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1 *Title page*

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

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18 **Running title:** Copper induced changes in antibiotic resistance

19

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

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

53 **Keywords**

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

55

56 **Introduction**

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

74 Agricultural soils are considered as an important environmental reservoir for ARGs and
75 pathogens (Nesme *et al.*, 2014), due to land application of animal manures containing residual
76 antibiotics and ARGs (Hu *et al.*, 2016). However, it was found that the concentrations of most
77 antibiotics in manure rapidly declined within 30 days after application to soils (Kopmann *et*
78 *al.*, 2013), and manure-derived ARGs cannot thrive in the soil environment and gradually
79 decreased over time after manure treatment (Heuer *et al.*, 2008; Hu *et al.*, 2016). Therefore,
80 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

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

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

131 **Results**

132 ***Diversity of ARGs and MGEs in Cu-contaminated soils***

133 The HT-QPCR approach was employed to assess the impact of 4-5 year Cu contamination on
134 the prevalence and diversity of ARGs and MGEs in two contrasting agricultural soils. Among
135 all the soil samples, a total of 157 and 149 unique ARGs (out of all 285 ARGs targeted) were
136 detected in the red soil (Fig. 1a) and the fluvo-aquic soil (Fig. 1b), respectively. As for the red
137 soil, the numbers of detected ARGs were significantly higher in the medium levels of Cu
138 contamination (Cu100 and Cu200 in 2011, and Cu200 in 2012) than those in other treatments,
139 but significantly decreased in the highest Cu-contaminated level (Cu800) in both years (Fig.
140 1a). By contrast, the numbers of ARGs detected in the control treatment of the fluvo-aquic
141 soil (ranging from 62 to 78) tended to increase along the increasing Cu concentrations, with
142 the highest values (ranging from 99 to 118) observed in the Cu400 and Cu800 treatments in
143 both years (Fig. 1b).

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

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

159 ***Relative abundance of ARGs and MGEs in Cu-contaminated soils***

160 The relative abundances of ARGs and MGEs were calculated by normalizing to the bacterial
161 16S rRNA gene in the same HT-QPCR array based on a comparative C_T method (Thomas and
162 Kenneth, 2008). In addition to shifts in the diversity of ARGs, changes in the relative
163 abundances of ARGs were also observed after 4-5 year Cu contamination (Fig. 2). As for the
164 red soil, the relative abundance of ARGs showed similar patterns along the gradient of Cu
165 concentrations as that observed for the numbers of detected ARGs (Fig. 1a), with the highest
166 values recorded in medium Cu-contaminated levels of Cu100 in 2011 and Cu200 in 2012 (Fig.
167 2a). By contrast, the relative abundance of ARGs in the fluvo-aquic soil was observed to
168 significantly increase in the high Cu-contaminated levels of Cu400 and Cu800 in 2011 and
169 Cu800 in 2012 (Fig. 2b). Of particular interest, ARGs encoding resistance to vancomycin,
170 belonging to the group of last resort of antibiotics in human medicine, remarkably increased
171 their abundances in high Cu concentrations of the fluvo-aquic soil (Fig. 2b).

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

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

180 ***Impacts of Cu contamination on the overall patterns of ARGs***

181 The relative abundances of ARGs along the gradient of Cu contamination were further
182 explored at a higher resolution level for individual ARG types in heat maps based on the
183 results of the HT-QPCR array (Fig. 3). All soil samples (columns in heat maps) could be
184 broadly separated into three clusters for both soils: low (dominated by control and Cu50),
185 medium (dominated by Cu100 and Cu200), and high (dominated by Cu400 and Cu800) Cu
186 contamination levels. The clear clustering of soil samples was further corroborated by non-
187 metric multidimensional scaling ordinations based on the Bray-Curtis dissimilarity matrices
188 (Fig. S1), which revealed that the low Cu-contaminated soil samples were significantly
189 separated from the medium and high Cu-contaminated soil samples (PerMANOVA analysis,
190 $P < 0.05$ for both soils) with a few exceptions. In general, the shifts in ARG profiles (rows in
191 heat maps) could be classified into four patterns (Fig. 3): (A) ARGs that are prevalent in all
192 samples with high abundance, and tended to be slightly more abundant in medium Cu-level
193 treatments in the red soil and in low Cu-level treatments in the fluvo-aquic soil; (B) ARGs
194 that are absent or less abundant in low Cu concentrations, but emergent or enriched in
195 medium and high Cu concentrations; (C) ARGs that are prevalent in low and medium Cu
196 concentrations, but not in high Cu concentrations; and (D) ARGs that persist in all samples
197 with low abundance and show no apparent responses to Cu contamination. It is notable that
198 the D cluster was only observed in the red soil, and comprised a majority of the detected
199 ARGs (Fig. 3a).

200 ***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

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

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

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

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

252 **Discussion**

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

254 A key finding of this study is that the two agricultural soils showed different patterns of
255 ARGs responding to 4-5 year Cu contamination. For example, the numbers and relative
256 abundance of ARGs tended to reach the maximum in the medium Cu concentrations (Cu100
257 and Cu200, with extractable Cu of 48.6 and 96.7 mg kg^{-1}, respectively) of the red soil,
258 compared to those found in the high Cu concentrations (Cu400 and Cu800, with extractable
259 Cu of 134.6 and 406 mg kg^{-1}, respectively) of the fluvo-aquic soil (Figs. 1 and 2). Because the
260 two soils were amended with the same gradient of Cu concentrations, we speculated that the
261 difference in soil basic properties (Table 2) might partially contribute to their different ARG
262 patterns, and the importance of soil properties was revealed by SEM analysis (Fig. 5). Among
263 these environmental factors, the toxicity of Cu is thought to be strongly dependent on soil pH
264 values which can influence the valence and bioavailability of metal ions (Seiler *et al.*, 2012),
265 and low pH values are known to increase the solubility of heavy metals (Schulz-Zunkel *et al.*,
266 2009). Appropriate levels of Cu are important for various physiological functions of the cell,
267 and are essential micronutrients for cellular components and enzymatic biochemical processes
268 in bacteria (Seiler and Berendonk, 2012). The high bioavailability of Cu, however, can be
269 toxic with metabolic relevance and cause damage to cellular membranes and DNA (Knapp *et*
270 *al.*, 2011; Seiler and Berendonk, 2012). The pH values (4.1~4.4) in the red soil are
271 significantly lower than those (7.7~8.1) in the fluvo-aquic soil, and therefore the same
272 concentrations of Cu would have higher bioavailability in the red soil. The red soil had the
273 optimum Cu concentration for development of antibiotic resistance in Cu100 and Cu200, but

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

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

290 Beyond the changes in the diversity and relative abundance of total ARGs, Cu contamination
291 also induced shifts in a wide spectrum of specific ARG types. In consistence with our findings,
292 previous freshwater microcosm studies found that exposure of bacterial communities to
293 individual heavy metals selected for multi-resistant microorganisms (Stepanauskas *et al.*,
294 2006), and Cu exposure was reported to co-select for increased bacterial resistance to various
295 antibiotics in soil environments (Berg *et al.*, 2010; Knapp *et al.*, 2011). Interestingly, these
296 diverse ARGs exhibited different patterns responding to Cu contamination which could be
297 clearly separated into four main clusters (Fig. 3). Cluster A was persistent with high
298 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

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

333 Despite the huge diversity of ARGs, however, recent metagenomic analysis found little
334 genetic potential for HGT of ARGs across soil communities in natural settings (Forsberg *et*
335 *al.*, 2014). The rate of horizontal transmission in soil was assumed to be very low, compared
336 with the vertical transmission of ARGs due to growth of resistant microorganisms (Heuer *et*
337 *al.*, 2011). In the present study, HT-QPCR array detected two integrase genes and eight
338 transposase genes in the two agricultural soils. The diversity and abundances of these MGEs
339 were highly responsive to Cu amendment, and significantly positive correlations were found
340 between the abundances of total MGEs and those of total ARGs and some specific types of
341 ARGs (e.g. FCA, aminoglycoside, β-lactam, MLSB, sulphonamide, vancomycin, and
342 multidrug resistance genes) in both soils (Table 1). The co-occurrence patterns of ARGs and
343 microbial taxa revealed that the same ARGs can be shared by different bacterial phyla (Fig.
344 4b), which might be attributed to the frequent HGT of ARGs. SEMs confirmed the important
345 role of MGEs in shaping the patterns of ARGs (Fig. 5), suggesting a potential for HGT of
346 these ARGs under selection pressure imposed by Cu contamination. Therefore, the
347 remarkable diversity of ARGs and MGEs in metal-contaminated environments together offers

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

353 **Conclusions**

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

367 **Experimental procedures**

368 ***Site description and soil sampling***

369 Soil samples were collected from two long-term experimental stations situated in Qiyang (QY,
370 26.75°N, 111.88°E), Hunan province, and Dezhou (DZ, 37.33°N, 116.63°E), Shandong
371 province, China. The QY site has a subtropical monsoon climate with a mean annual rainfall

372 of 1,408 mm and a mean annual temperature of 18.1°C. The QY soil has a pH value of 4.3,
373 and is classified as red soil (Paleudults in USDA Soil Taxonomy) with a ratio of clay, silt and
374 sand (46-35-19%). The DZ site is characterized by a temperate continental climate with a
375 mean annual rainfall of 548 mm and a mean annual temperature of 12.9°C. The DZ soil has a
376 pH value of 7.9, and is classified as fluvo-aquic soil (Ustochrept in USDA Soil Taxonomy)
377 with a ratio of clay, silt and sand (18-18-64%). In July 2007, six sets of treatments (including
378 control without Cu addition and treatments with 50, 100, 200, 400, and 800 mg Cu kg^{-1} soil,
379 denoted as Control, Cu50, Cu100, Cu200, Cu400 and Cu800, respectively) were established
380 in four randomized replicate plots for both sites. In each site, copper chloride powders were
381 thoroughly mixed with surface soils taken from the field and then applied back to the plots.
382 Copper concentrations used in this study are representative of those found in a wide range of
383 soils contaminated with heavy metals from anthropogenic sources (Seiler and Berendonk,
384 2012). The both experimental sites were planted with maize-wheat rotations under
385 conventional agricultural management practices. Inorganic fertilizers were supplied the QY
386 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
387 rate of 450 kg N, 120 kg P and 150 kg K per hectare per year during the trial period. Soil
388 samples were taken from the two sites by mixing five soil cores (2.5 cm in diameter and 10
389 cm in depth) for each plot in July 2011 and July 2012. All the soil samples were transported
390 on ice to the laboratory, sieved through 2 mm mesh, and stored at 4°C before
391 physicochemical analyses and at -80°C before DNA extraction.

392 ***DNA extraction and physicochemical analysis***

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

397 quality and quantity of the isolated DNA were assessed using a NanoDrop® ND-2000c UV-
398 Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The DNA
399 concentrations varied between 20-30 ng μl^{-1} for the red soil and between 30-40 ng μl^{-1} for the
400 fluvo-aquic soil. The A260/A280 ratios were greater than 1.8 for all the DNA extracts, and we
401 did not observe any obvious effect of Cu contamination on the DNA extraction efficiency.
402 Soil pH was determined using a fresh soil to water ratio of 2.5 with a Delta pH-meter
403 (Mettler-Toledo Instruments, Columbus, OH, USA). Soil moisture content (H_2O%) was
404 measured by oven-drying soil samples at 105°C for 24 h. Total nitrogen (TN) was measured
405 on an Element analyser (Vario EL III, Elementar, Hanau, Germany). Soil organic matter
406 (SOM) was determined by the $K_2Cr_2O_7$ oxidation-reduction colorimetric method. Soil
407 microbial biomass carbon (SMBC) was determined by using a $CHCl_3$-fumigation-extraction
408 procedure as described previously (Li *et al.*, 2015b). Copper was extracted from soils with
409 0.11 M CH_3COOH, and measured on ICP-OES (PerkinElmer, Waltham, MA, USA). The
410 detailed information about the basic soil properties is shown in Table 2.

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

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

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

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

434 ***Quantitative PCR analysis of the bacterial 16S rRNA gene***

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

445 ***High-throughput profiling of the bacterial community by Illumina sequencing***

446 Changes in the soil bacterial community compositions across different Cu-contaminated
447 treatments were determined by targeting the bacterial 16S rRNA gene spanning the
448 hypervariable V4 region using the primers 515f and barcoded-806r (Bates *et al.*, 2011). The
449 50 µl PCR reaction mixture contained 25 µl of Premix Ex Taq (TaKaRa), 0.5 µl of each
450 primer (10 µM), and 2 µl of five-fold diluted DNA template. Amplification conditions were
451 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
452 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
453 PCR products were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, San
454 Luis Obispo, CA, USA), and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen,
455 Carlsbad, CA, USA). The purified PCR products were combined in approximately equimolar
456 amount into a single tube, and sent for sequencing on the Miseq platform (Illumina, San
457 Diego, CA, USA), at Novegene, Beijing, China.

458 The obtained raw sequences were split by samples, quality filtered and de-noised using
459 Quantitative Insights Into Microbial Ecology (QIIME) 1.7.0 following the standard operation
460 procedure (Caporaso *et al.*, 2010a). The resultant high-quality reads were clustered into
461 operational taxonomic units (OTUs) at a 97% identity level using Uclust clustering (Edgar,
462 2010). A set of representative sequences from each OTUs were aligned against the
463 Greengenes reference set (DeSantis *et al.*, 2006) using PyNAST (Caporaso *et al.*, 2010b), and
464 the taxonomic classification was conducted using the Ribosome Database Project Classifier
465 with a confidence threshold of 0.80 (Wang *et al.*, 2007). To correct for differences in the
466 sequencing efforts, sequences were rarefied to the same number of reads per sample before
467 the downstream analysis.

468 ***Network analysis and visualization***

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

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

489 ***Statistical analysis***

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

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

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

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

522

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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).