commonly found on the same plasmid, integron, or
98 transposon (Summers *et al.*, 1993), and such structural linkage is defined as co-resistance
99 (Chapman, 2003). In other cases, single genes can encode generic detoxifying mechanisms to
100 reduce intracellular contents of both metals and antibiotics, which is defined as cross-
101 resistance (Chapman, 2003). Heavy metals are also known to up-regulate ARGs and decrease
102 microbial susceptibility to antibiotics, consequently resulting in enhanced antibiotic resistance
103 (Baker-Austin *et al.*, 2006). To date, elevated frequencies of bacterial resistance to a limited
104 number of well-studied antibiotics have been observed in metal-contaminated freshwater
105 ecosystems (McArthur and Tuckfield, 2000; Graham *et al.*, 2011), freshwater microcosms

(Stepanauskas *et al.*, 2006), activated sludge bioreactor (Peltier *et al.*, 2010), and waters in industrial environments (Stepanauskas *et al.*, 2005). However, relatively less effort was devoted to explicitly explore the metal-induced changes of antibiotic resistance in the soil environment (Berg *et al.*, 2005; Knapp *et al.*, 2011). Among the multiple types of heavy metals examined, Cu was found to have stronger impacts on ARGs than other heavy metals such as nickel, lead, zinc and iron (Knapp *et al.*, 2011; Zhu *et al.*, 2013). Meanwhile, the majority of previous studies failed to directly assess the role of metals in complex settings, where multiple factors (e.g. metals, antibiotics and other antimicrobial agents) may function additively or synergistically to select for resistant microbes, which hinders our ability to reliably assess ARG patterns in contaminated soil environments.

The main objective of this study is to examine the impact of 4-5 year Cu contamination on the composition and resistance profiles of soil bacterial community in two contrasting agricultural soils. Soils were collected from two long-term well-manipulated experimental sites where independent plots were amended with different levels of Cu, providing a unique opportunity to assess the Cu contamination as a direct selection pressure for antibiotic resistance. High-throughput quantitative PCR (HT-QPCR) array was employed to target a broad spectrum of ARGs conferring resistance against almost all major classes of antibiotics and various types of mobile genetic elements (MGEs) (Looft *et al.*, 2012). We tested the following hypotheses: (i) Cu contamination can significantly impact the abundance and diversity of ARGs, and the ARG levels are enhanced with the increasing Cu concentrations; (ii) the effects of Cu contamination on ARGs and MGEs are different between the two agricultural soils, as the bioavailability and toxicity of heavy metals are highly dependent on soil properties; and (iii) changes in ARGs profiles can be closely associated with changes of the total bacterial community, which has been recognized as an important determinant of soil ARG content (Forsberg *et al.*, 2014).

Results

Diversity of ARGs and MGEs in Cu-contaminated soils

The HT-QPCR approach was employed to assess the impact of 4-5 year Cu contamination on the prevalence and diversity of ARGs and MGEs in two contrasting agricultural soils. Among all the soil samples, a total of 157 and 149 unique ARGs (out of all 285 ARGs targeted) were detected in the red soil (Fig. 1a) and the fluvo-aquic soil (Fig. 1b), respectively. As for the red soil, the numbers of detected ARGs were significantly higher in the medium levels of Cu contamination (Cu100 and Cu200 in 2011, and Cu200 in 2012) than those in other treatments, but significantly decreased in the highest Cu-contaminated level (Cu800) in both years (Fig. 1a). By contrast, the numbers of ARGs detected in the control treatment of the fluvo-aquic soil (ranging from 62 to 78) tended to increase along the increasing Cu concentrations, with the highest values (ranging from 99 to 118) observed in the Cu400 and Cu800 treatments in both years (Fig. 1b).

The detected ARGs across different treatments could potentially confer resistance to a broad spectrum of antibiotics (Fig. 1). The multidrug resistance genes were the most frequently encountered ARGs, accounting for 23.6% and 24.2% (an average of all soil samples) of the total number of ARGs in the red and fluvo-aquic soil, respectively. Other frequently detected ARGs included resistance genes to β-lactam (21.0% and 17.4%), MLSB (16.6% and 13.4%), vancomycin (10.2% and 13.4%), aminoglycoside (12.7% and 11.4%), and tetracycline (10.2% and 12.1%) for the red and fluvo-aquic soil, respectively. The numbers of each class of ARGs responded sensitively to Cu amendment and most of them showed the similar patterns as that observed for total ARGs along the gradient of Cu contamination (Fig. 1). Among all the soil samples, the 10 targeted MGEs including two integrase genes and eight transposase genes were detected in both soils (Fig. 1). The numbers of detected MGEs varied slightly across

different treatments in the red soil in both years, and no clear tendency of the MGE patterns was observed along the gradient of Cu concentrations (Fig. 1a). By contrast, the numbers of MGEs detected in the fluvo-aquic soil tended to increase along the increasing Cu concentrations, showing a similar pattern to that of ARGs in both years (Fig. 1b).

Relative abundance of ARGs and MGEs in Cu-contaminated soils

The relative abundances of ARGs and MGEs were calculated by normalizing to the bacterial 16S rRNA gene in the same HT-QPCR array based on a comparative C_T method (Thomas and Kenneth, 2008). In addition to shifts in the diversity of ARGs, changes in the relative abundances of ARGs were also observed after 4-5 year Cu contamination (Fig. 2). As for the red soil, the relative abundance of ARGs showed similar patterns along the gradient of Cu concentrations as that observed for the numbers of detected ARGs (Fig. 1a), with the highest values recorded in medium Cu-contaminated levels of Cu100 in 2011 and Cu200 in 2012 (Fig. 2a). By contrast, the relative abundance of ARGs in the fluvo-aquic soil was observed to significantly increase in the high Cu-contaminated levels of Cu400 and Cu800 in 2011 and Cu800 in 2012 (Fig. 2b). Of particular interest, ARGs encoding resistance to vancomycin, belonging to the group of last resort of antibiotics in human medicine, remarkably increased their abundances in high Cu concentrations of the fluvo-aquic soil (Fig. 2b).

The relative abundance of MGEs showed sensitive responses to Cu contamination, and generally exhibited a similar pattern to those of total ARGs, with the exception of the red soil in 2011 (Fig. 2). Significantly positive correlations between the relative abundances of total MGEs and total ARGs were found in both soils as revealed by the Spearman's correlation analysis (Table 1). The dynamics of MGEs were also significantly and positively correlated with those of specific classes of ARGs, for example, FCA, aminoglycoside, β-lactam, MLSB,

multidrug, and other resistance genes in the red soil, and FCA, aminoglycoside, β-lactam, sulphonamide, tetracycline, vancomycin, and multidrug in the fluvo-aquic soil (Table 1).

Impacts of Cu contamination on the overall patterns of ARGs

The relative abundances of ARGs along the gradient of Cu contamination were further explored at a higher resolution level for individual ARG types in heat maps based on the results of the HT-QPCR array (Fig. 3). All soil samples (columns in heat maps) could be broadly separated into three clusters for both soils: low (dominated by control and Cu50), medium (dominated by Cu100 and Cu200), and high (dominated by Cu400 and Cu800) Cu contamination levels. The clear clustering of soil samples was further corroborated by non-metric multidimensional scaling ordinations based on the Bray-Curtis dissimilarity matrices (Fig. S1), which revealed that the low Cu-contaminated soil samples were significantly separated from the medium and high Cu-contaminated soil samples (PerMANOVA analysis, $P < 0.05$ for both soils) with a few exceptions. In general, the shifts in ARG profiles (rows in heat maps) could be classified into four patterns (Fig. 3): (A) ARGs that are prevalent in all samples with high abundance, and tended to be slightly more abundant in medium Cu-level treatments in the red soil and in low Cu-level treatments in the fluvo-aquic soil; (B) ARGs that are absent or less abundant in low Cu concentrations, but emergent or enriched in medium and high Cu concentrations; (C) ARGs that are prevalent in low and medium Cu concentrations, but not in high Cu concentrations; and (D) ARGs that persist in all samples with low abundance and show no apparent responses to Cu contamination. It is notable that the D cluster was only observed in the red soil, and comprised a majority of the detected ARGs (Fig. 3a).

Co-occurrence patterns among the detected ARG types

Network analysis was conducted to explore the co-occurrence patterns among the detected ARG types based on strong ($\rho > 0.8$) and significant ($P < 0.05$) correlations (see supplementary results for more information). The resultant network consisted of 91 nodes (ARG subtypes) and 307 edges, and could be clearly separated into seven modules (Fig. 4a). The most densely connected node in each module was defined as 'hub', for example, the 'matA-mel' and 'acrA-01' encoding multidrug resistance were the hubs for modules I and II, respectively, and it has been suggested that hubs could act as ARG indicators to represent the quantity of other co-occurring ARGs in the same module (Li *et al.*, 2015a).

Co-occurrence between ARG types and microbial taxa

Changes in the total soil bacterial community compositions across different Cu-contaminated treatments were determined by targeting the bacterial 16S rRNA gene on the Miseq Illumina sequencing platform. The high-throughput Illumina sequencing yielded a total of 11,604,122 quality-filtered bacterial sequences for the 96 soil samples, corresponding to an average of 120,876 sequences per sample. The co-occurrence patterns between the ARG types and microbial taxa (at the phylum level) were also explored using network analysis (Fig. 4b). It was hypothesized that strong ($\rho > 0.8$) and significant ($P < 0.05$) co-occurrence patterns between ARGs and microbial taxa might indicate the possible host information of ARGs (Li *et al.*, 2015a), which means that some specific microbial taxa are speculated to carry some specific ARGs. In this network, Cyanobacteria, Actinobacteria, Firmicutes, Proteobacteria, Gemmatimonadetes, OD1, Planctomycetes, and Armatimonadetes were the most prevalent predicted taxa, and each contained ARGs conferring resistance to different classes of antibiotics (Fig. 4b). Armatimonadetes and OD1 were found to have more connections with multidrug resistance genes, whereas Gemmatimonadetes and Bacteroidetes encompassed more diverse MLSB resistance genes. Multidrug, MLSB, and β-lactam resistance genes showed the strongest relationships with the bacterial communities, and they were predicted to

be carried in different microbial taxa. Procrustes analysis based on unweighted UniFrac dissimilarity matrices revealed that the ARG content and the bacterial community compositions consistently displayed highly significant goodness-of-fit measures in both soils (Fig. S2).

Relationships among Cu concentrations, soil properties, bacterial abundance and community compositions, MGEs, and ARGs

Structural equation models (SEMs) were constructed to explore the direct and indirect effects of Cu concentrations, soil properties, bacterial communities and MGEs on the patterns of ARGs in both soils (Fig. 5). SEM is an *a priori* approach offering the ability to separate multiple pathways of influence and view them as a system, and is useful to explore the complex networks of relationships found in ecosystems (Eisenhauer *et al.*, 2015). Our SEMs explained 49% and 24% of the variance found in the patterns of ARGs in the red and fluvo-aquic soil, respectively. In the red soil (Fig. 5a), Cu contamination was found to have significantly positive influences on soil properties ($\lambda = 0.68$, $P < 0.001$) and bacterial community composition ($\lambda = 0.31$, $P < 0.05$). We also observed strongly positive effects of bacterial community composition ($\lambda = 0.43$, $P < 0.001$), MGEs ($\lambda = 0.68$, $P < 0.001$) and bacterial abundance ($\lambda = 0.21$, $P < 0.05$) on ARGs. Overall, bacterial community composition and Cu contamination were the two most important drivers of ARGs in the red soil, followed by MGEs (Fig. 5c). Similarly in the fluvo-aquic soil (Fig. 5b), Cu contamination was found to have a significant influence on bacterial community composition ($\lambda = 0.64$, $P < 0.001$), and soil properties could significantly affect bacterial abundance ($\lambda = 0.45$, $P < 0.001$) and community composition ($\lambda = 0.35$, $P < 0.001$). Strongly positive effects of bacterial community composition ($\lambda = 0.48$, $P < 0.05$) and MGEs ($\lambda = 0.22$, $P < 0.05$) on ARGs were also observed in the fluvo-aquic soil. Overall, the patterns of ARGs in the fluvo-aquic soil

were primarily regulated by bacterial community composition, Cu contamination, and MGEs (Fig. 5d).

Discussion

Impacts of long-term Cu contamination on the diversity and abundance of soil ARGs

A key finding of this study is that the two agricultural soils showed different patterns of ARGs responding to 4-5 year Cu contamination. For example, the numbers and relative abundance of ARGs tended to reach the maximum in the medium Cu concentrations (Cu100 and Cu200, with extractable Cu of 48.6 and 96.7 mg kg^{-1}, respectively) of the red soil, compared to those found in the high Cu concentrations (Cu400 and Cu800, with extractable Cu of 134.6 and 406 mg kg^{-1}, respectively) of the fluvo-aquic soil (Figs. 1 and 2). Because the two soils were amended with the same gradient of Cu concentrations, we speculated that the difference in soil basic properties (Table 2) might partially contribute to their different ARG patterns, and the importance of soil properties was revealed by SEM analysis (Fig. 5). Among these environmental factors, the toxicity of Cu is thought to be strongly dependent on soil pH values which can influence the valence and bioavailability of metal ions (Seiler *et al.*, 2012), and low pH values are known to increase the solubility of heavy metals (Schulz-Zunkel *et al.*, 2009). Appropriate levels of Cu are important for various physiological functions of the cell, and are essential micronutrients for cellular components and enzymatic biochemical processes in bacteria (Seiler and Berendonk, 2012). The high bioavailability of Cu, however, can be toxic with metabolic relevance and cause damage to cellular membranes and DNA (Knapp *et al.*, 2011; Seiler and Berendonk, 2012). The pH values (4.1~4.4) in the red soil are significantly lower than those (7.7~8.1) in the fluvo-aquic soil, and therefore the same concentrations of Cu would have higher bioavailability in the red soil. The red soil had the optimum Cu concentration for development of antibiotic resistance in Cu100 and Cu200, but

the treatments Cu400 and Cu800 are supposed to have higher Cu bio-availability which is toxic to the soil resident bacteria carrying ARGs, and thus we observed a decrease in ARG diversity and abundances in Cu400 and Cu800 of the red soil. By contrast, due to the relatively low bio-availability of Cu in the fluvo-aquic soil, available Cu in the treatments of Cu400 and Cu800 might have not exceeded the minimum threshold which is significantly toxic to soil resident bacteria, and therefore the fluvo-aquic soil could develop increasing resistance with the increasing Cu concentrations.

Apart from soil pH, other environmental parameters such as vegetation, geographical location, organic matter contents, and nitrogen contents, climatic factors such as temperature and precipitation, as well as agricultural practices can also affect the bioavailability of heavy metals and ARG contents in soil (Schulz-Zunkel *et al.*, 2009; Forsberg *et al.*, 2014). These factors together might have contributed to the different patterns of ARGs between the two Cu-contaminated soils. These findings suggest that the degree of metal-induced changes of ARGs differ across soil types, which necessitates more investigations on how soil properties influence the metal-induced effects on environmental ARGs by covering different soil/climate conditions and spatial variability in heavy metal-contaminated areas.

Beyond the changes in the diversity and relative abundance of total ARGs, Cu contamination also induced shifts in a wide spectrum of specific ARG types. In consistence with our findings, previous freshwater microcosm studies found that exposure of bacterial communities to individual heavy metals selected for multi-resistant microorganisms (Stepanauskas *et al.*, 2006), and Cu exposure was reported to co-select for increased bacterial resistance to various antibiotics in soil environments (Berg *et al.*, 2010; Knapp *et al.*, 2011). Interestingly, these diverse ARGs exhibited different patterns responding to Cu contamination which could be clearly separated into four main clusters (Fig. 3). Cluster A was persistent with high abundance while Cluster D was present with low abundance in all samples for both soils,

299 suggesting that these ARGs might reside in microbial assemblages which are resistant to
300 heavy metal pressure (Li *et al.*, 2015b). The high abundance of ARGs in Cluster A might be
301 originated from ancient soil indigenous bacteria which may have had developed antibiotic
302 resistance before the introduction of heavy metals (D'Costa *et al.*, 2011) or from soil ARGs
303 introduced by agricultural practices (Hu *et al.*, 2016). Cluster B was found to be highly
304 responsive to Cu contamination, and tended to increase with increasing Cu concentrations in
305 both soils. This cluster might represent the acquisition of new ARGs in Cu-resistance bacteria
306 co-selected by Cu contamination or the enrichment of soil indigenous bacteria bearing ARGs
307 stimulated by Cu amendment. Cluster C was favoured in low and medium levels of Cu
308 contamination but decreased in high Cu levels, suggesting that these ARGs might be located
309 on bacteria sensitive to high Cu concentrations. Therefore, Cu-induced changes of antibiotic
310 resistance could occur in a wide range of Cu concentrations, but would select different ARG
311 types in different Cu concentrations.

312 ***Soil bacterial community as a significant determinant of ARG contents***

313 The abundant and diverse ARGs found in the two agricultural soils and their remarkable
314 responses to Cu contamination prompted us to explore the main factors influencing soil ARG
315 patterns.

316 ***Potential for horizontal gene transfer of ARGs in Cu-contaminated soils***

317 The high abundance and diversity of ARGs observed in Cu-contaminated soils provide a high
318 likelihood of dispersal and HGT of ARGs to other bacteria and pathogens. The HGT of ARGs
319 among environmental microbial assemblages is an important factor in resistance
320 dissemination and the acquisition of antibiotic resistance by human pathogens (Heuer *et al.*,
321 2011; Forsberg *et al.*, 2012). Most of ARG cassettes are found in MGEs such as integrons
322 frequently located on transposons and broad-host range plasmids with a wide distribution in

agricultural systems (Gogartion and Townsend, 2005; Wolters *et al.*, 2015). Of these MGEs, Class 1 integrons are assumed to facilitate co-selection because they are frequently reported to be influenced by heavy metals (Seiler and Berendonk, 2012; Gillings *et al*., 2015). This assumption is supported by the observation of increased abundance and diversity of class 1 integrons in aquatic environments and freshwater sediments contaminated by heavy metals (Wright *et al.*, 2008; Abella *et al.*, 2015). The other well-documented co-selection case involves Tn21 and Tn21-like transposons, in which located a metal-resistance operon and an integron containing multiple ARGs (Wireman *et al.*, 1997; Baker-Austin *et al.*, 2006). These studies suggested the critical role of MGEs in the adaptation and evolution of bacterial communities against heavy metal perturbations.

Despite the huge diversity of ARGs, however, recent metagenomic analysis found little genetic potential for HGT of ARGs across soil communities in natural settings (Forsberg *et al.*, 2014). The rate of horizontal transmission in soil was assumed to be very low, compared with the vertical transmission of ARGs due to growth of resistant microorganisms (Heuer *et al.*, 2011). In the present study, HT-QPCR array detected two integrase genes and eight transposase genes in the two agricultural soils. The diversity and abundances of these MGEs were highly responsive to Cu amendment, and significantly positive correlations were found between the abundances of total MGEs and those of total ARGs and some specific types of ARGs (e.g. FCA, aminoglycoside, β-lactam, MLSB, sulphonamide, vancomycin, and multidrug resistance genes) in both soils (Table 1). The co-occurrence patterns of ARGs and microbial taxa revealed that the same ARGs can be shared by different bacterial phyla (Fig. 4b), which might be attributed to the frequent HGT of ARGs. SEMs confirmed the important role of MGEs in shaping the patterns of ARGs (Fig. 5), suggesting a potential for HGT of these ARGs under selection pressure imposed by Cu contamination. Therefore, the remarkable diversity of ARGs and MGEs in metal-contaminated environments together offers

a high probability of dispersal, selection, and HGT of ARGs. However, it should be noted that some other abiotic factors (e.g. temperature, pH, nutrient, soil types, oxygen and moisture contents) and biotic factors (e.g. antagonistic, commensal, mutualistic and other relationships among the soil inhabitants) can also affect the frequencies of HGT for ARGs in the environment (Koonin *et al.*, 2001; Aminov, 2011).

Conclusions

In conclusion, by combining long-term field experiments with HT-QPCR arrays, we provided comprehensive field-based evidence that 4-5 year Cu contamination could significantly affect the abundance, diversity, and mobility potential of a broad spectrum of ARGs in two contrasting agricultural soils. Although the pervasive use of antibiotics is the principal factor for global spread of ARGs, our results suggested that the Cu-induced changes in antibiotic resistance might be an important pathway for development of environmental ARGs particularly in countries where antibiotics uses as growth promoters have been banned. Our findings are alarming, as metals like Cu can accumulate to selective concentrations in the environment and might trigger the proliferation and evolution of environmental antibiotic resistance. Our findings have public health implications considering the potential for HGT of environmental ARGs to human commensals, and highlight the necessity to understand the risk of antibiotic resistance in the polluted environments, before we could design management options to control the transport of environmental resistome.

Experimental procedures

Site description and soil sampling

Soil samples were collected from two long-term experimental stations situated in Qiyang (QY, 26.75°N, 111.88°E), Hunan province, and Dezhou (DZ, 37.33°N, 116.63°E), Shandong province, China. The QY site has a subtropical monsoon climate with a mean annual rainfall

of 1,408 mm and a mean annual temperature of 18.1°C. The QY soil has a pH value of 4.3, and is classified as red soil (Paleudults in USDA Soil Taxonomy) with a ratio of clay, silt and sand (46-35-19%). The DZ site is characterized by a temperate continental climate with a mean annual rainfall of 548 mm and a mean annual temperature of 12.9°C. The DZ soil has a pH value of 7.9, and is classified as fluvo-aquic soil (Ustochrept in USDA Soil Taxonomy) with a ratio of clay, silt and sand (18-18-64%). In July 2007, six sets of treatments (including control without Cu addition and treatments with 50, 100, 200, 400, and 800 mg Cu kg^{-1} soil, denoted as Control, Cu50, Cu100, Cu200, Cu400 and Cu800, respectively) were established in four randomized replicate plots for both sites. In each site, copper chloride powders were thoroughly mixed with surface soils taken from the field and then applied back to the plots. Copper concentrations used in this study are representative of those found in a wide range of soils contaminated with heavy metals from anthropogenic sources (Seiler and Berendonk, 2012). The both experimental sites were planted with maize-wheat rotations under conventional agricultural management practices. Inorganic fertilizers were supplied the QY site at a rate of 300 kg N, 120 kg P and 120 kg K per hectare per year, and to the DZ site at a rate of 450 kg N, 120 kg P and 150 kg K per hectare per year during the trial period. Soil samples were taken from the two sites by mixing five soil cores (2.5 cm in diameter and 10 cm in depth) for each plot in July 2011 and July 2012. All the soil samples were transported on ice to the laboratory, sieved through 2 mm mesh, and stored at 4°C before physicochemical analyses and at -80°C before DNA extraction.

DNA extraction and physicochemical analysis

The total genomic DNA was isolated from 0.25 g of soil using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to manufacturer's instructions, with a slight modification that the initial cell-lysis step was performed on a FastPrep bead beating system (Bio-101, Vista, CA, USA) at a speed of 5.5 m s^{-1} for 30 s. The

quality and quantity of the isolated DNA were assessed using a NanoDrop® ND-2000c UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA concentrations varied between 20-30 ng μl^{-1} for the red soil and between 30-40 ng μl^{-1} for the fluvo-aquic soil. The A260/A280 ratios were greater than 1.8 for all the DNA extracts, and we did not observe any obvious effect of Cu contamination on the DNA extraction efficiency. Soil pH was determined using a fresh soil to water ratio of 2.5 with a Delta pH-meter (Mettler-Toledo Instruments, Columbus, OH, USA). Soil moisture content (H_2O%) was measured by oven-drying soil samples at 105°C for 24 h. Total nitrogen (TN) was measured on an Element analyser (Vario EL III, Elementar, Hanau, Germany). Soil organic matter (SOM) was determined by the $K_2Cr_2O_7$ oxidation-reduction colorimetric method. Soil microbial biomass carbon (SMBC) was determined by using a $CHCl_3$-fumigation-extraction procedure as described previously (Li *et al.*, 2015b). Copper was extracted from soils with 0.11 M CH_3COOH, and measured on ICP-OES (PerkinElmer, Waltham, MA, USA). The detailed information about the basic soil properties is shown in Table 2.

Profiling of ARGs by high-throughput quantitative PCR (HT-QPCR)

The occurrence of ARGs was analysed by HT-QPCR array as described previously (Looft *et al.*, 2012; Zhu *et al.*, 2013) on a Bio-Rad CFX384^{TM} Real-Time PCR Detection System (Bio-Rad, Herculers, CA, USA) with slight modifications. The HT-QPCR array used in the current study can simultaneously target 285 ARGs conferring resistance to all major classes of antibiotics [including tetracyclines, sulfonamides, macrolide-lincosamide-streptogramin B (MLSB), aminoglycosides, vancomycin, β-lactam, FCA (fluoroquinolone, quinolone, florfenicol, chloramphenicol, and amphenicol), multidrug, and others], eight transposon-transposase genes, two class 1 integron-integrase genes, and one 16S rRNA gene (Table S1). The targeted ARGs are associated with the antibiotics intensively used as veterinary and human medicine, or veterinary growth promoters. The HT-QPCR reactions were optimized to

meet the assumptions of the comparative C_T method that the PCR efficiency of the target ARGs is similar to the internal control 16S rRNA gene (Thomas and Kenneth, 2008).

The 5 µl reaction mixture consisted of 2.5 µl of SYBR Premix Ex Taq (TaKaRa Biotechnology, Otsu, Shiga, Japan), 0.4 µl of each primer (10 µM), 1 µl of 10-fold diluted template DNA, and microbial DNA-free water. The thermal-cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 30 s at 95°C and 30 s at 60°C. The baseline and threshold fluorescence values were manually adjusted to the same levels across all HT-QPCR runs, and a threshold cycle value (C_T) of 31 was used as the detection limit. Only samples with three or four replicates above the limit of quantification were regarded as positive. The relative abundances of ARGs and MGEs were calculated by normalizing to the bacterial 16S rRNA gene abundance in the same HT-QPCR run based on a comparative C_T method (Thomas and Kenneth, 2008).

Quantitative PCR analysis of the bacterial 16S rRNA gene

The bacterial 16S rRNA gene abundance was quantified on a Bio-Rad CFX384^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories) using the primer pair BACT1369F/PROK1492R with the probe TM1389F (Suzuki *et al.*, 2000). Amplification was performed in a total volume of 10 µl including 5 µl of SYBR Premix Ex Taq (TaKaRa), 0.5 µl of each primer (10 µM), 0.5 µl of probe (10 µM), and 2 µl of template DNA. Amplification conditions were as follows: 95°C for 10 s, followed by 35 cycles of 95°C for 15 s and 56°C for 1 min. Standard curves were generated from 10-fold serial dilutions of plasmids containing correct inserts of the bacterial 16S rRNA gene sequence. The specificity of PCR amplicons was verified by performing melting curve analysis following each QPCR run, and PCR efficiency ranged between 86% and 94%.

High-throughput profiling of the bacterial community by Illumina sequencing

Changes in the soil bacterial community compositions across different Cu-contaminated treatments were determined by targeting the bacterial 16S rRNA gene spanning the hypervariable V4 region using the primers 515f and barcoded-806r (Bates *et al.*, 2011). The 50 µl PCR reaction mixture contained 25 µl of Premix Ex Taq (TaKaRa), 0.5 µl of each primer (10 µM), and 2 µl of five-fold diluted DNA template. Amplification conditions were as follows: 94°C for 10 s, six touch-down cycles of 94°C for 45 s, 65°C to 58°C for 60 s, and 72°C for 70 s, followed by 22 cycles of 94°C for 45 s, 58°C for 60 s, and 72°C for 60 s. The PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, San Luis Obispo, CA, USA), and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). The purified PCR products were combined in approximately equimolar amount into a single tube, and sent for sequencing on the Miseq platform (Illumina, San Diego, CA, USA), at Novegene, Beijing, China.

The obtained raw sequences were split by samples, quality filtered and de-noised using Quantitative Insights Into Microbial Ecology (QIIME) 1.7.0 following the standard operation procedure (Caporaso *et al.*, 2010a). The resultant high-quality reads were clustered into operational taxonomic units (OTUs) at a 97% identity level using Uclust clustering (Edgar, 2010). A set of representative sequences from each OTUs were aligned against the Greengenes reference set (DeSantis *et al.*, 2006) using PyNAST (Caporaso *et al.*, 2010b), and the taxonomic classification was conducted using the Ribosome Database Project Classifier with a confidence threshold of 0.80 (Wang *et al.*, 2007). To correct for differences in the sequencing efforts, sequences were rarefied to the same number of reads per sample before the downstream analysis.

Network analysis and visualization

The co-occurrence/interaction patterns among the detected ARGs and between the ARGs and microbial taxa were explored in network analysis using the CoNet Cytoscape plug-in (Soffer

et al. 2014). Prior to the network construction, the detected ARGs and microbial taxa with a minimum occurrence of 30 across all the samples were discarded to minimize the artificial association bias (Li *et al.*, 2015a). The network analysis was performed at the phylum level for soil bacteria to reduce the complexity in calculation, and rare species were discarded to avoid introduction of spurious correlations. Briefly, for all the pairwise interactions, correlation scores were calculated using Spearman correlation, Pearson correlation, Kullback-Leibler dissimilarity and Bray-Curtis dissimilarity, followed by a combination of *P*-values of these correlation measures. The ReBoot procedure with 100 permutations was conducted to eliminate the potential false-positive correlations and compositionality biases, and the resultant distribution was further refined with 1000 bootstraps. The *P* values for correlations were combined from the five correlation measures using the *Simes* method, and only correlations found to be significant by at least two correlation methods were included (Soffer *et al.* 2014). The resultant pairwise correlations were utilized to construct the co-occurrence networks. Network topology was visualized using Cytoscape and the plug-in Network Analyzer and explored using the Frucherman Reingold algorithm on the open-source interactive platform Gephi (Bastian *et al.* 2009). Only correlations with a correlation coefficient (ρ-value) above 0.8, and a significance level (*P*-value) below 0.05 were considered statistically robust and displayed in the networks (Junker and Schreiber, 2008).

Statistical analysis

One-way analysis of variance followed by Student-Newman-Keuls test was performed to compare the numbers and relative abundances of ARGs and MGEs and the bacterial 16S rRNA gene abundance across different treatments in SPSS 20 (IBM, Armonk, NY, USA). The 16S rRNA gene copies were log-transformed prior to statistical analysis to satisfy the normality assumptions. Spearman's rank correlation test was performed to assess the correlations between the relative abundance of ARGs and MGEs in both soils. Statistically

significant differences were accepted at $P < 0.05$. Non-metric multidimensional scaling (NMDS) analysis was performed to visualize the Bray-Curtis dissimilarity matrix based on the relative abundance of ARGs. The effects of Cu-contaminated treatments on the patterns of ARGs were tested by permutational multivariate analysis of variance (PerMANOVA) using the 'adonis' function in the 'vegan' package of the R platform with 999 permutations. The heat maps illustrating the HT-QPCR results of ARGs with log-transformed normalized abundances were generated using the 'gplots' package in R, and the dendrograms for columns and rows were constructed from the Bray-Curtis dissimilarity distance. Procrustes test for correlation analysis between ARGs and the bacterial communities based on unweighted UniFrac dissimilarity matrices was performed in QIIME.

Finally, we constructed structural equation models (SEMs) to evaluate how Cu contamination (extractable Cu) influences soil properties (including soil pH, H_2O%, SOM, TN, and SMBC; Table 2), bacterial abundance, bacterial community composition, MGEs and ARGs. Prior to the SEM analysis, the pairwise correlations among these variables were examined by the Mantel test using the *Ecodist* package in R. The matrix of *R* values derived from the Mantel test was imported into AMOS (SPSS Inc., Chicago, IL, USA) for construction of SEM. The *a priori* and theoretical assumptions (Fig. S4) made to establish the SEM were (i) Cu contamination could directly influence the patterns of MGEs and ARGs through metal-induced co-selection process; (ii) Cu contamination could also indirectly affect the patterns of MGEs and ARGs by altering the abundance and compositions of bacterial communities conferring resistance to antibiotics; and (iii) Cu contamination can lead to changes in soil properties, which consequently affected bacterial community, MGEs and ARGs. The data matrix was fitted to the model using the maximum-likelihood estimation method. The overall goodness-of-fit of model fits was indicated by a non-significant chi-square test ($P > 0.05$),

high goodness-of-fit index (> 0.90), low Akaike information criteria (AIC), and low root square mean errors of approximation (RMSEA < 0.05).

Acknowledgements

This work was financially supported by Melbourne Early Career Researcher Grant Scheme, Chinese Academy of Sciences (XDB15020200), and Australian Research Council (DE150100870). The authors declare that there is no conflict of interest.

References

Abella, J., Fahy, A., Duran, R., and Cagnon, C. (2015) Integron diversity in bacterial communities of freshwater sediments at different contamination levels. *FEMS Microbiol Ecol* **91:** fiv120.

Alonso, A., Sanchez, P., and Martinez, J.L. (2001) Environmental selection of antibiotic resistance genes. *Environ Microbiol* **3:** 1-9.

Aminov, R.I. (2011) Horizontal gene exchange in environmental microbiota. *Front Microbiol* **2:** 158.

Ashbolt, N.J., Amezquita, A., Backhaus, T., Borriello, P., Brandt, K.K., Collignon, P., *et al.* (2013) Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ Health Perspect* **121:** 993-1001.

Baker-Austin, C., Wright, M.S., Stepanauskas, R., and McArthur, J.V. (2006) Co-selection of antibiotic and metal resistance. *Trends Microbiol* **14:** 176-182.

Bastian, M., Heymann, S., and Jacomy, M. (2009) Gephi: an open source software for exploring and manipulating networks. In: *International AAAI Conference on Weblogs and Social Media: San Jose, California*.

544 Bates, S.T., Berg-Lyons, D., Caporaso, J.G., Walters, W.A., Knight, R., and Fierer, N. (2011)
545 Examining the global distribution of dominant archaeal populations in soil. *ISME J* **5:** 908-
546 917.
547 Berendonk, T.U., Manaia, C.M., Merlin, C., Fatta-Kassinos, D., Cytryn, E., Walsh, F., *et al.*
548 (2015) Tackling antibiotic resistance: the environmental framework. *Nat Rev Microbiol* **13:**
549 310-317.
550 Berg, J., Thorsen, M.K., Holm, P.E., Jensen, J., Nybroe, O., and Brandt, K.K. (2010) Cu
551 exposure under field conditions coselects for antibiotic resistance as determined by a novel
552 cultivation independent bacterial community tolerance assay. *Environ Sci Technol* **44:**
553 8724-8728.
554 Berg, J., Tom-Petersen, A., and Nybroe, O. (2005) Copper amendment of agricultural soil
555 selects for bacterial antibiotic resistance in the field. *Lett Appl Microbiol* **40:** 146-151.
556 Bolan, N.S., Adriano, D.C., and Mahimairaja, S. (2004) Distribution and bioavailability of
557 trace elements in livestock and poultry manure by-products. *Crit Rev Environ Sci Technol*
558 **34:** 291-338.
559 Boucher, H.W., Talbot, G.H., Bradley, J.S., Edwards, J.E., Gilbert, D., Rice, L.B., *et al.* (2009)
560 Bad drugs, no drugs: no ESKAPE! *Clin Inf Diseases* **48:** 1-12.
561 Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et*
562 *al.* (2010a) QIIME allows analysis of high-throughput community sequencing data. *Nat*
563 *Methods* **7:** 335-336.
564 Caporaso, J.G., Bittinger, K., Bushman, F.D., De Santis, T.Z., Anderson, G.L., and Knight, R.
565 (2010b) PyNAST: a flexible tool for aligning sequences to a template alignment.
566 *Bioinformatics* **26:** 266-267.
567 Chapman, J.S. (2003) Disinfectant resistance mechanisms, cross-resistance and co-resistance.
568 *Int Biodeterior Biodegradation* **51:** 271-276.

569 Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.F., Yannarell, A.C., *et al.*
570 (2009) Fate and transport of antibiotic residues and antibiotic resistance genes following
571 land application of manure waste. *J Environ Qual* **38:** 1086-1108.
572 D'Costa, V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W., Schwarz, C., *et al.* (2011)
573 Antibiotic resistance is ancient. *Nature* **477:** 457-461.
574 D'Costa, V.M., McGrann, K.M., Hughes, D.W., and Wright, G.D. (2006) Sampling the
575 antibiotic resistome. *Science* **311:** 374-377.
576 DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., *et al.* (2006)
577 Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with
578 ARB. *Appl Environ Microbiol* **72:** 5069-5072.
579 Edgar, R.C. (2010) Search and clustering orders of magnitude faster than BLAST.
580 *Bioinformatics* **26:** 2460-2461.
581 Eisenhauer, N., Bowker, M.A., Grace, J.B., and Powell, J.R. (2015) From patterns to causal
582 understanding: structural equation modelling (SEM) in soil ecology. *Pedobiologia* **58:** 65-
583 72.
584 Forsberg, K.J., Reyes, A., Wang, B., Selleck, E.M., Sommer, M.O.A., and Dantas, G. (2012)
585 The shared antibiotic resistome of soil bacteria and human pathogens. *Science* **337:** 1107-
586 1111.
587 Forsberg, K.J., Patel, S., Gibson, M.K., Lauber, C.L., Knight, R., Fierer, N., *et al.* (2014)
588 Bacterial phylogeny structures soil resistomes across habitats. *Nature* **509:** 612-615.
589 Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, A., Tiedje, J.M., and Zhu, Y.G. (2015)
590 Using the class 1 integron integrase gene as a proxy for anthropogenic pollution. *ISME J* **9**:
591 1269-1279.
592 Gogartion, J.P., and Townsend, J.P. (2005) Horizontal gene transfer, genome innovation and
593 evolution. *Nat Rev Microbiol* **3:** 679-687.

Graham, D.W., Olivares-Rieumont, S., Knapp, C.W., Lima, L., Werner, D., and Bowen, E. (2011) Antibiotic resistance gene abundances associated with waste discharges to the Almendares River near Havana, Cuba. *Environ Sci Technol* **45:** 418-424.

Han, X.M., Hu, H.W., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., *et al.* (2016) Impacts of reclaimed water irrigation on soil antibiotic resistome in urban parks of Victoria, Australia. *Environ Pollut* **211:** 48-57.

Heuer, H., Focks, A., Lamshoft, M., Smalla, K., Matthies, M., and Spiteller, M. (2008) Fate of sulfadiazine administrated to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol Biochem* **40:**1892–1900.

Heuer, H., Schmitt, H., and Smalla, K. (2011) Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr Opin Microbiol* **14:** 236-243.

Hu, H.W., Han, X.M., Shi, X.Z., Wang, J.T., Han, L.L., Chen, D., *et al.* (2016) Temporal changes of antibiotic resistance genes and bacterial communities in two contrasting soils treated with cattle manure. *FEMS Microbiol Ecol* **92:** fiv169.

Ji, X., Shen, Q., Liu, F., Ma, J., Xu, G., Wang, Y., *et al.* (2012) Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai, China. *J Hazard Mater* **235-236:** 178-185.

Junker, B.H., and Schreiber, F. (2008) Correlation networks. In: *Analysis of Biological Networks*. New York: Wiley-Interscience.

Knapp, C.W., McCluskey, S.M., Singh, B.K., Campbell, C.D., Hudson, G., and Graham, D.W. (2011) Antibiotic resistance gene abundances correlated with metal and geochemical conditions in archived Scottish soils. *PLoS One* **6:** e27300.

617 Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., et al.
618 (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US
619 strams, 1999-2000: a national reconnaissance. *Environ Sci Technol* **36:** 1202-1211.
620 Koonin, E.V., Makarova, K.S., and Aravind, L. (2001) Horizontal gene transfer in
621 prokaryotes: quantification and classification. *Annu Rev Microbiol* **55:** 709-742.
622 Kopmann, C., Jechalke, S., Rosendahl, I., Groeneweg, J., Krogerrecklenfort, E., Zimmerling,
623 U., *et al.* (2013) Abundance and transferability of antibiotic resistance as related to the fate
624 of sulfadiazine in maize rhizosphere and bulk soil. *FEMS Microbiol Ecol* **83:** 125–134.
625 Li, B., Yang, Y., Ma, L.P., Ju, F., Guo, F., Tiedje, J.M., *et al.* (2015a) Metagenomic and
626 network analysis reveal wide distribution and co-occurrence of environmental antibiotic
627 resistance genes. *ISME J* **9:** 2490-2502.
628 Li, J., Ma, Y.B., Hu, H.W., Wang, J.T., Liu, Y.R., and He, J.Z. (2015b) Field-based evidence
629 for consistent responses of bacterial communities to copper contamination in two
630 contrasting agricultural soils. *Front Microbiol* **6:** 31.
631 Looft, T., Johnson, T.A., Allen, H.K., Bay, D.O., Alt, D.P., Stedtfeld, R.D., *et al.* (2012) In-
632 feed antibiotic effects on the swine intestinal microbiome. *Proc Nat Acad Sci USA* **109:**
633 1691-1696.
634 Marshall, B.M., and Levy, S.B. (2011) Food animals and antimicrobials: impacts on human
635 health. *Clin Microbiol Rev* **4:** 718-733.
636 Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., *et al.* (2013) Impact of
637 manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of
638 detection of antibiotic resistance genes in soil and on vegetables on harvest. *Appl Environ*
639 *Microbiol* **79:** 5701-5709.
640 McArthur, J.V., and Tuckfield, R.C. (2000) Spatial patterns in antibiotic resistance among
641 stream bacteria: effects of industrial pollution. *Appl Environ Microbiol* **66:** 3722-3726.

642 Nesme, J., Cecillon, S., Delmont, T.O., Monier, J.M., Vogel, T.M., and Simonet, P. (2014)
643 Large-scale metagenomics-based study of antibiotic resistance in the environment. *Curr*
644 *Biol* **10**:1096–1100.
645 Peltier, E., Vincent, J., Finn, C., and Graham, D.W. (2010) Zinc-induced antibiotic resistance
646 in activated sludge bioreactors. *Water Res* **44:** 3829-3836.
647 Pruden, A., Arabi, M., and Sorteboom, H.N. (2012) Correlation between upstream human
648 activities and riverine antibiotic resistance genes. *Environ Sci Technol* **46:**11541–11549.
649 Pruden, A., Pei, R., Storteboom, H., and Carlson, K.H. (2006) Antibiotic resistance genes as
650 emerging contaminants: studies in Northern Colorado. *Environ Sci Technol* **40:** 7445-7450.
651 Schulz-Zunkel, C., and Krueger, F. (2009) Trace metal dynamics in floodplain soils of the
652 River Elbe: a review. *J Environ Qual* **38:** 1349-1362.
653 Seiler, C., and Berendonk, T.U. (2012) Heavy metal driven co-selection of antibiotic
654 resistance in soil and water bodies impacted by agriculture and aquaculture. *Front*
655 *Microbiol* **3:** 399.
656 Soffer, N., Zaneveld, J., and Thurber, R.V. (2014) Phage-bacteria network analysis and its
657 implication for the understanding of coral disease. *Environ Microbiol* **17:**1203–1218.
658 Stepanauskas, R., Glenn, T.C., Jaqoe, C.H., Tuckfield, R.C., Lindell, A.H., and McAethur,
659 J.V. (2005) Elevated microbial tolerance to metals and antibiotics in metal-contaminated
660 industrial environments. *Environ Sci Technol* **39:** 3671-3678.
661 Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., King, C.J., *et al.*
662 (2006) Coselection for microbial resistance to metals and antibiotics in freshwater
663 microcosms. *Environ Microbiol* **8:** 1510-1514.
664 Su, J.Q., Wei, B., Ou-Yang, W.Y., Huang, F.Y., Zhao, Y., Xu, H.J., *et al.* (2015) Antibiotic
665 resistome and its association with bacterial communities during sewage sludge composting.
666 *Environ Sci Technol* **49:** 7356-7363.

667 Summers, A.O., Wireman, J., Vimy, M.J., Lorscheider, F.L, Marshall, B., Levy, S.B., *et al.*
668 (1993) Mercury released from dental silver fillings provokes an increase in mercury-
669 resistant and antibiotic-resistant bacteria in oral an intestinal floras of primates. *Antimicrob*
670 *Agents Chemother* **37:** 825-834.
671 Suzuki, M.T., Taylor, L.T., and Delong, E.F. (2000) Quantitative analysis of small-subunit
672 rRNA genes in mixed microbial populations via 5′-nuclease assays. *Appl Environ*
673 *Microbiol* **66:** 4605-4614.
674 Thomas, D.S., and Kenneth, J.L. (2008) Analyzing real-time PCR data by the comparative C_T
675 method. *Nat Protoc* **3:** 1101-1108.
676 Wang, C., Sui, Z., Leclercq, S.O., Zhang, G., Zhao, M., Chen, W., *et al.* (2015) Functional
677 characterization and phylogenetic analysis of acquired and intrinsic macrolide
678 phosphotransferases in the Bacillus cereus group. *Environ Microbiol* **17:** 1560-1573.
679 Wang, Q., Garrity, G.M., Tiedje, J.M., and Cole, J.R. (2007) Native Bayesian classifier for
680 rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ*
681 *Microbiol* **73:** 5261-5267.
682 Wireman, J., Liebert, C.A., Smith, T., and Summers, A.O. (1997) Association of mercury
683 resistance with antibiotic resistance in the Gram-negative fecal bacteria of primates. *Appl*
684 *Environ Microbiol* **63:** 4494-4503.
685 Wolters, B., Kyselkova, M., Krogerrecklenfort, E., Kreuzig, R., and Smalla, K. (2015)
686 Transferable antibiotic resistance plasmids from biogas plant digestates often belong to the
687 IncP-1ε subgroup. *Front Microbiol* **5:** 765.
688 World Health Organization. Antimicrobial Resistance: Global Report on Surveillance.
689 Geneva: WHO Press, 2014.

690 Wright, M.S., Peltier, G.L., Stepanauskas, R., and McArthur, J.V. (2006) Bacterial tolerance
691 to metals and antibiotics in metal-contaminated and reference streams. *FEMS Microbiol*
692 *Ecol* **58:** 293-302.
693 Wright, M.S., Baker-Austin, C., Lindell, A.H., Stepanauskas, R., Stokes, H.W., and McArthur,
694 J.V. (2008) Influence of industrial contamination on mobile genetic elements: class 1
695 integron abundance and gene cassette structure in aquatic bacterial communities. *ISME J* **2:**
696 417-428.
697 Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld, R.D., *et al.* (2012)
698 Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc Nat Acad*
699 *Sci USA* **110:** 3435-3344.

Table 1 Spearman correlations between the relative abundance of ARGs and MGEs in the red and fluvo-aquic soil.

	Red soil	Fluvo-aquic soil
	Relative abundance of MGEs	Relative abundance of MGEs
Total ARGs	**0.328 (0.023)**	**0.660 (<0.001)**
FCA	**0.311 (0.031)**	**0.364 (0.011)**
Aminoglycoside	**0.304 (0.036)**	**0.656 (<0.001)**
Beta Lactamase	**0.317 (0.028)**	**0.512 (<0.001)**
MLSB	0.136 (0.358)	0.136 (0.357)
Sulfonamide	0.081 (0.583)	**0.738 (<0.001)**
Tetracycline	0.089 (0.548)	**0.317 (0.028)**
Vancomycin	0.189 (0.199)	**0.495 (<0.001)**
Multidrug	**0.375 (0.009)**	**0.374 (0.009)**
Other	**0.299 (0.039)**	0.195 (0.163)

Values showed in this table are the *R*-value exported from the Spearman correlation analysis, followed by the *P*-value in the brackets. The bold numbers indicate significant correlations ($P < 0.05$).

Table 2 Basic properties of the red and fluvo-aquic soil samples collected from Qiyang (QY) and Dezhou (DZ), China, respectively.

	Treatment	Soil pH	H_2O %	SOM (g kg^{-1})	TN (g kg^{-1})	SMBC (mg kg^{-1})	Added Cu (mg kg^{-1})	Extractable Cu (mg kg^{-1})
Red soil (QY)	Control	4.3	20.2	16.2	0.92	400	0	1.4
	Cu-50	4.1	19.3	15.6	0.88	239	50	22.0
	Cu-100	4.1	18.6	13.7	0.82	228	100	48.6
	Cu-200	4.2	10.1	15.0	0.86	126	200	96.7
	Cu-400	4.3	11.3	15.3	0.86	127	400	211.3
	Cu-800	4.4	12.7	16.5	0.78	170	800	377.9
Fluvo-aquic soil (DZ)	Control	7.8	14.1	13.4	0.46	368	0	0.5
	Cu-50	7.7	14.2	11.3	0.5	330	50	3.6
	Cu-100	8.1	13.6	12.3	0.46	278	100	12.9
	Cu-200	8.0	15.8	11.5	0.44	268	200	38.7
	Cu-400	8.0	15.8	12.8	0.4	194	400	134.6
	Cu-800	7.9	15.5	12.3	0.44	137	800	406

Figure legends

Fig. 1 The number of unique ARGs and MGEs detected across different Cu-contaminated treatments in the red (a) and fluvo-aquic soil (b). Error bars indicate standard errors (n = 4). Different letters above the bars indicate a significant difference for ARGs. (Abbreviations: FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance genes)

Fig. 2 The abundance of ARGs and MGEs relative to the bacterial 16S rRNA gene across different Cu-contaminated treatments in the red (a) and fluvo-aquic soil (b). Error bars indicate standard errors (n = 4). Different letters above the bars indicate a significant difference for ARGs. (Abbreviations: FCA, fluoroquinolone, quinolone, florfenicol, chloramphenicol and amphenicol resistance genes; MLSB, Macrolide-Lincosamide-Streptogramin B resistance genes)

Fig. 3 The heat maps showing the distribution profiles of ARGs across the different Cu-contaminated treatments in the red (a) and fluvo-aquic soil (b). The labels under each column denote the treatment name followed by the replicate number and the sampling years, for example, Cu200.3-11 represents the third repliate sample collected in 2011 from the Cu200 treatment. Each row is the results from a specfic type of ARG primer set. The plotted values are the natural logarithm transformed proportion of each ARG to the bacterial 16S rRNA gene abundance. The dendrograms for the columns and rows were constructed from the Bray-Curtis dissimilarity distance. Black boxes represent different patterns of ARGs: (A) prevalent in all samples with high abundance; (B) emergent or enriched in medium and high Cu concentrations; (C) prevalent in low and medium Cu concentrations, but not in high Cu concentrations; and (D) non-responsive to Cu contamination.

Fig. 4 (a) The network analysis showing the co-occurrence patterns among the detected ARG types across all the treatments. The nodes with different colors represent different modularity classes, and the edges correspond to strong ($\rho > 0.8$) and significant ($P < 0.05$) correlations between nodes. (b) The network analysis showing the co-occurrence patterns between ARG types and microbial taxa across all the treatments. The nodes with different colors represent different ARG types (diamonds) and microbial taxa (squares), and the edges correspond to strong ($\rho > 0.8$) and significant ($P < 0.05$) correlations between nodes. The size of each node is proportional to the number of significant correlations between nodes.

Fig. 5 Structural equation models showing the direct and indirect effects of Cu contamination, soil properties, bacterial abundance, bacterial community composition, MGEs on ARG patterns in the red (a,c) and fluvo-aquic soil (b,d). 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. Continuous and dashed arrows indicate positive and negative relationships, respectively. Significance levels are indicated as follows: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. (c,d) Standardized total effects (direct plus indirect effects) derived from the structural equation models depicted above. The hypothetical models fit our data well, as suggested by $\chi^2 = 0.5$, $P = 0.92$, GFI = 0.99, AIC = 36.48, and RMSEA = 0.00 for the red soil (a) and $\chi^2 = 0.1$, $P = 0.99$, GFI = 0.99, AIC = 36.12, and RMSEA = 0.00 for the fluvo-aquic soil (b).